SYMPOSIUM ON MOLLUSCA

PART II

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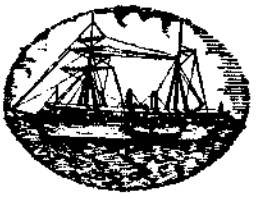


MARINE BIOLOGICAL ASSOCIATION OF INDIA
MARINE FISHERIES P.O., MANDAPAM CAMP
INDIA

PROCEEDINGS

OF THE

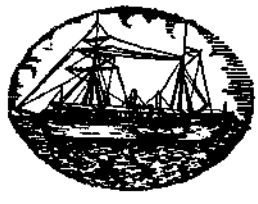
SYMPOSIUM ON MOLLUSCA

HELD AT

COCHIN

FROM JANUARY 12 TO 16, 1968

PART II



SYMPOSIUM SERIES 3

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PART II

ON THE PROBABLE CHANGE OF FORM IN THE CLAM *MERETRIX CASTA* (CHEMNITZ) DURING ITS GROWTH[1]

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ABSTRACT

While examining large samples of the clam *Meretrix casta* of different sizes from Athankarai estuary near Mandapam for its biological studies, it was noticed that the form of the clam was not constant in different size-groups. The smallest clams were more or less regular and equilateral in form while the largest had their posterior regions slightly produced thereby giving an impression of inequilaterality.

To verify this, 40 clams from each of the three size-groups were opened and five selected external and internal dimensions of the right valves of their shells measured. The regressions of each of these characters with respect to length were compared for the three size-groups by the method of analysis of covariance.

The results tend to indicate that the clam grows rapidly at its posterior region than at the anterior region and there is no equality of growth rate in the three size-groups of the clam. These suggest a change of form during growth in the case of the clam *M. casta* inhabiting Athankarai estuary. This may perhaps be due to the younger clams preferring the soft soil while the older ones generally inhabiting a hard soil.

INTRODUCTION

THE change of form due to the different environmental conditions and habits has long been known in the animal world. The relative growth of different parts of the body changes, thereby bringing about the change in form. Huxley (1932) has contributed greatly to the study of such differential growth processes. He recognised heterogonic and isogonic growth depending upon whether the rate of growth of a body part is different from or similar to that of the body.

A study on the change in form in the case of molluscs has been attempted by Hamai (1934) who found local variations in the shell of *Meretrix meretrix*. Those, he felt, were the effects of environmental conditions such as temperature, physical nature of sand, and salinity. In a later publication, Hamai (1935) observed the change of shape to be related to the substratum. This he confirmed by actual transplantation experiments (Hamai, 1938). Durve and Dharma Raja (1965) investigated the differences in the dimensional relationship in the clam *M. casta* collected from the marine fish farm and Athankarai estuary near Mandapam.

The change in form during ontogeny associated with the change in life habits has been worked out by Kristensen (1957) who showed that the burrowing ability is critical in juveniles of *Cardium edule* L., and that it decreases with increasing size. This is accompanied by a change in form from juvenile to adult cockles. Pohlo (1964) observed the same phenomenon in the case of the bivalve *Tresus nuttalli*.

The present study deals with such a change of form in the case of *Meretrix casta* from small to large size. The phenomenon of the change of form in this bivalve was first noticed when large samples of this clam belonging to different size-groups were being collected from Athankarai estuary near Mandapam, for biological studies.

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[1] Published with the permission of the Director, Central Marine Fisheries Research Institute, Mandapam Camp.

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Material and Methods

Clams of different sizes were collected from Athankarai estuary from stations 2, 3, and 4 (refer Durve and Alagarswami, 1964). The smallest clams occur in station 2 and the size increases as one proceeds to station 4 where the largest clams occur. The clams at any station belong to more or less the same size-group. The size-groups collected were 16·0–18·3, 27·0–30·3, and 38·0–43·6 mm. in length (or width) and were designated as smallest, larger and largest groups respectively. The clams were cleaned, opened, and the meat scooped out. The empty shells with both the valves attached together were allowed to dry for some time and a few selected measurements were taken of the right valve alone, with vernier callipers and a fine mathematical divider correct to 0·1 mm. The dimensions measured were as follows (*see* also Fig. 1):

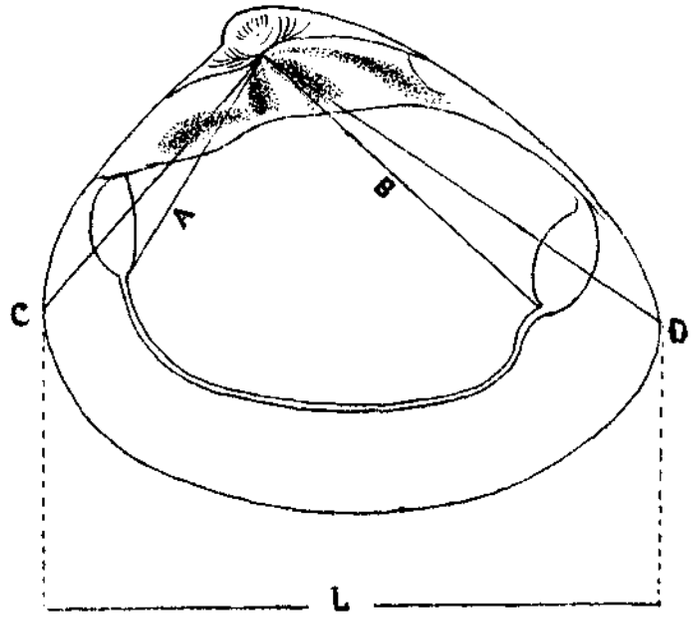

Fig. 1. The right valve of *Meretrix casta* showing dimensions used for the study. (For explanation, please see the text)

L : The maximum linear dimension along the antero-posterior axis of the shell.

A : The linear distance between the umbo and the origin of the pallial line at the base of the anterior adductor impression.

B : The linear distance between the umbo and the point of merging of the pallial line with the posterior adductor impression.

C : The maximum distance from the umbo to the anterior edge.

D : The maximum distance from the umbo to the posterior edge.

It was felt that these dimensions will alone give some idea about the change of form, if any, in the clam under investigation. The measurements A, B, C and D were independently plotted against the measurement L for each size-group on separate bivariate scatter diagrams. A straight line appeared to be the best fit for each scatter diagram of the three groups. For plotting the straight line, the regression equation of the form $y = a + bx$ where y represents the length L and x the four dimensions A, B, C and D, was employed. The regressions of each of these four dimensions with regard to length were compared for the three size-groups by the method of analysis of covariance with the significance of all tests judged at 5% level of probability.

Results and Discussion

The regression relations of the four dimensions A, B, C and D to the length L for the three size-groups are:

Dimension A

Smallest : A = 2·8245 + 0·2885 L
Larger : A = 6·4443 + 0·2099 L
Largest : A = 9·3996 + 0·1787 L

Dimension B

Smallest : B = 3·9654 + 0·3176 L
Larger : B = 2·6587 + 0·5068 L
Largest : B = 8·2296 + 0·4197 L

Dimension C

Smallest : C = 2·0122 + 0·5372 L
Larger : C = 2·4668 + 0·5052 L
Largest : C = 11·0360 + 0·2811 L

Dimension D

Smallest : D = 5·2084 + 0·4557 L
Larger : D = 3·8241 + 0·6693 L
Largest : D = 9·0533 + 0·6273 L

The regression lines are plotted in Fig. 2.

Comparisons were made by the analysis of covariance to test the significant difference of the regressions for the three size-groups. *Appendix I* (*a* to *d*) and *Appendix II* (*a* to *d*) give the statistical calculations of Sums of Squares and Products, Mean Squares, and the test of analysis of covariance. The Table showing F values based on the results given in Appendices I and II is given below.

Table showing F values

1 Dimension		2 Between S_3 and $(S_2+S_3+S_4)$	3 Between S_1 and S_2	4 Between (S_1+S_2) and S_3
1.	A	$\frac{1\cdot7893}{0\cdot3191} = 5\cdot61$‡ (4,114)	$\frac{0\cdot3191}{0\cdot08825} = 3\cdot62$ (114,2)	$\frac{3\cdot3485}{0\cdot3151} = 10\cdot63$‡ (1,116)
2.	B	$\frac{1\cdot9362}{0\cdot3313} = 5\cdot84$‡ (4,114)	$\frac{0\cdot3313}{0\cdot19185} = 1\cdot73$ (114,2)	$\frac{0\cdot3289}{0\cdot0096} = 34\cdot26$ (116,1)
3.	C	$\frac{1\cdot0756}{0\cdot3977} = 2\cdot70$* (4,114)	$\frac{0\cdot7883}{0\cdot3977} = 1\cdot98$ (2,114)	$\frac{1\cdot2988}{0\cdot4044} = 3\cdot21$ (1,116)
4.	D	$\frac{4\cdot9928}{0\cdot3880} = 12\cdot69$‡ (4,114)	$\frac{0\cdot3880}{0\cdot27045} = 1\cdot43$ (114,2)	$\frac{8\cdot3662}{0\cdot3860} = 21\cdot67$‡ (1,116)

* indicates significance at 5% probability level.
‡ indicates significance at 1% probability level.
Figures within brackets indicate the respective degrees of freedom.

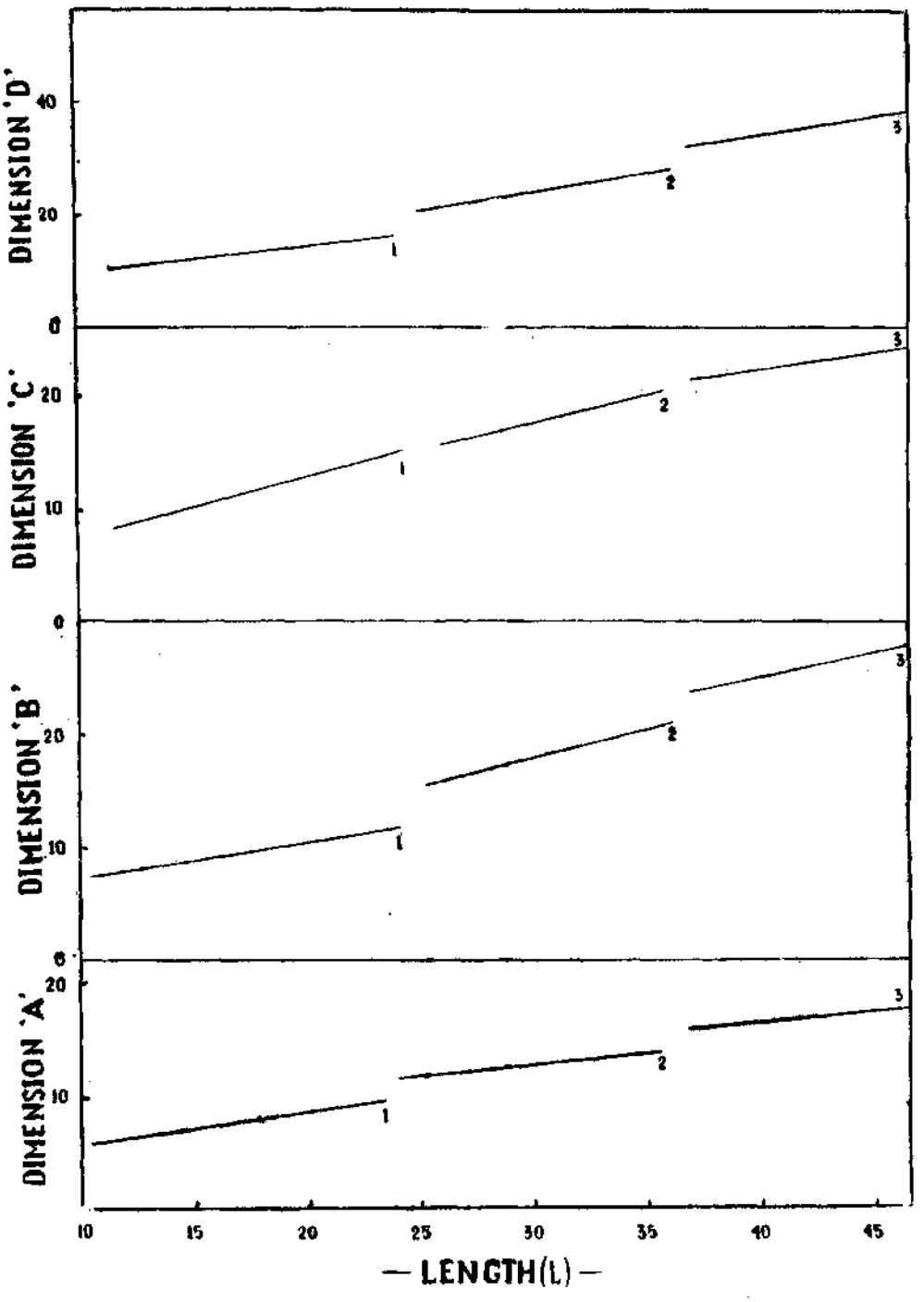

FIG. 2. Regressions of different dimensions on length in the three size-groups of *Meretrix casta*. (Size-groups: 1—smallest, 2—larger, 3—largest)

Column 2 in the previous table gives observed F values and all the values are found to be significant at 5% probability level. Further, excepting for dimension C, the F values are significant even at 1% probability level. This indicates that there are real differences between groups and a single line will not represent all the three groups (Fig. 2).

Column 3 in the previous table gives the observed F values for comparing the deviations from within-group regressions with the differences of regresssions among the groups. All the F values are found to be non-significant at 5% level and hence the hypothesis of equality of regressions within groups cannot be rejected. The F values corresponding to comparison of deviations from average within-group regressions with those from regression through group means are given in column 4. The F values are significant for the dimensions A and D, but non-significant for the dimensions B and C. This means that while for the dimensions A and D, the hypothesis of linearity of regressions of group means should be rejected; the same cannot be rejected for the dimensions B and C.

Thus for dimensions A and D, heterogeneity is suspected among the 3 groups, *i.e.*, the regressions of these dimensions are different among the 3 groups. In the case of dimensions B and C, the adjusted mean values for the three groups are different and this is to be expected since the size ranges of the 3 groups are different but the hypothesis of equality of regressions within the 3 groups cannot be rejected. From a comparison of *b* values in A and D dimensions, it is found that for A dimension, *b* decreases from the smallest to the largest group, while for the dimension D, *b* increases from the smallest to largest groups, and as for the other dimensions, no statistically significant from the noticed.

The above observations, especially the significant differences between the regressions of dimensions A and D discussed above, indicate that the clam *M. casta* grows more rapidly at its posterior region than at the anterior one during its growth span. This results in the change of form in this clam from its small to large size, the posterior end becoming more wedge-shaped and the anterior remaining roundish. In short, the clam which is almost equilateral during its early life becomes more or less inequilateral as it grows. However, it is necessary to confirm this by more detail study. It is further felt that this change of form may not be continuous. It stops after a certain stage as no large *M. casta* of abnormally elongated or different form has so far been observed in the locality. There is a possibility, as Hamai (1938) has observed, that the change of form depends upon the adaptability in each case to the respective environment. Thus, once the form best suited for the environment is attained, there is no further change in it. However, this hypothesis needs confirmation.

The studies made on the density of clams of different sizes in relation to substratum reveal that small-sized clams invariably prefer a soft soil with more water content, finer grade particles and silt; while the larger clams were found to be more in the areas where the soil contained all the above ingradients in lesser quantity (Durve, unpublished). This may perhaps be the reason for the change of form in the clam *M. casta* as it grows. It is likely that burrowing and reorientation after disturbance is easier in soft soil than in the one containing less of water and finer grade particles with the result, active smaller clams need not have sharper and more pointed posterior ends. These features, perhaps, are necessary for the larger, more or less stationary clams, in order to help them in anchorage and keep their siphonal regions above the hard soil of coarse particles. This, however, needs verification. Hamai (1935) has observed, as stated earlier, that the elongated type of *M. meretrix* grows on a sandy shore facing the open sea and the roundish type in a calm sea with a slightly muddy bottom.

ACKNOWLEDGEMENTS

Authors record with pleasure their thanks to Dr. S. Jones, Director, Central Marine Fisheries Research Institute, Mandapam Camp, for his interest in this work. They are also grateful to

Dr. R. Raghu Prasad and Shri S. K. Banerji for going through the manuscript critically and suggesting improvements.

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* Not referred to in original.

Discussion

Dr. A. D. Ansell: Do the animals on different substrates while in migration from one type of environment to another involve in change of form ?

Dr. V. S. Durve: Here we have to distinguish between two types of migrations. First, a temporary migration and second a permanent migration involving a change in the mode of life. The possibility of the change of form is only in the animals involved in the second type of migration. Such a change of form is evident in certain pelagic crabs which migrate from shallow to deeper waters and also in the bivalves *Cardium edule* and *Tresus nuttalli* which change their substrata and thereby the mode of life during early period of their life. Hamai has confirmed this change of form due to change in the substratum by actual transplantation experiments in the case of *Meretrix meretrix*.

Further, I feel, the change in form need not necessarily be due to the migrations alone, but may even be caused by the change in the substratum itself by several natural causes such as shifting sands or mud banks, currents, cyclones, etc., However, this needs confirmation.

(Answer subsequently obtained.)

APPENDIX I (*a*)

Statistics describing sums of squares and products for the dimension A in relation to length L

Size-group	D.F.	Sx^2	Sxy	Sy^2	b	D.F.	S.S.	
Within smallest group	39	17·8875	5·1602	5·7698	0·2885	38	4·2812	36·3787 (S_1)
Within bigger group	39	26·2190	5·5045	12·2198	0·2099	38	11·0642	
Within biggest group	39	75·5110	13·4920	23·4440	0·1787	38	21·0333	
Within groups	117	119·6175	24·1567	41·4336	0·2019	116	36·5552	(S_1+S_2)
Between groups	2	11243·7517	4240·4453	1602·5811	0·3771	1	3·3485	(S_2)
TOTAL	119	11363·3692	4264·6020	1644·0147	0·3753	118	43·5360	(S_3)

S.S.—Sum of Squares $Sy^2 - (Sxy)^2/Sx^2$. D.F.—Degrees of Freedom.

APPENDIX I (*b*)

Statistics describing sums of squares and products for the dimension B in relation to length L

Size-group	D.F.	Sx^2	Sxy	Sy^2	b	D.F.	S.S.	
Within smallest group	39	17·8875	5·6818	8·5378	0·3176	38	6·7330	37·7735 (S_1)
Within bigger group	39	26·2190	13·2890	15·1590	0·5068	38	8·4235	
Within biggest group	39	75·5110	31·6930	35·9190	0·4197	38	22·6170	
Within groups	117	119·6175	50·6638	59·6158	0·4235	116	38·1572	($S_1 + S_2$)
Between groups	2	11243·7517	7563·4687	5087·8201	0·6727	1	0·0096	(S_2)
TOTAL	119	11363·3692	7614·1325	5147·4359	0·6701	118	45·5181	(S_3)

S.S.—Sum of Squares $Sy^2 - (Sxy)^2/Sx^2$. D.F.—Degrees of Freedom.

APPENDIX I (*c*)

Statistics describing sums of squares and products for the dimension C in relation to length L

Size-group	D.F.	Sx^2	Sxy	Sy^2	b	D.F.	S.S.	
Within smallest group	39	17·8875	9·6092	12·2478	0·5372	38	7·0857	45·3356 (S_1)
Within bigger group	39	26·2190	13·2465	16·1878	0·5052	38	9·4953	
Within biggest group	39	75·5110	21·2235	34·7198	0·2811	38	28·7546	
Within groups	117	119·6175	44·0792	63·1554	0·3685	116	46·9122	($S_1 + S_2$)
Between groups	2	11243·7517	5376·2956	2572·0245	0·4782	1	1·2988	(S_2)
TOTAL	119	11363·3692	5420·3748	2635·1799	0·4770	118	49·6382	(S_3)

S.S.—Sum of Squares $Sy^2 - (Sxy)^2/Sx^2$ D.F.—Degrees of Freedom.

APPENDIX I (*d*)

Statistics describing sums of squares and products for the dimension D in relation to length L

Size-group	D.F.	Sx^2	Sxy	Sy^2	b	D.F.	S.S.	
Within smallest group ..	39	17·8875	8·1505	10·4390	0·4557	38	6·7252	44·2332 (S_1)
Within bigger group ..	39	26·2190	17·5495	22·6698	0·6693	38	10·9232	
Within biggest group ..	39	75·5110	47·3650	56·2950	0·6273	38	26·5848	
Within groups ..	117	119·6175	73·0650	89·4038	0·6108	116	44·7741	($S_1 + S_2$)
Between groups ..	2	11243·7517	10263·3248	9376·7461	0·9128	1	8·3662	(S_3)
TOTAL ..	119	11363·3692	10336·3898	9466·1499	0·9096	118	63·9245	(S_5)

S.S.—Sum of Squares $Sy^2 - (Sxy)^2/Sx^2$. D.F.—Degrees of Freedom.

APPENDIX II (*a*)

Analysis of Covariance—Dimension A

Source of variation	D.F.	S.S.	M.S.
Deviation from individual within group regressions ..	114	36·3787 (S_1)	0·3191
Difference between regressions	2	0·1765 (S_2)	0·08825
Deviation from average within group regressions (b_a) ..	116	36·5552 (S_1+S_2)	0·3151
Deviation from regression through group means (b_m)	1	3·3485 (S_3)	3·3485
Deviation between b_a and b_m	1	3·6323 (S_4)	3·6323
Deviation from common (Total) regression (b_o)	118	43·5360 (S_5)	

D.F. = Degrees of Freedom. S.S. = Sum of Squares. M.S. = Mean Square.

APPENDIX II (*b*)

Analysis of Covariance—Dimension B

Source of variation	D.F.	S.S.	M.S.
Deviation from individual within group regressions	114	37·7735 (S_1)	0·3313
Difference between regressions	2	0·3837 (S_2)	0·19185
Deviation from average within group regressions (b_a) ..	116	38·1572 (S_1+S_2)	0·3289
Deviation from regression through group means (b_m)	1	0·0096 (S_3)	0·0096
Deviation between b_a and b_m	1	7·3513 (S_4)	7·3513
Deviation from common (Total) regression (b_o)	118	45·5181 (S_5)	

D.F. = Degrees of Freedom. S.S. = Sum of Squares. M.S. = Mean Square.

APPENDIX II (*c*)

Analysis of Covariance—Dimension C

Source of variation	D.F.	S.S.	M.S.
Deviation from individual within group regressions	114	45·3356 (S_1)	0·3977
Difference between regressions	2	1·5766 (S_2)	0·7883
Deviation from average within group regression (b_a)	116	46·9122 (S_1+S_2)	0·4044
Deviation from regression through group means (b_m)	1	1·2988 (S_3)	1·2988
Deviation between b_a and b_m	1	1·4272 (S_4)	1·4272
Deviation from common (Total) regression (b_t)	118	49·6382 (S_5)	

D.F. = Degrees of Freedom. S.S. = Sum of Squares. M.S. = Mean Square.

APPENDIX II (*d*)

Analysis of Covariance—Dimension D

Source of variation	D.F.	S.S.	M.S.
Deviation from individual within group regressions	114	44·2332 (S_1)	0·3880
Difference between regressions	2	0·5409 (S_2)	0·27045
Deviation from average within group regressions (b_a) ..	116	44·7741 (S_1+S_2)	0·3860
Deviation from regression through group means (b_m) ..	1	8·3662 (S_3)	8·3662
Deviation between b_a and b_m	1	10·7842 (S_4)	10·7842
Deviation from common (Total) regression (b_t)	118	63·9245 (S_5)	

D.F. = Degrees of Freedom. S.S. = Sum of Squares. M.S. = Mean Square.

AN EXPERIMENT WITH OVERCROWDED COCKLES (*CARDIUM EDULE*)

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ABSTRACT

The occurrence during 1963 of a very heavy and widespread spatfall of the cockle (*Cardium edule*) on intertidal sand flats in South Wales was used to study the effect on growth and subsequent spatfall of experimental local reduction in their density. It was found that at the higher densities the mean size and density of cockles were inversely correlated as were also the numbers of spat and the numbers of older cockles per unit area of bottom.

Thinning out accompanied by transplantation of overcrowded cockles resulted in a greatly increased yield of larger cockles.

EARLIER experiments by the author on high-density patches of cockles have shown that competition may occur, leading to reduction in shell growth and reduction in spat numbers. During 1963, following the previous severe winter when most cockles died on the Llanrhidian Sands, South Wales (Hancock and Urquhart, 1964), there was a heavy and widespread settlement of spat (Hancock and Urquhart, 1966). The resulting high densities provided an opportunity to investigate more fully the relationships between overcrowded cockles, as well as the changes in yield produced by thinning them out. The experiment described here is just one of a series conducted during 1964-65, all of which will be presented more fully at a later date.

METHOD

In August, 1964, a twelve-metre square plot was fenced off as in Fig. 1. A metre-wide border was left between the fencing stakes and the sampling area, to reduce any disturbance due to water movement round the stakes. The area was divided into two parts, and five 1/20 m^2 samples were taken in each half (shown by open circles in Fig. 1). The western half (A) was marked into five strips measuring 10 metres $\times$ 1 metre, and the surface material containing cockles was removed from three of them with a spade to a depth of a few centimetres (Fig. 1 and Plate I). The material removed was transplanted to a marked area (C) which was previously barren of cockles, and spread over its surface (Plate II); later these cockles also were examined for growth. On the following day, after coverage by two high tides, the depressions from which cockles had been removed were still visible (Plate III), and ten samples were taken from the five strips, as indicated by the triangles in Fig. 1. The treated western half of the area was sampled again in October, 1964, and in July and November, 1965, *i.e.*, during and at the end of each of two growing seasons, while the untreated eastern half was sampled more frequently for mortality observations. The area of each sample taken in 1964 was 1/20 m^2, but with decreasing numbers the area was increased to 1/10 m^2 during 1965. In order to rule out the chance of a sample falling on a position previously sampled, lines of samples were positioned differently on each occasion (Fig. 1). Shell length measurements were made to the nearest millimetre below, using vernier calipers, and the total volume of each sample was measured in a litre cylinder.

RESULTS

(*a*) *Numbers*

Table I shows the individual sample results (all expressed per 1/10 m^2) and it can be seen that although the numbers in the cleared strips increased only slightly in the first two months after treat-

ment, there was a marked increase by the following July. A graph of logarithms of numbers against time (Fig. 2), shows that the rate of fall of total numbers per m^2 in the treated western half as a whole was very similar to the rate of loss in the untreated area. This suggests that most of the increase in the cleared strips was derived from cockles washed in from neighbouring untreated strips, rather than from outside the sampling area. It would be useful to know whether crowded areas have a higher than normal natural mortality, but any difference in rate of loss between the thinned-out area and the untreated area could not be properly assessed because of the continuous infiltration of cockles into the cleared areas.

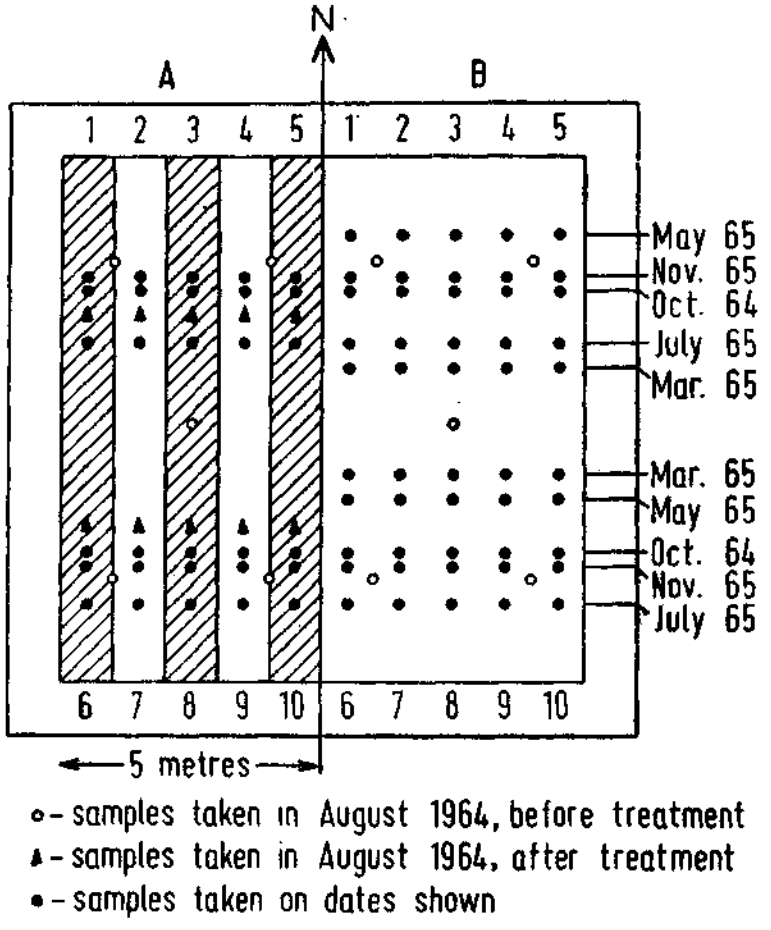

Fig. 1. Plan of experimental area, showing Plots A and B, and the cleared areas (shown shaded). The positions of samples taken on various dates are shown.

(b) Mean size

In October, 1964, July, 1965, and November, 1965, length measurements were made on cockles in most of the samples; the relationships between these lengths and density are shown in Figs. 3 *a* and 3 *b*, but it should be noted that for October, 1964, only the mean values are given. By October, 1964, only small differences were noted between samples from the treated and untreated areas (Fig. 3 *a*), but already shells from the untreated area were showing the "bent" margins

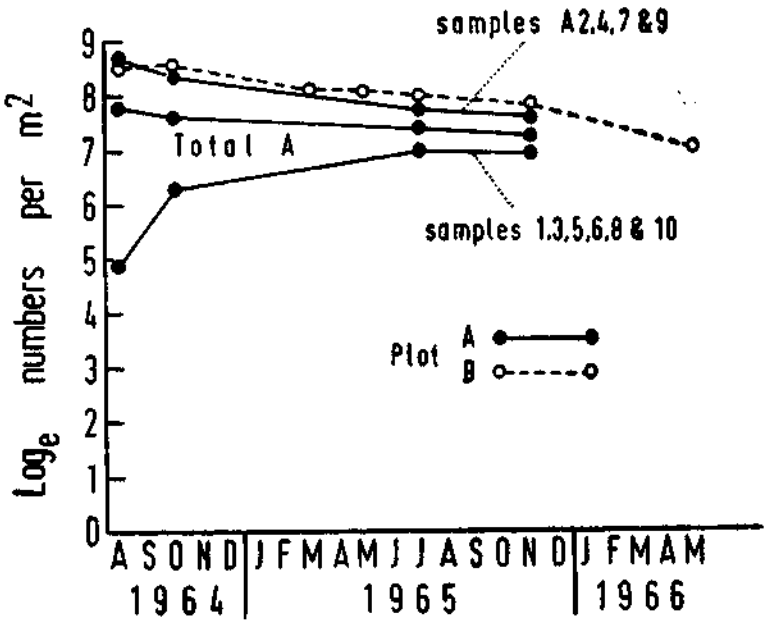

FIG. 2. Natural logarithms of numbers of cockles to compare changes in density in treated (A) and untreated (B) plots.

which are typical signs of overcrowding (Plate IV). Deformation of shells of cockles in overcrowded conditions was also previously described by Kreger (1940). In July, 1965, there was a slightly more pronounced inverse relationship between mean length and density, and by November, 1965, this had become much more marked at higher densities. On Fig. 3 is also given a theoretical "maximum density curve" expressing the relationship between density and size of cockles found experimentally to completely fill a surface, and it can be seen that the higher densities in July and November, 1965, come quite close to this line. The slight slope of the relationship between mean length and lower densities suggests competition for food. The increased slope at higher densities, at which the shells show bending as a result of pressure from overcrowding, appears to result from competition for both food and space. This result was repeated even more convincingly in other experiments to be reported at a later date. The fact that closely situated samples within a plot can have a growth rate varying with the density clearly has an important bearing on sampling procedure.

(*c*) *Transplanted cockles*

In Fig. 3 *a* is also related the mean size and density in samples taken in July and November 1965, from cockles transplanted to area C from the experimental area A in August, 1964. There had been a spectacular increase in growth rate compared with cockles from the original plots, both treated (A) and untreated (B). Possible reasons for this may be that: (1) in the transplant plot the cockles were at lower average densities; (2) the transplant plot was surrounded by fairly barren ground and not overcrowded by cockles; (3) it was naturally a better area for growth. The relationship between mean size and density from these samples did not show any obvious slope, but no very high densities were represented. It must be remembered also that mean size estimates from samples of a density less than 1,000 per m^2 are subject to greater sampling error, because there will be fewer than 100 per 1/10 m^2 sample. That mean size and density are in fact correlated also in areas surrounded by barren ground was shown by samples taken in August and October, 1964, and July,

1965, from a plot D which was adjacent to plot C. These cockles had been transplanted from another overcrowded area in May, 1964, and the inverse correlation between mean size and density can again be clearly seen in Fig. 3 *b*.

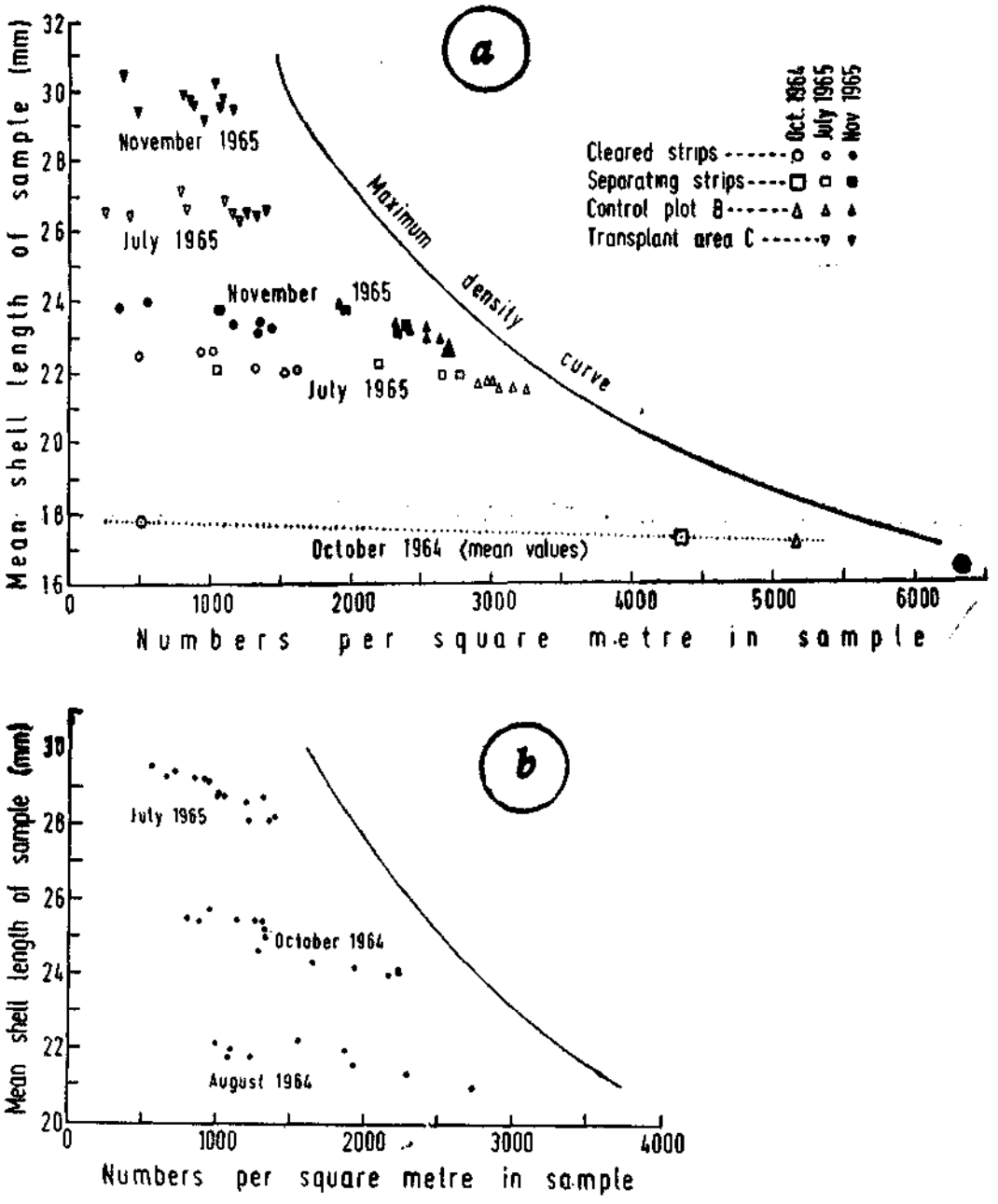

FIG. 3. Relationship between mean size and density of cockles: (*a*) From the treated Plot A (cleared strips and separating strips), the control Plot B, and the transplant area C. (*b*) From the transplant area D.

In Figure 4, the mean size of cockles from the untreated plot B in 1964 (measured in October, which is very near the end of the growing season) is compared with the mean sizes and densities of second-year cockles from the same area in previous years. Once again faster growth is seen to be associated with lower densities.

(d) Spat settlement

During 1964 there was virtually no spat settlement, but in 1965 the spatfall on the experimental area was good. In Fig. 5 the numbers of spat present in November have been plotted against

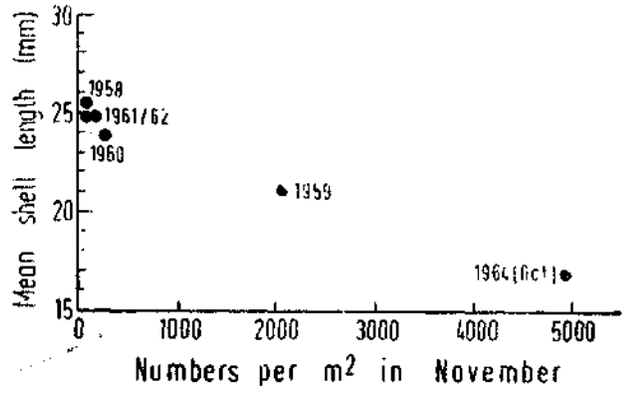

FIG. 4. Relationship between mean shell length and numbers of second-year cockles in November of each year.

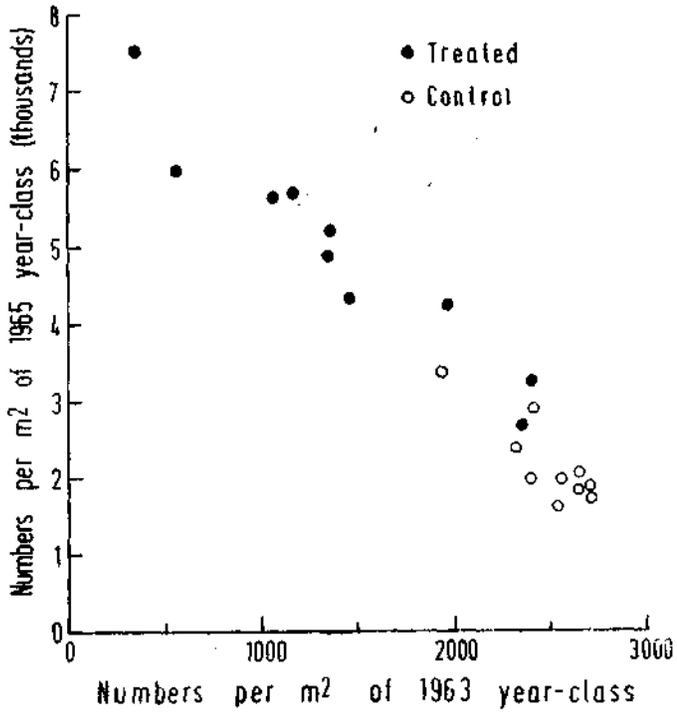

FIG. 5. Relationship between numbers of the 1965 year-class of cockles in samples from treated (*a*) and control (*b*) plots in November 1965.

the numbers of older cockles present in the same samples, and there was a marked inverse correlation. It was noticeable that the loss of spat during the following winter (Table I) was much higher than usual, but it is not known whether this was in any way associated with the high density of older cockles. From measurements of spat there was a suggestion (to be investigated further) that, in contrast to the situation in older cockles, spat size decreased slightly with decreasing density of spat, but this in fact was also associated with higher density of older cockles (Table I).

TABLE I

Numbers of cockles from the 1963 *and* 1965 *year-classes in samples from treated plot (A) and its control plot (B)*
[Samples A 1, 3, 5, 6, 8 and 10 were from cleared strips, and A 2, 4, 7 and 9 from areas between them (*see* Fig. 1). All samples are expressed per 1/10 m²]

Samples	Numbers of 1963 year-class								1965 year-class	
	August 1964		October 1964	March 1965	May 1965	July 1965	November 1965	May 1966	November 1965	May 1966
	Before treatment	After treatment								
A 1	588*	6	50	NS	NS	49	35	NS	759	NS
3	518	28	46	,,	,,	103	117	,,	569	,,
5	538	8	32	,,	,,	133	146	,,	434	,,
6	578	8	40	,,	,,	96	56	,,	603	,,
8	358	30	72	,,	,,	154	137	,,	522	,,
10	NS	2	68	,,	,,	163	137	,,	488	,,
Mean per m²		137	513			1,163	1,047		5,625	
A 2	NS	572	420	NS	NS	221	198	NS	423	NS
4	,,	550	444	,,	,,	277	241	,,	324	,,
7	,,	596	418	,,	,,	105	107	,,	566	,,
9	,,	572	452	,,	,,	265	235	,,	271	,,
Mean per m²		5,725	4,335			2,170	1,953		3,960	
Mean A 1–10	5,160	2,372	2,042			1,566	1,409		4,959	
B 1	536*	NS	484	445	332	309	240	155	198	6
2	480	,,	482	403	343	325	242	101	289	75
3	544	,,	432	260	305	314	254	95	162	16
4	398	,,	516	332	304	298	270	153	186	3
5	470	,,	504	236	357	291	265	105	206	4
6	NS	,,	478	417	314	298	194	126	340	3
7	,,	,,	510	396	333	318	252	106	240	3
8	,,	,,	516	301	314	317	266	101	179	9
9	,,	,,	504	278	277	298	271	101	173	9
10	,,	,,	448	(77)	(135)	277	256	103	197	5
Mean per m²	4,856		4,874	3,409†	3,199†	3,045	2,490	1,156	2,170	133

* Five samples in plots A and B before treatment were not related to subsequent sampling pattern.

† Excluding B 10 which may have been affected by turbulence around a fencing post (figures in parentheses).
NS Not sampled.

(e) Yield

Changes in yield have been represented in terms of whole volume per m², and the results have been summarized for November, 1965, in Table II:

TABLE II

Plot	Number of cockles per m²	Mean length (mm.)	Whole volume (ml.)	Volume per cockle (ml.)	Number of spat per m²
		(mm.)			
Untreated (B) ..	2,490	22·9	15,945	6·4	2,170
Treated (A) ..	1,409	23·4	9,798	7·0	4,959
Transplanted (C) ..	872	29·7	13,176	15·1	7
TOTAL (A+C)	2,281		22,974	10·1	

Actual differences in mean size and volume per cockle between the treated and untreated plots were small (compared with those obtained from other methods used for thinning out cockles, to be described at a later date), and this may have resulted from slowness of spreading out of high and low densities within the treated plot. However, when transplanted cockles were also taken into consideration, although the total number of survivors in the treated and transplanted plots (A + C) was slightly less than in the untreated plot (B) the yield showed an increase of 58 per cent. Another benefit to be considered is the higher proportion of cockles in plots A and C which would reach the legal minimum size of 23 mm. earlier. Those cockles which fail to reach the minimum size before the onset of winter are subject to natural losses throughout 6-7 winter months before growth recommences and they can legally be fished.

SUMMARY AND CONCLUSIONS

1. At high densities mean size and density of cockles are inversely correlated.
2. By thinning out of overcrowded cockles the mean size can be increased.
3. Numbers of spat and numbers of older cockles are inversely correlated.
4. Thinning out accompanied by transplanting of overcrowded cockles resulted in a greatly increased yield of larger cockles.
5. Speculation about the biological mechanisms responsible for the relationships described will be deferred until a later date, when a full account of the whole series of experiments to investigate this topic will be presented.

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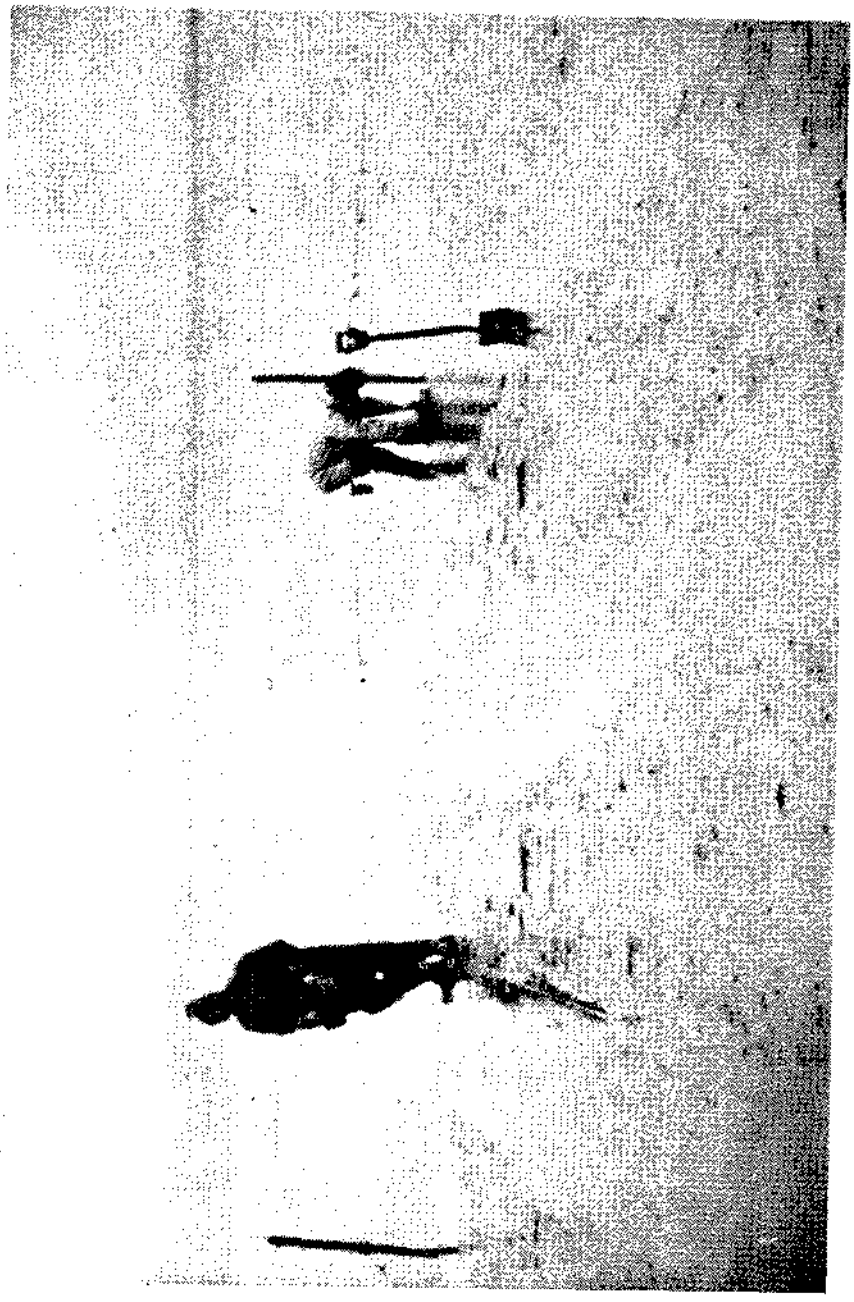
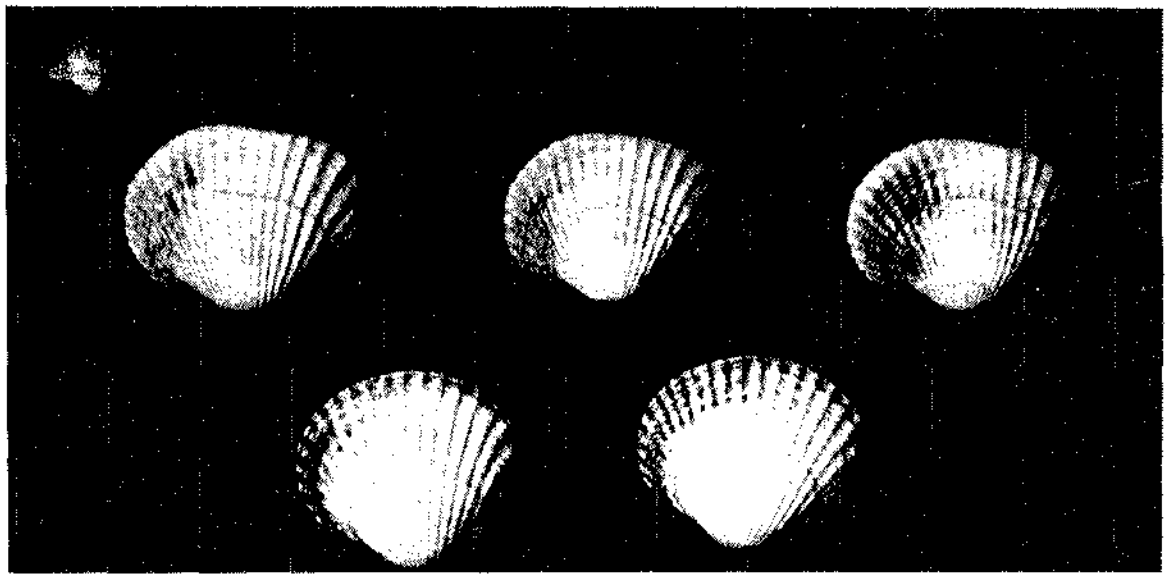
Removing the surface material containing cockles, in metre wide strips with a spade.

The cockles mixed with sand were spread with a rake over an area previously burnt of cockles



Sampling treated and untreated strips after two tides.



Second-year cockles showing "bending" of the valve margin (top row) compared with those which had grown normally.

STUDIES ON THE RATE OF FEEDING OF ESTUARINE BIVALVE *ARCA GRANOSA* (LINNE)

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ABSTRACT

Arca granosa lives in continually varying environmental conditions. The study of the response and adjustment of the animal to some of the more important variables of the environment has been in progress. The present paper describes the variations in filtering and feeding rates of the animal in relation to variations in salinity, temperature and pH. Observations were made on the feeding rates of the animals maintained in media of different salinities, temperature and pH. The salinity employed in the experiments varied from 10‰ to 28‰, the temperature from 20° to 32° C. and pH from 6·5 to 8·4. Maximum feeding rate was observed with media of salinity of 28‰, temperature of 30° C. and pH of 8·2. Minimum feeding rate was noticed with media of salinity 10‰, temperature of 20° C. and pH of 6·5.

INTRODUCTION

Arca granosa lives in estuaries and brackish water lagoons, where many of the environmental factors are continually varying. It may be expected that several functions in the animal, such as ciliary activity of the gills, gape of the shell, and rate of pumping water, will vary in accordance with these environmental conditions. The amount of food available to a suspension feeding bivalve is determined by the rate of transport of the water through the gills. A number of environmental factors are known to influence the flow rate such as salinity, temperature, and composition of the surrounding water. These have been investigated in a few bivalves such as *Crassostrea virginica* and the clam *Meretrix casta*. Hopkins (1936) studied the adaptation of the feeding mechanism of an oyster in relation to different salinities. Loosanoff and Tommers (1947) studied the effect of pH upon the rate of pumping of oysters. Durve (1963) studied the rate of filtration of the Clam *Meretrix casta* in relation to size and salinity. Different species of bivalves may be expected to differ in their sensitivity to these external factors. The present study relates to *Arca granosa* which shows a variable distribution in the estuarine waters at Porto Novo. With a view to finding out the factors governing their distribution in the estuary a preliminary study has been made of the effect of salinity, temperature, and pH on the rate of feeding.

MATERIAL AND METHODS

Specimens of uniform size, measuring 22·4 mm. in length, were used for experimental work in the present study. They were maintained in active condition in the laboratory. Each specimen was placed in a finger bowl which was then immersed in a trough containing sea-water. At the beginning of the experiment the water in the trough was adjusted so that the upper edge of the bowl was exposed. The water in the finger bowl was completely replaced by a dilute solution of freshly prepared colloidal graphite. At intervals of about fifteen minutes, five ml. of the solution was pipetted out from the finger bowl and the rate of removal of graphite particles as measured by turbidity was read in Klett Summerson colorimeter using red filter. The rate of feeding was computed from the formula

$$pt = po \text{ exp. } \frac{m}{Mt}$$

Where *M* is the quantity of water used for the experiment, *m* the rate of filtration, *po* initial, and *pt* final concentration, and *t* time.

The feeding rate was determined under three different sets of conditions:—(*a*) In five different salinities, 10 ppm, 15 ppm, 20 ppm, 25 ppm, and 28 ppm, at a temperature of 30° C. and pH 8·2, (*b*) at four different temperatures, *viz.*, 20° C., 25° C., 30° C., and 32° C., keeping salinity constant at 28 ppm and pH 8·2, and (*c*) in media with four different pH values, 6·5, 7·5, 8·2, and 8·4, keeping temperature constant at 30° C. and salinity at 28 ppm.

Observations and Results

Effect of salinity.—The results are represented graphically (Fig. 1, A, B and C). It will be seen that feeding rate is directly related to salinity, that is, it increases with salinity. This increase is not, however, at a uniform rate, as can be discerned by the slope of the line relating salinity and feeding rate. The feeding rates in the different salinities investigated were:

Salinity ppm	..	10	15	20	25	28
Feeding rate (ml./15 min./ animal) mean	..	10·2	14·8	29·9	37·1	41·7
Range	..	(9·8–11·3)	(14·2–15·8)	(28·2–30·7)	(35·2–38·2)	(40·2–43·4)

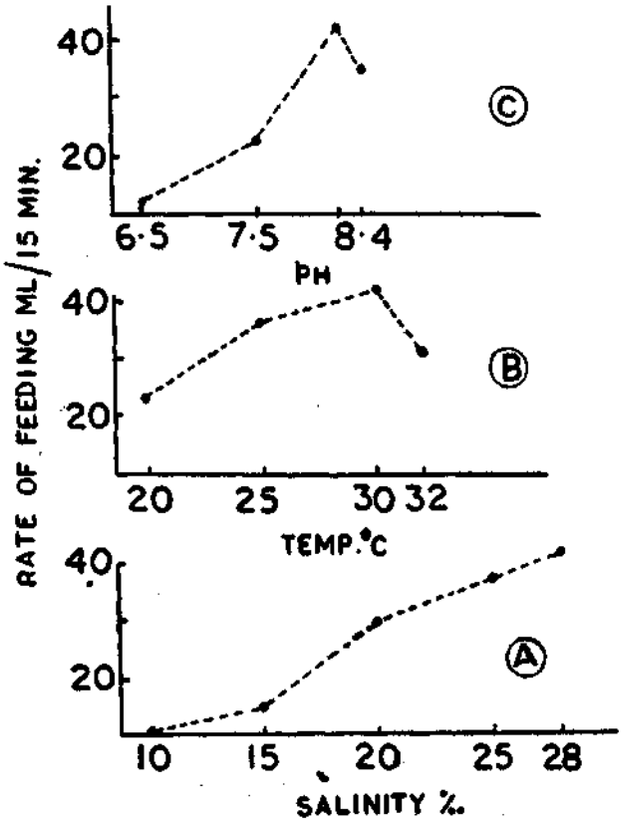

Fig. 1. Effect of salinity, temperature and pH on the rate of feeding *Area granosa*.

Effect of Temperature.—From 20 to 30° C. temperature, the increase in feeding rate is related to increase in temperature, but above 30° C. the feeding rate declines. The feeding rates in different temperatures were:—

Temperature °C.	..	20·0	25·0	30·0	32·0
Feeding rate (ml./15 min./animal) mean	..	23·2	36·2	41·7	31·2
Range	..	(22·1–24·3)	(34·2–37·8)	(40·2–43·4)	(28·6–34·2)

Effect of pH.—From pH 6·5 to 8·2 the feeding rate is directly related to pH, but from 8·2 to 8·4 it is inversely related. The feeding rates in the media of different pH investigated were:

pH	..	6·5	7·5	8·2	8·4
Feeding rate (ml./15 min./animal) mean	..	12·2	22·4	41·7	34·7
Range	..	(4·2–26·3)	(8·1–46·2)	(40·2–43·4)	(32·8·37·2)

Discussion

Hopkins (1936) observed normal pumping rates at salinities ranging from 25 to 39 ppm. But effective pumping was not observed below 13 ppm. Durve (1963) studied the rate of filtration in *Meretrix casta* in different salinities. He found that the rate of filtration decreases in extreme low and high salinities, but the clams can adapt themselves for a wide range of salinities. In *Arca granosa*, as we have seen, only 24·5% of the maximum feeding rate is observed at the salinity of 10 ppm. The increase in feeding rate in higher salinities is not uniform. From salinity 10 ppm to 15 ppm the increase in feeding rate is only 11·0% while from 15 ppm to 20 ppm it is 36·3% and this declines to 27·1%, from salinity 20 to 25 ppm. From salinity 25 to 28 ppm the increase in feeding rate is only 1·1%.

Active feeding is found only within a certain temperature range. Galtstoff (1928) showed that the optimum temperature for the activity of gills was about 30° C. Hopkins (1936) also found that maximum work was performed by the gills at this temperature. Loosanoff (1950) observed no marked fluctuation in the rate of pumping of *Crassostrea virginica* between temperature 16 to 28° C. but he observed maximum rates of pumping between 28 and 32° C. In *Arca granosa* the increase in feeding rate is directly related to temperature from 20 to 30° C., the maximum rate being at 30° C. but above this temperature the feeding rate declines. The increase in the rate of feeding is greater from 20 to 25° C. than between 25 to 30° C. At 32° C. the mantle borders begin to close slightly, thus diminishing the gape between the values. The slight fall in feeding rate might be partly due to this. The optimum temperature for active feeding is evidently 30° C.

Loosanoff and Tommers (1947) studied the effect of changes in pH of the medium on *Crassostrea virginica*. Lowering of pH from 7·75 to 6·75 was followed by a temporary increase in pumping rate, which was later followed by a decrease to below the normal values. In *Arca granosa* the maximum feeding rate was observed in a medium of pH 8·2 and minimum at 6·5. From pH 6·5 to 8·2 the feeding rate was directly related to pH, but in a medium of pH 8·4 it declines. Active feeding is at the medium of pH 8·2.

The water temperature in the natural environment varies from about 22·2° C. during January to 32·2° C. during May. Salinity varies from a minimum 0·21‰ during December and to a maximum 31·8‰. pH varies from a minimum of about 7·9 during October to a maximum of 8·4.

Observations show that when the salinity in the estuary is higher than average, as in summer months, *Arca granosa* migrates higher up in the estuary, but during floods it migrates into more saline waters. In the estuary, where diurnal, and seasonal variations, particularly in salinity and temperature, prevail, the feeding rate of the bivalve may be expected to show corresponding variations.

ACKNOWLEDGEMENTS

We have great pleasure in expressing our indebtedness to Prof. R. V. Seshaiya, Director, U.G.C. Centre for Advanced Study and Research in Marine Biology, for suggesting the problem, guidance, and instructions. Our thanks are also due to Dr. R. Natarajan for reading through the manuscript and to the University Grants Commission, New Delhi, for the award of Junior Research Fellowships.

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STUDIES ON SOME ASPECTS OF BIOLOGY AND FISHERY OF THE COCKLE, *ANADARA GRANOSA* (LINNAEUS), FROM KAKINADA BAY[1]

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ABSTRACT

Age and growth of *A. granosa* has been studied by an examination of the size-frequencies of random samples for the period from April 1965 to September 1966. The cockles measure 31·5 mm. in length at the end of the first year and 49·5 mm. at the end of the second year of life. No growth rings were noticed to be of use in age determination. The commercial catches chiefly consisted of '0' and 1+ age groups in the year April 1965 to March 1966. The cockles reach sexual maturity when they measure 21 mm. and about 7 months old. They appear to breed throughout the year. There is a peak spawning activity in January-April period. The length-weight relationship has been studied.

Brief accounts are given regarding the location of the fishing villages, fishing season, fishing methods, extraction of lime, its marketing and utility. An estimate of the lime sold in the market by weight and value from Yetimoga village for one year is made. The factors that are in operation to check depletion of the cockle beds are discussed.

INTRODUCTION

THE culture of *Anadara* spp. is practised in China and Japan (Cahn, 1951). It has considerable economic importance in the Philippines, Thailand, Malaysia, and Borneo (Pathansali and Soong, 1958). At Kakinada Bay (Fig. 1) *Anadara granosa* is regularly fished along with species of *Placuna, Meretrix, Paphia, Oliva, Umbonium, Murex, Cerithium,* etc., for burning into lime. An organised lime burning industry exists in several villages, situated along the western and southern side of Kakinada Bay. The commercially exploited area in the Bay is about 100 square Km. and is characterised mostly by soft muddy bottom, with a good amount of silt deposition due to the flow of water through irrigation canals and small rivers like the Koringa river and the Gaderu river. Among the molluscs fished in the bay, *A. granosa* ranks first in price, for its lime is said to be of a very high quality. A number of fishermen depend on this fishery for additional income to the usual earnings. In view of the considerable economic importance of *A. granosa*, the present study was taken up and an attempt has been made in the following account to furnish particulars of the fishery along with information on some aspects of biology of the species.

MATERIAL AND METHODS

The biological studies were based on random samples, taken for a period of 18 months from April, 1965 to September, 1966, from Kakinada Bay. Collections were made once every month, except for December, 1965 and June, 1966, when no samples were obtained. During low tide a plank-built boat was engaged with one diver. The large specimens were hand-picked, while the smaller ones were obtained by sieving the bottom with a sieve having 2 mm. mesh size. Each random

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sample usually consisted of more than 150 cockles. In the laboratory, length of the cockles was measured in the greatest antero-posterior direction correct to 0·1 mm. with vernier calipers. Age and growth have been studied by analysis of length-frequency distribution. The data were arranged in size groups with class intervals of 3 mm. and their percentages calculated. A total of 3,250 cockles were measured for this purpose. For one year, from April, 1965, to March, 1966, a *Kuncham* (about 5 litres) of the cockles landed by fishermen were regularly obtained every month and their length was measured for age composition of the commercial catch. These sample values were not raised to represent the size composition of the entire commercial catch; only the sample

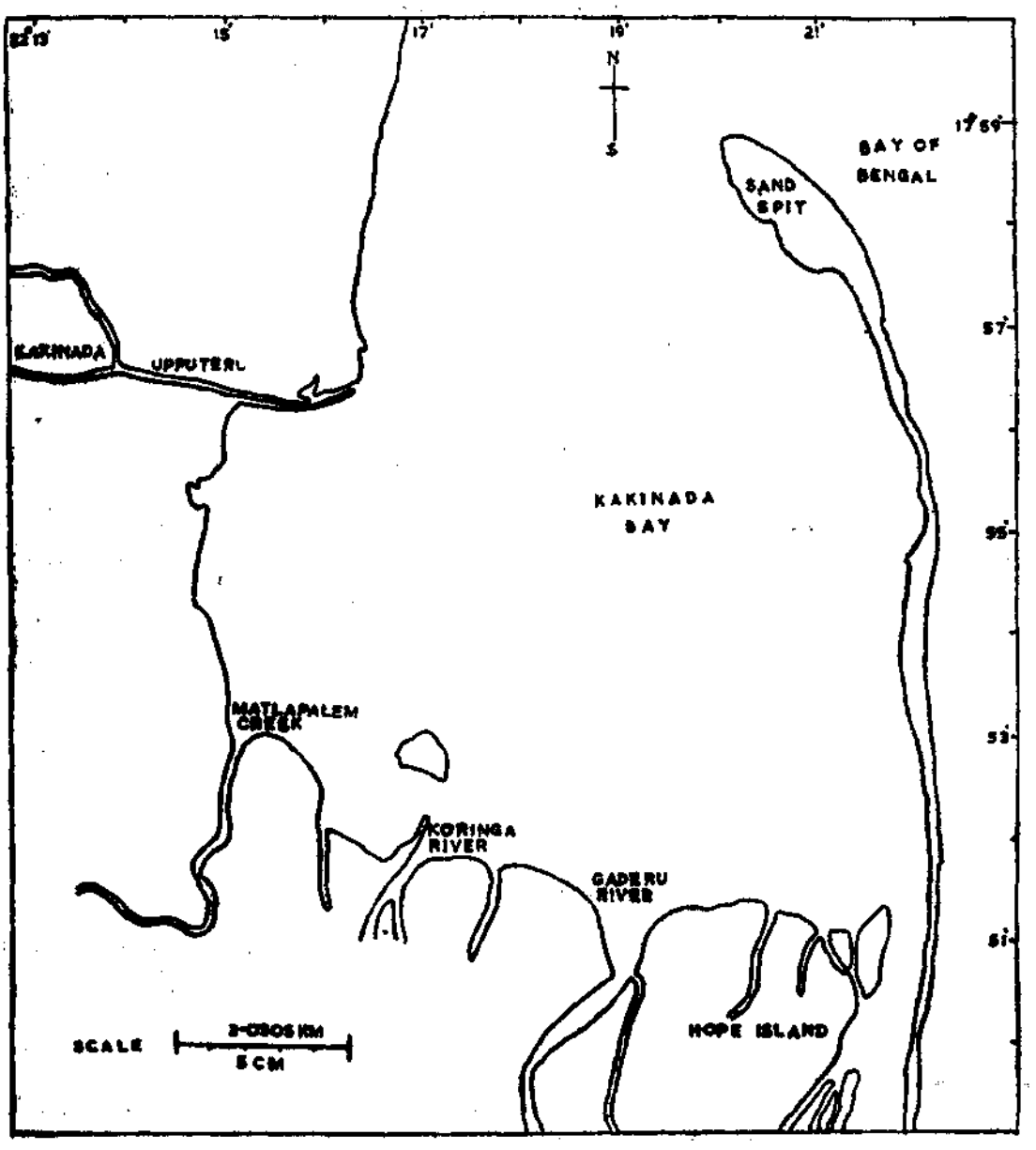


FIG. 1. Map showing the vicinity of Kakinada Bay.

values were pooled for each quarter and for the whole year. The length-weight relationship was studied by using specimens collected in April, May and June, 1965, and preserved in 5% formalin. The excess moisture was removed by filter-paper, and weights were recorded to the nearest milligram. For maturity studies the gonads were examined microscopically. A survey of some fishing villages around Kakinada Bay was undertaken to collect information about the economic importance of the fishery, fishing methods, season, and the method of lime preparation.

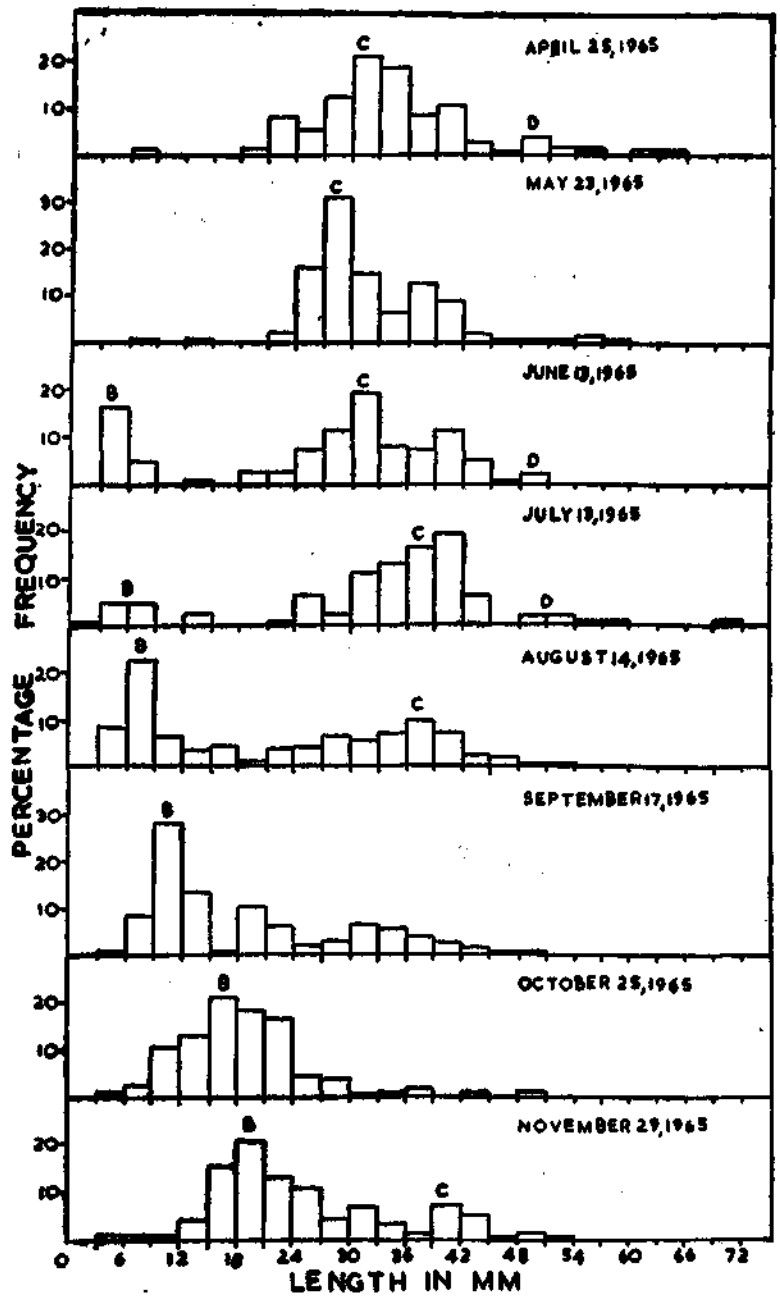


FIG. 2. Length-frequency distribution of *Anadara granosa*.

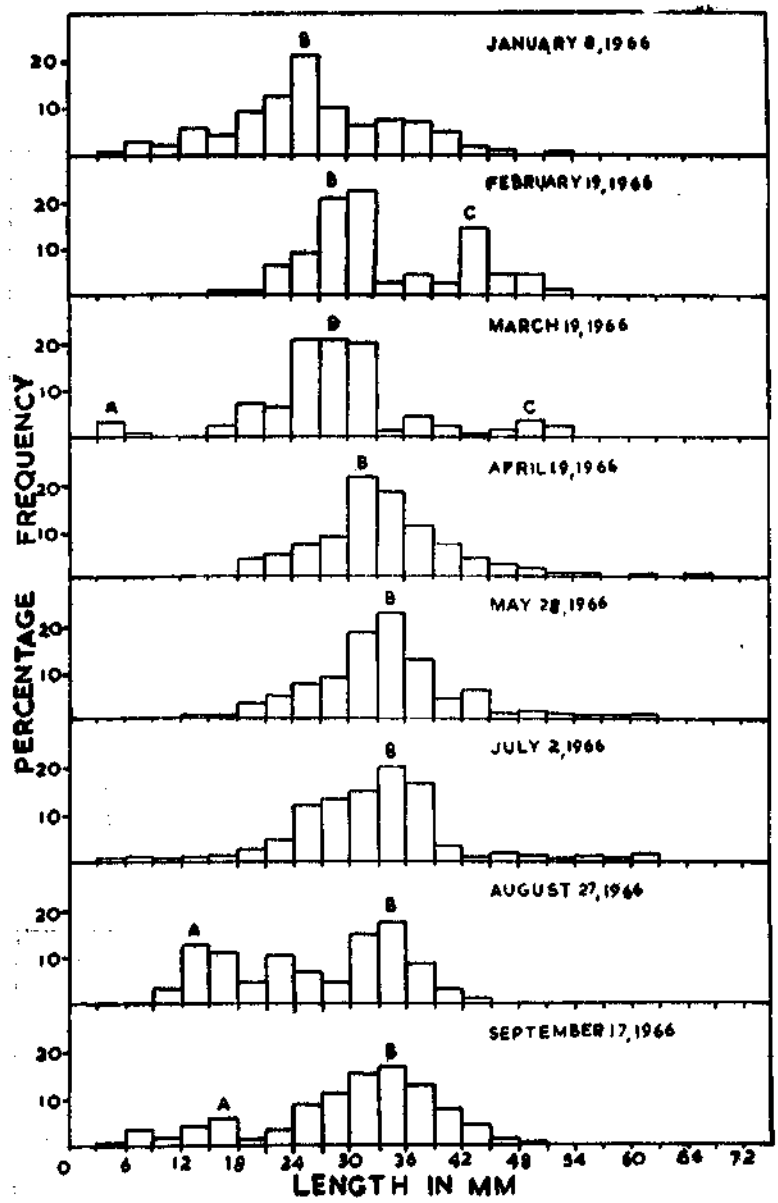


FIG. 3. Length-frequency distribution of *Anadara granosa* (*continued*).

AGE AND GROWTH

The length-frequency distribution based on random samples collected is shown in Figs. 2 and 3. In June, 1965, 3 modes, namely, B, C, and D were present at 4·5, 31·5, and 49·5 mm. respectively. Mode B moved to 31·5 mm. by April, 1966, *i.e.*, it grew to the size of C in about one year's time. Hence the time interval between B and C can be regarded as one year. In April, 1965, mode C was at 31·5 mm, and D at 49·5 mm, and in March, 1966, C moved to 49·5 mm. In other

words it occupied the position of D in one year's time. Consequently the difference in age between C and D may be considered as one year.

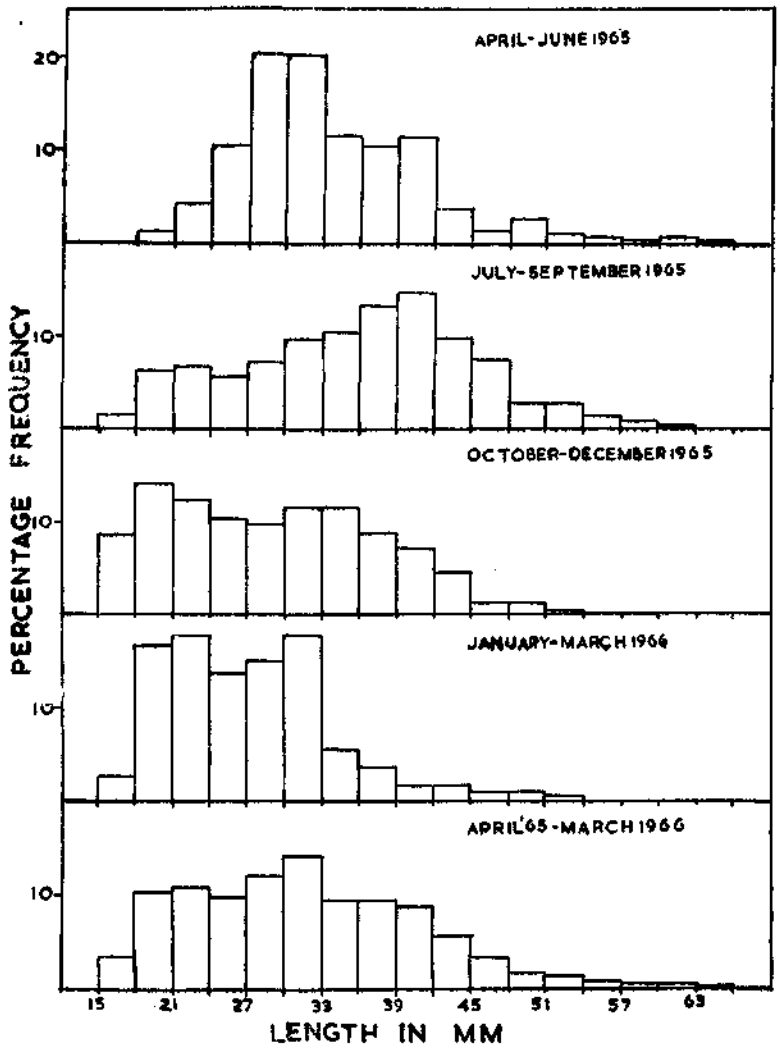


FIG. 4. Size-frequency distribution of commercial catch of *Anadara granosa*.

Reference to Fig. 2 shows that B, which was 4·5 mm. in June, 1965, moved to 7·5 mm. by August, 1965, thereby showing a size increment of 3 mm. in 2 months time. Assuming the same growth rate prior to June, 1965, B would have stood at 1·5 mm. or more likely less, in April, 1965, forming the 1965 year class. From the above it can be reasonably concluded that the cockles indicated by B at 31·5 mm. in April, 1966, have just completed one year of life. Mode B remained stationary at 34·5 mm. for 5 months from May to September, 1966. Whether this is due to cessation of growth or due to the mixing of smaller size groups is not clear.

It has already been shown that B, C, and D have a time interval of one year each. Since B represents the 1965 year class, it would follow that C and D represent the 1964 and 1963 year classes respectively. C and D stood at 31·5 mm. and 49·5 mm. in April, 1965, and as 31·5 mm. is the

length attained at the end of the first year of life, it would lead to the conclusion that the cockles measure 49·5 mm. at the end of the second year of life.

In March, 1966, mode A was present at 4·5 mm. which shifted to 16·5 mm. by September, 1966. By allowing a growth rate of 3 mm. in 2 months, as was done for B in the early part of life, A would have measured 1·5 mm. or less in January, 1966, and represents the 1966 year class. In April and May, 1966, considerable number of empty shells measuring 4 mm. to 11 mm., with both the valves open and attached, were collected. Judged from the freshness of the colour, these shells belonged to the 1966 year class. There was no indication of any external damage and the cause of the motality is not known.

Cockles measuring 30 to 70 mm. in length were studied in detail and growth rings were not observed to be of any utility in age determination (Plate I, Fig. 1).

Age Composition of the Commercial Catch

The size frequency distribution of the commercial catch for one year from April, 1965 to March, 1966, on a quarterly basis, and also for the whole year, is indicated in Fig. 4. It will be seen that the commercial catch consisted of cockles belonging mainly to '0' and 1+ age groups. The '0' age group is exploited in large numbers from October to June while 1+ age group formed the majority of the catch in July–September. For the whole year '0' age group formed 59·6%, 1+ age group 37·9%, and the rest 2·5%. The smallest cockle obtained from the commercial catches measured 16·2 mm. and the largest 65·4 mm.

Size and Age at Sexual Maturity and Spawning Period

A few individuals measuring around 18 mm. showed mature gonads and a majority of cockles from 21 mm. onwards revealed ripe reproductive elements in both the sexes. As the cockles grow to 21 mm. length in about 7 months time, it is concluded that *A. granosa* reaches sexual maturity when it is about 7 months old.

Between 15 and 20 adult cockles were examined microscopically for their gonadal condition from monthly samples. In this study maturing, ripe, and spent individuals were encountered throughout the period of investigation. From this, it would appear that *A. granosa* spawns throughout the year. A perusal of the length-frequency data (Figs. 2 and 3) reveals that 4·5 mm. modal group was present in varying numbers from June, 1965, to March, 1966, for a period of 10 months excepting February, 1966 (December 1965 no collection). This fact indicates that spawning is very much prolonged and lends support to the duration of spawning period arrived at by gonadal study.

Reference to Figs. 2 and 3 also shows that the modes A and B are well demarcated as isolated or nearly isolated groups. Hence there is every reason to believe that, in spite of breeding activity throughout the year, a definite peak occurs in the spawning. Such peak spawning appears to have occurred in January, 1966, and April, 1965, as was shown in size-frequency analysis for the modes A and B respectively.

Length-Weight Relationship

A preliminary plot of the logarithmic values of length and the corresponding weight showed that the same regression line would not fit the data for the entire length range examined. There appeared to be a break in the relationship at a length of about 20 mm. Two regression lines were therefore fitted by the method of least squares, one to the length range 3 to 19 mm. and the other

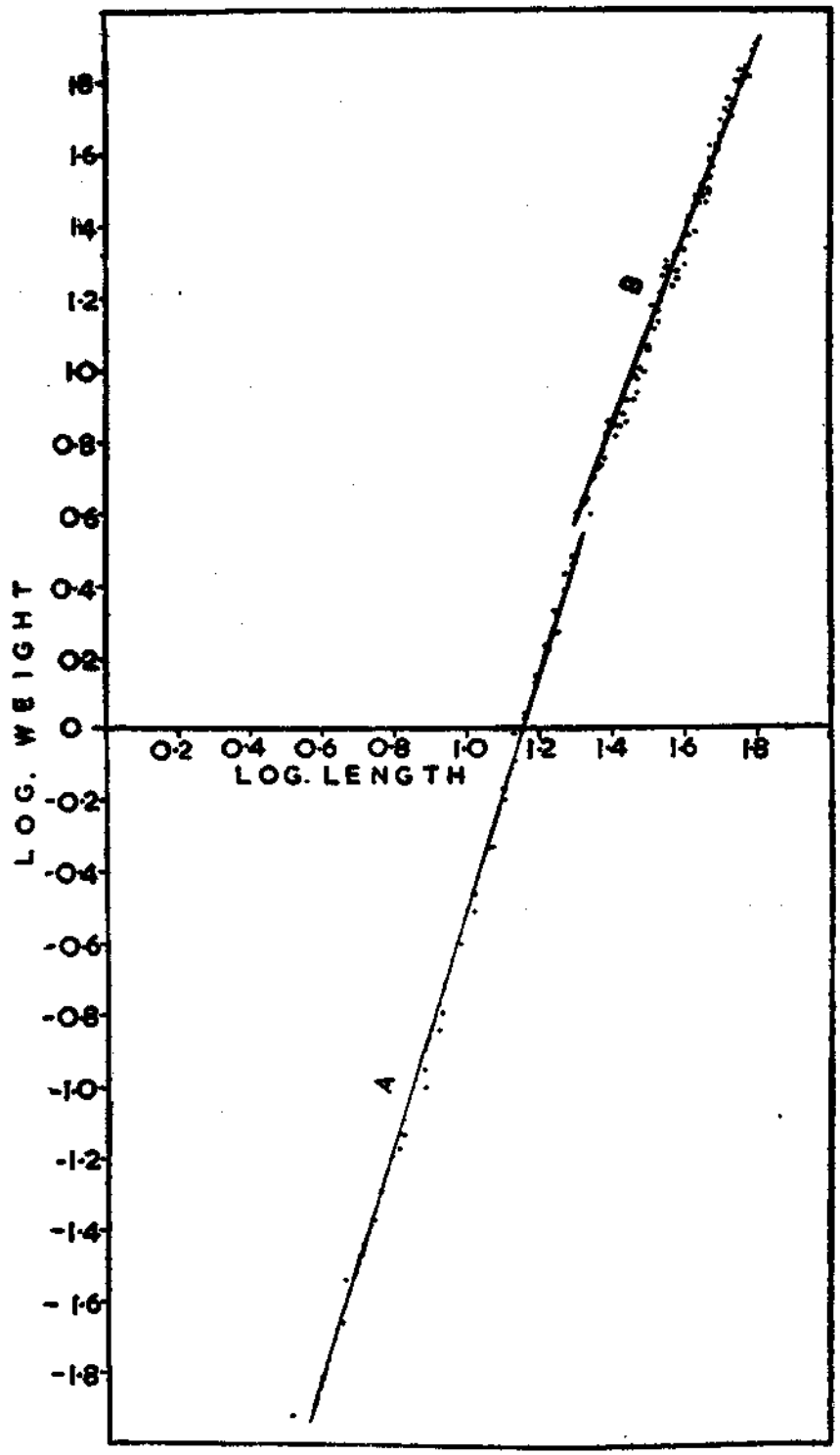


FIG. 5. Logarithmic relation of length and weight of *Anadara granosa*. Length range 3 to 19 mm. is represented by the regression line A and 20 to 63 mm. by B.

to the length range 20 to 63 mm. The number of specimens in the former group was 34 and in the latter group 78. The relationship obtained was

3 to 19 mm. length group: $Y = -3{\cdot}7130 + 3{\cdot}2096\ X$
20 to 63 mm. length group: $Y = -2{\cdot}8732 + 2{\cdot}6459\ X$

where Y = log weight and X = log length.

The difference between the two regression coefficients was tested by the method of analysis of covariance. The relevant data are given in Table I.

TABLE I

Analysis of Covariance of the length-weight relationship of A. granosa *from Kakinada Bay*

Length range	N	N − 1	$\Sigma(x-\bar{x})(y-\bar{y})$	$\Sigma(x-\bar{x})^2$	$\Sigma(y-\bar{y})^2$	$b\,\Sigma(x-\bar{x})(y-\bar{y})$	$\Sigma(y-Y)^2$	N − 2
3 to 19 mm. ..	34	33	5·2961	1·6501	17·0651	16·9984	0·0667	32
20 to 63 mm. ..	78	77	3·6767	1·3896	10·6734	9·7282	0·9452	76
TOTAL ..	112	110	8·9728	3·0397	27·7385	26·7266	1·0119	108

N = Number of observations. $\Sigma(y-Y)^2$ = Sum of squares due to deviation from regression.

Test of heterogeneity of regressions within the samples

Source of variation	Degrees of freedom	Sum of squares	Mean squares
Deviation from average total regression ..	109	1·2517	—
Deviations from individual regressions within samples ..	108	1·0119	0·009369
Difference ..	1	0·2398	0·2398

F = 25·595. $F_{1\%} = 6{\cdot}89$.

The F test showed that the difference between the two regression coefficients is significant at 1% level. The two regression lines marked as A for the length 3 to 19 mm. and as B for the length range 20 to 63 mm. are shown in Fig. 5.

It will be seen from the figure and text that the smaller cockles upto 19 mm. length increase in weight in relation to length at a higher rate than the larger ones.

FISHERY

Fishing villages.—Among the fishing villages, where the cockle fishery is practised, mention may be made of Yetimoga, Gadimoga, Bhairavipalem, Balusutippa, and Masanitippa. In Yerragaruvu village, where a lucrative lime burning industry exists, no fishing is done, but the fishermen regularly buy the cockles from nearby villages.

Fishing season.—The cockles are fished thoughout the year with maximum catches in January due to heavy demand for lime in this month.

Fishing method.—Plank-built boats, with 5 to 8 divers on each boat, depending on the size of the boat, go to the bay about 3 hours after the high-tide. Each diver carries with him a bamboo basket and a rope about 8 metres long. After anchoring the boat at the collection spot, the diver ties one end of the rope to the basket and the other end to his waist. The cockles, along with other molluscs, are hand-picked and transferred to the basket. The rope helps him to keep track of the basket as he often comes to surface for breathing. After the basket is full, the catch is transferred to the boat and the operation is repeated for four to five hours by which time the water begins to swell due to the approaching high-tide. Generally fishing is confined to less than four metres depth during low-tides at daytime. The catch is auctioned and each fisherman gets Rs. 2 to 5 as his share for the day's fishing.

Method of lime extraction.—The cockles are burnt into lime in an oven, locally known as *batty* (Plate I, Fig. 2). The *batties* are hollow and cylindrical; small ones have 110 cm. diameter, height 75 cm. and the wall 15 cm. thick with 9 ventilation holes at the base; big ovens have 155 cm. diameter, 85 cm. height, the wall at base 18 cm. thick and has 14 ventilation holes. There are also *batties* of intermediate sizes.

The *batty* is constructed on levelled ground, elevated 2 to 3 metres. Bamboo pieces of the required height are fixed in a circle and woven horizontally 15 to 20 cm. from the ground to top with thin bamboo sticks. A thick layer of mud is plastered both inside and outside of this bamboo structure, after making the required number of ventilation holes at the base. Care is taken to see that mud plastering is sufficiently thick inside the bamboo structure so as to avoid contact with fire. The *batty* is allowed to dry for 4 to 5 days before it is operated.

The cockles, with meat intact, are dried for about 4 days before they are transferred to the *batty*. By this method the valves open and the moisture is evaporated from the meat. However, when there is heavy demand for lime, the cockles are burnt within hours of landing, in which case the time taken for burning would be longer, requiring more coal. Also, the quality of the lime is said to be poor. Different species of molluscs are burnt separately to ensure uniform burning into lime.

Inside the *batty* pieces of broken tiles and earthen pots are kept to allow free passage of air. Upon this a layer of dried coconut or palm fibre is placed. Over this, layers of coal and cockles are filled alternately up to the top of the *batty*. Again coconut fibre is placed on this and in turn covered superficially with little mud so that the top fibre layer is not blown by the wind. Now the *batty* is lit at the bottom through the ventilation holes and the latter are closed with mud excepting those 3 or 4 facing the wind. If the direction of the wind changes, those facing the wind are opened and the remaining ones closed.

During rainy season an umbrella made of palm leaves is suspended above the top of the *batty* from bamboo poles erected for the purpose. The elevated platform on which the *batty* is constructed prevents the flow of ground water into the *batty* and at the same time ensures a rich supply of air.

After the *batty* is lit, it is allowed to burn for five to six hours by which time the fire goes out. The lime is collected after eight to ten hours. The burning operation usually begins at 12 noon, the fire goes out by 5 P.M., and the lime is taken out at 4 A.M. next morning for marketing. At present there are 23 *batties* at Kakinada.

Marketing and economics of the fishery.—The cockles burnt into lime maintain their shape (Plate I, Fig. 3) and are sold in the market by a local measure known as *kuncham*, which is equivalent to about five litres. A *kuncham* of burnt cockles weighs approximately 2 kg. and fetches 25 to 35 paise. 'Pongal', which comes in January every year, is an important festival in Andhra Pradesh when lime-washing of the houses is customary. In this month the cost of a *kuncham* of burnt cockles goes up to 50–60 paise due to the prevailing demand.

Based on the information furnished by merchants, an approximate estimate was made of the lime sold in the year 1966. The figures given pertain to the lime prepared out of cockles from Yetimoga fishing village only. About 160 tons of lime was sold valued at Rs. 32,000 and January alone accounted for 70 tons priced at Rs. 18,000. From the figures it would appear that the fishing intensity is dependent upon the local demand and prevailing prices of lime.

Utility.—The cockle meat is said to be good for pregnant women and except for this occasional medicinal use it is not eaten locally. As already stated the cockles are burnt into lime with meats intact.

Discussion

The growth of *A. granosa* from a commercial bed at Penang Island, Malaysia, was studied by Pathansali (1964). From his figures it would appear that the length of one-year-old cockle is 27·5 mm., two-year-old 37·5 mm, and three-year-old 43·3 mm. The present study from Kakinada shows that one-year-old measures 31·5 mm. which compares favourably with Pathansali's estimate. But the size of two-year-olds at 49·5 mm. in the present investigation is very much higher than that estimated by Pathansali. It may be mentioned here that Pathansali himself stated that his estimate of the growth of cockles above 25·4 mm. in length is not reliable due to commercial exploitation on the culture bed. It is also possible that the disparity observed in the growth may be due to differences in the environmental conditions.

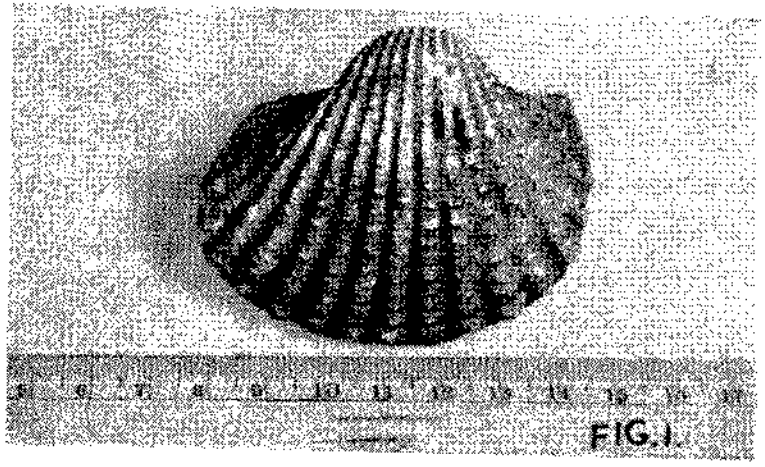
Pathansali (*op. cit.*) found that *A. granosa* reaches sexual maturity between 18–20 mm. length when the cockles are about 6 months old. These findings closely correspond to what is observed at Kakinada.

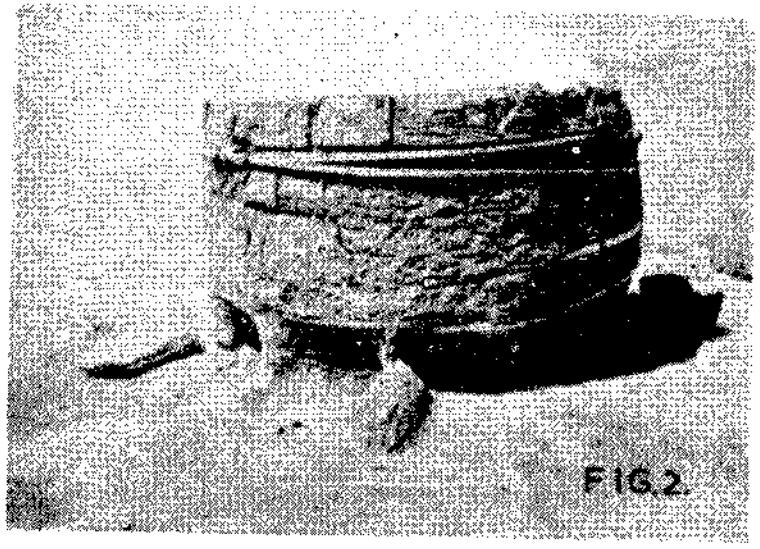
On the spawning of *A. granosa* from Malaysia, Pathansali (1964) wrote "....the major breeding season is spread over a period of time, and some breeding takes places throughout the year." He collected larvae throughout the year for three years and by gonadal study observed that peak breeding took place in late August–September in 1958 and in late September–early October in 1959. In the present study the author found that *A. granosa* spawns throughout the year with peak spawning in April, 1965, and in January, 1966. The results obtained at Kakinada regarding the duration of the spawning period and the occurrence of a definite peak in the breeding activity are in agreement with Pathansali's findings. While off Penang Island *A. granosa* has major breeding period in August–October, at Kakinada the peak spawning seems to take place some time in January–April period.

Among the bivalves which showed prolonged spawning period, investigated from India, mention may be made of *Crassostrea madrasensis*, which under marine conditions breeds throughout the year with two peaks, once in about November–December and again about May (Rao, 1951). *Meretrix casta* from Adayar estuary appears to breed throughout the year with peak spawning activity in certain months only (Abraham, 1953). In *Solen kempi*, Rao *et al.* (1962) noted that spawning is prolonged, commencing in October–November and extending until the end of March, with vigorous spawning activity at the beginning of the spawning period. It would appear, that in those species which have a prolonged spawning period in a given environment, there exists a definite peak or peaks in the breeding activity, restricted to certain months only.

It has been stated in the length-weight relationship study that the smaller cockles up to about 19 mm. length increase in weight in relation to length at a higher rate than the larger ones. This change in weight is quite likely due to the onset of sexual maturity at 21 mm. length.

A. granosa is cultured for its food value in some 3,500 acres in Malysia. Its meat is equal to other shellfish in food value and compares well with beef (Pathansali and Soong, 1958). The meat





FIGS. 1–3. Fig. 1. Photograph of *Anadara granosa*. Fig. 2. A *batty* in operation. Fig. 3. Marketing of the cockle lime. The local measure, *kunchum* is also shown.

of the above species is not eaten at Kakinada, except for medicinal use, although a number of species of bivalves are used for culinary purpose both along the east and west coasts of India. This is because of traditional dislike; moreover, the red colour of the cockle meat does not find favour with the people.

In the present condition of the cockle fishery, three factors strongly operate to check depletion. Firstly, there are many areas in Kakinada Bay where the depth is more than 4 metres, which are not exploited, thus leaving large-sized individuals to replenish the stock. Secondly, the prolonged spawning habit of *A. granosa* should help considerably in providing recruits to the fishery for a major part of the year. Lastly, except for January every year, there is not much inducement for fishermen to catch cockles in large quantities due to low market value of lime. However, once the meat finds utility it can be reasonably expected that the picture will change.

ACKNOWLEDGEMENTS

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A PRELIMINARY STUDY OF GROWTH IN *CERITHIDEA (CERITHIDEOPSILLA) FLUVIATILIS* (POTIEZ AND MICHAUD), (PROSOBRANCHIA—GASTROPODA)

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ABSTRACT

Observations on the growth of *Cerithidea (Cerithideopsilla) fluviatilis* in the Vellar estuary have been in progress for over six months. Weekly samples were collected and linear measurements were recorded for a total number of 13,300 specimens. The samples showed unimodal frequency. The heavy aggregation of young ones during July and August and the unimodal frequency distribution would indicate that there is only one breeding season in the year. In the interior parts of inlets and creeks during flood time and at other times also over-sized specimens above 23 mm. were observed whereas only large number of dead ones infested with crabs were noticed during flood time in the sampling area. This is apparently due to differential survival, the over-sized specimens taking shelter in creeks.

INTRODUCTION

THE study of growth in any of its different aspects is a problem of great importance and a comparative study in any group of organisms is a promising field of investigation. The gastropod molluscs are particularly interesting for studies of growth and are also very suitable material. In recent years some important contributions have been made on the growth of a few prosobranchs, in particular *Gibbula*, *Opisthostoma*, and *Littorina*. The latter has been studied by more than one investigator. Hayes (1927, 1929) studied the effect of environmental factors on the development, growth, and behaviour in *Littorina*. Moore (1937) investigated growth of the shell in *Littorina littorea* and Smith and Newell (1955) studied the dynamics of zonation. Williams (1964 *a*) investigated growth and distribution of *Littorina littorea* (L.) on a rocky shore with a view to determining which section of the population was responsible for the maintenance of the population as a whole. Green and Green (1932) compared growth rates in *Littorina littorea* using weight and height as size indicators. Of the Indian forms, *Trochus* and *Cerithidea* have been studied so far. Rao (1939) investigated rate of growth in *Trochus niloticus* found in the Andamans. Sadasivan (1948) studied the rate of growth in *Cerithidea cingulatus* from the backwaters of Madras. The present paper records the results of a preliminary study of growth in *Cerithidea (C.) fluviatilis* based on length-frequency data for a period of seven months.

MATERIAL AND SAMPLING METHOD

Cerithidea (C.) fluviatilis occurs in abundance along the banks of estuaries and is able to live under the varying conditions prevailing in these regions. The species is particularly abundant in backwater creeks and in mud-flats where it is densely distributed from high-water level to low-water. The abundance of populations of *Cerithidea fluviatilis* throughout the year is a striking feature of estuarine areas.

The genus *Cerithidea* Swainson, according to Thiele (1931), contains two subgenera *Cerithidea* s.s. and *Cerithideopsis*. *Cerithideopsis* s.s. contains two sections *Cerithideopsis* s.s. and *Cerithideopsilla*. *Cerithidea (Cerithidea) decollata* (Linne) and *C. (Cerithideopsilla) fluviatilis* (Potiez and Michaud) are distributed in the Indo-Pacific regions. *Cerithidea (C.) fluviatilis* (Potiez and Michaud) is synonymous with *Cerithidea cingulatus* Gmelin, and *Potamidea cingulatus* Hornell.

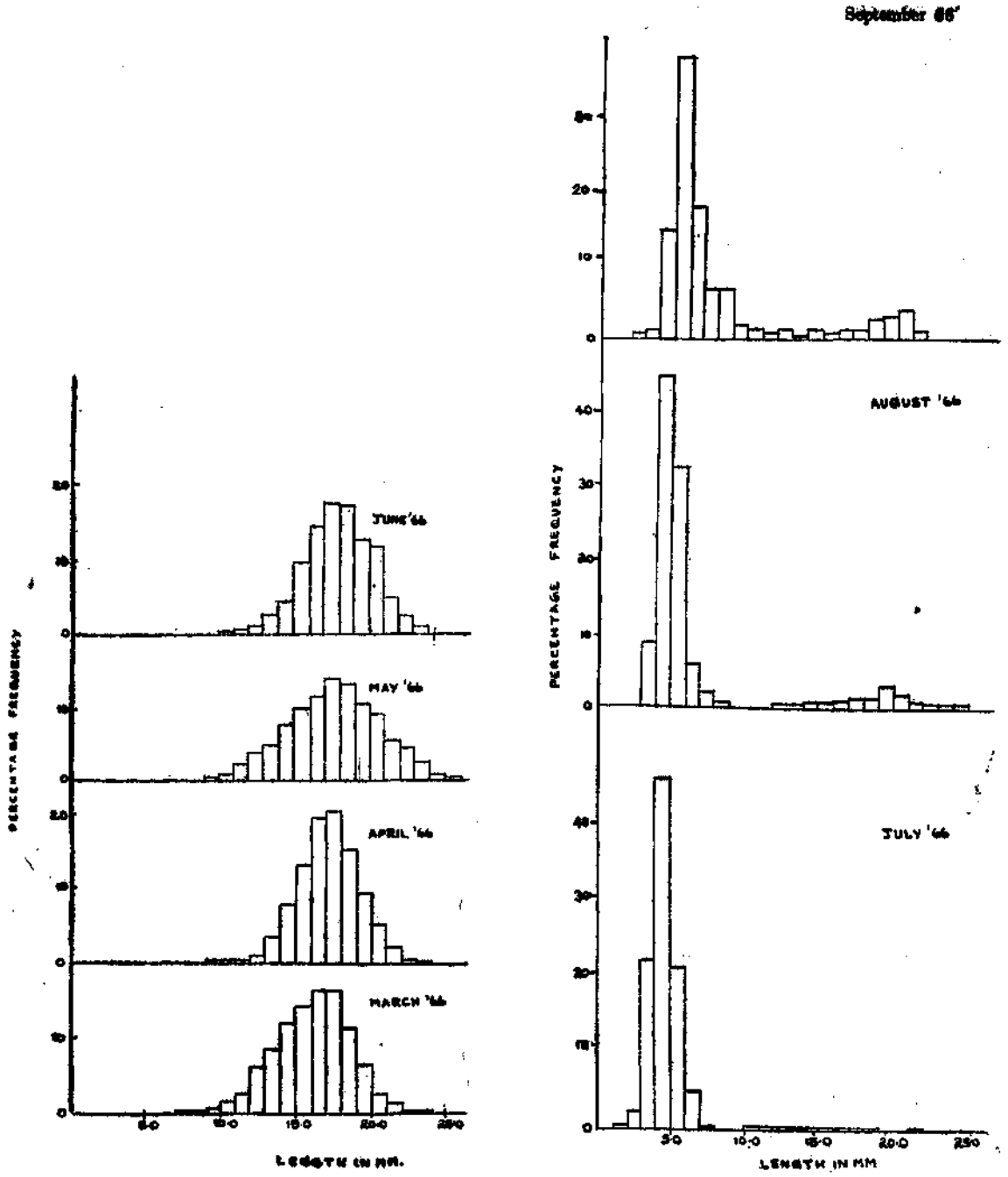

FIG. 1. Size frequency distribution of *Cerithidea fluviatilis* during March to September, 1966, at a station on the bank.

Samples of *Cerithidea (C.) fluviatilis* were collected once a week for a period of seven months, extending from March to September, 1966, at a defined station in the Vellar estuary. Specimens

were also collected from backwaters for about three months. On the whole, fifty samples with a total of 13,300 specimens, giving an average of 266 specimens per sample, were collected.

Cerithidea (*C.*) *fluviatilis* is relatively semi-sedentary, showing very restricted movement. Thus sampling could be effectively carried out by the "quadrat method". Wooden frames measuring 1 metre square, were used to obtain random samples. The specimens included in each quadrat were taken to the laboratory for measurement. The heights of the specimens were recorded to the nearest 0·1 mm. with vernier calipers. Shell height was considered as age/size indicator.

Observations and Discussion

It was found convenient to group the animals into two categories, those which are 7 mm. and less, and those which are above 7 mm. The former will be referred to in this paper as small-sized groups and the latter as the large-sized. Histograms (Fig. 1) showing the relative frequency of the size groups were drawn for each month and the frequency patterns of the different months compared.

The main features of the frequency distribution of the size groups are as follows:

(*a*) The range of variation in size is more or less similar from March to June. Specimens below 7 mm. are lacking, and there are only few below 10 mm. The predominant group is the 17-17·9 mm. group. The coefficient of variation for the population during these months is low.

(*b*) The predominant size groups during July and August is 4–4·9 mm. and during September 5–5·9 mm. During these months the large-size groups, *i.e.*, above 7 mm., show a low frequency. The frequency for these groups is mostly 0·1 per cent, and in August and September slightly higher. The large-size groups and small-size groups are discrete during July and August, but in September they show a continuous frequency. However, during August and September the large-size groups have distinct modes. The slight increase in the frequency of the large-size groups might have been due to a few immigrants from other areas, or due to sampling error or both. But this does not alter the overall picture, which is the great preponderance of small-size groups that were entirely lacking in the earlier months.

Field observations extending over a few years also indicated that the juvenile specimens occurred only during July to September and in very large numbers. Sadasivan (1948) reported on *Cerithidea cingulatus* from the backwaters of Madras that breeding season extended from January until June. The present study based on specimens from Porto Novo does not confirm this.

Sadasivan (1948) also reported that the maximum size observed by him was 22 mm. and the rate of growth in young shells was 1·17 mm. per month.

In the Vellar estuary specimens of *Cerithidea* (*C.*) *fluviatilis* were found to attain greater size. On the banks of the estuary 26 mm. size was occasionally met with and in the backwater and creeks specimens which were 31 mm. were collected.

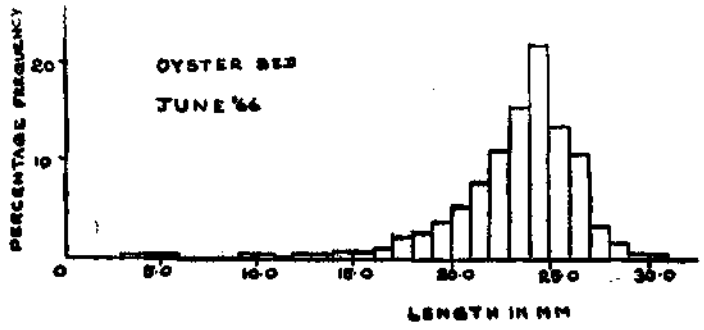

Fig. 2. Size frequency distribution of *Cerithidea fluviatilis* during the month of June at oyster bed region.

Sadasivan (1948) also reported that the average rate of growth in young shells was 1·17 mm. per month. In the present study the maximum size of juvenile specimens observed in July was 8 mm., in August 9 mm., and in September 10 mm. Thus it would appear that the average rate of growth was 1 mm. per month. However, this is only a provisional inference and will have to be corroborated by further observations which are in progress.

Another interesting feature which was observed was the high incidence of barnacles during March on the shells ranging in height from 12 to 18 mm. The percentage of incidence during this period was 54·71%. The incidence in other months was limited.

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RATE OF WATER FILTRATION IN *MARTESIA FRAGILIS* IN RELATION TO BODY SIZE AND OXYGEN CONSUMPTION

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ABSTRACT

Martesia fragilis, though a wood-borer, has none of the adaptations for subsisting on wood, on the other hand shows well-developed plankton feeding mechanisms as in the rock-boring pholads. Both in the young and adult stages they can live outside wood for varying periods and feed exclusively on plankton. The filtering activity of *Martesia fragilis* of different sizes of body during growth has been estimated and presented as index of metabolic rate. It is found that the filtration rates of *Martesia fragilis* are higher than in *Mytilus californianus*, *Pecten irradians* and *Crossostrea virginica*.

The total filtration shows a linear relationship with size up to 30 mg. (wet) of body weight, beyond which this was not maintained. Similarly the rate of filtration per mg. weight shows a sharp fall up to this size with increasing body weight. A correlation of filtering rates with the rate of oxygen uptake shows that the filtering rates decrease with decreasing oxygen uptake. However, this decrease appears to be only up to the level of the mature adult. This suggests that the smallest *Martesia* studied filters more and respires more intensely than larger ones. When the filtration rate is expressed as litres of water transported for each c.c. of oxygen consumed in comparable weights the rates varied from 1·280 to 4·465 (for each cc. of oxygen taken up). Higher rates of filtration relative to oxygen consumption were found in the young active forms, and they gradually decrease with growth.

THERE is a complex interrelationship between the rates of water transport, oxygen consumption, and food requirements of organisms (Jørgensen, 1960). During the course of a study of the ciliary currents and associated organs of *Martesia fragilis*, a common pholadid wood-borer of Madras, Srinivasan (1960) suggested that though a wood-borer it has none of the adaptations for subsisting on wood. On the other hand it shows well-developed plankton feeding mechanisms as in the rock-boring pholads. That the animal can live and grow even when it is denied access to wood has been shown by experimental observations, indicating that both in the young and adult stages these forms can live outside of wood for varying periods and feed exclusively on plankton (Srinivasan, 1962). Srinivasan (1965) studied the rate of oxygen consumption in relation to body size and nitrogen content in *M. fragilis*. It has been shown by previous workers that smaller forms filtered at a faster rate than larger ones (Chipman and Hopkins, 1954; Jørgensen, 1949 *a*, 1960; Willemsen, 1952). In *M. fragilis* the filtration rates were studied in relation to their body size to observe whether a similar phenomenon exists and also to help in understanding its relationship with oxygen consumption.

Nelson (1936), Fox, Sverdrup, and Cunningham (1937), Galtsoff, Chipman, Hasler, and Engle (1938), and Jørgensen (1943) have carried out extensive work on the rate of water transport of a number of lamellibranchs especially oysters and mussels using either direct measurements or indirect methods based on the reduction of number of particles in a suspension in which the animals have been placed. The rate of filtration and its relation to the metabolic activity of lamellibranchs was studied by Jørgensen (1949 *a*, *b*, 1952, 1955) using colloidal graphite. Rao (1953, 1954) used the above technique to study the rate of water transport of *Mytilus californianus* as a function of latitude and also correlated to different tidal cycles. Chipman and Hopkins (1954), Van Dam (1954),

Ballentine and Morton (1956), Morton, Boney, and Corner (1957) and Jørgensen (1960) have studied the rate of water transport and oxygen consumption in *Pecten irradians, Lasaea rubra, Crassostrea virginica, Mytilus edulis* and *Pecten latiauratus*. The filtering activity of the edible clam *Meretrix casta* in relation to size and salinity has been studied by Durve (1963) who observed that while the rate increases with size, it falls in excessive low and high salinities.

It will be seen from the reviews of Jørgensen (1955, 1960, 1966) that studies hitherto made relate mostly to mussels, scallops, and oysters and comparatively little is known about the filtration rates of pholads, both wood-boring and rock-boring, except for brief observations made on *Martesia striata* by Nagabushanam (1956) who employed the neutral red technique and found that neutral red has a great depressing effect upon the filtration rate of *Martesia*.

Methods

It was observed by earlier worker (Jørgensen, 1960) that larger mussels are more sensitive to environment than the smaller ones (less than about 4·5 cm.), which can only remain open during experimental periods and hence yielded normal rates of water transport. With *Martesia* it was found that all animals used were small (within 18 mm. in length) and they were keeping their siphons open throughout the experimental period. However, even when the greatest care was exercised, the animals did react by retracting the siphons and closing the valves which affected the water transport.

Hence, it was essential to conduct experiments on animals which were kept undisturbed. The animals were placed in troughs (15·5" in diameter) and the sea-water changed every morning. As soon as the water is changed the animals reacted quickly and extruded the pseudofaces at a faster rate than before for about an hour. They were left in filtered sea-water for over two hours to adjust themselves to outside changes.

About 25 mg. of colloidal graphite (Prodag—5·15 μ in size—Rao, 1953) was mixed well with 50 c.c. of filtered sea-water (filtered through Seitz filter). After vigorous shaking the solution was left undisturbed for about 15 minutes by which time the larger particles had settled down. The supernatant was increased to 250 c.c., a uniform suspension was made by rapid shaking, and this was used in the experiments. Suspensions of 1/2 to 3 hours old have been used throughout these experiments and the filtration was found efficient. One animal was transferred to each beaker containing 100 c.c. of the solution. Though the animals closed their shell valves immediately after being removed from the aquarium tanks, they protruded their siphons on being placed in the experimental beakers and started clearing the suspension and behaved normally over four to five hours. The beakers were left in large troughs which contained water at least up to the neck to prevent any temperature variations. Experiments were performed during 10 a.m. to 1 p.m. every day. The temperature varied from 29° C. to 29·8° C. and salinity of the water for all the experiments ranged only from 33·1 ‰ to 34·2 ‰. An hour after the animal had been left in the beakers, 10 c.c. of the suspension was carefully taken out using an ink-filler and its concentration determined using the Unicam colorimeter. For the second hour the rate of filtration in 90 c.c. volume of water was studied. Concentrations which gave more than about 67% transmission were used, since at (*i.e.*, about 35% of the arbitrary high concentrations) lesser concentrations the clearances tended to decrease.

Clearances were read out from a standard graph prepared with suspensions of colloidal graphite. The rate of water propulsion was calculated using Jørgensen's formula and values obtained for animals of different sizes (wet weight) plotted.

Results

Rate of water filtration

Clearance of graphite particles.—The rate of clearing of the suspended graphite particles at different times were plotted on a semilogarithmic scale (Fig. 1) in order to visualize the various

changes in the suspension, which varied somewhat, but followed the same pattern. There was a gradual decrease in the rate of reduction during the experiment as could be seen from Table I.

TABLE I

Clearance of colloidal graphite by Martesia fragilis

No.	Initial concentration %	1 hr. %	2 hr. %	3 hr. %
1	30	23	18·0	13
2	25	18	13·0	8
3	25	9	0·5	—

Maximum clearances were observed in the third hour. In one experiment (Ex. No. 3), the suspension was completely filtered within 2 hours. During this time of complete removal the observed rate of decrease in concentration represents the rate of water filtration by *Martesia*.

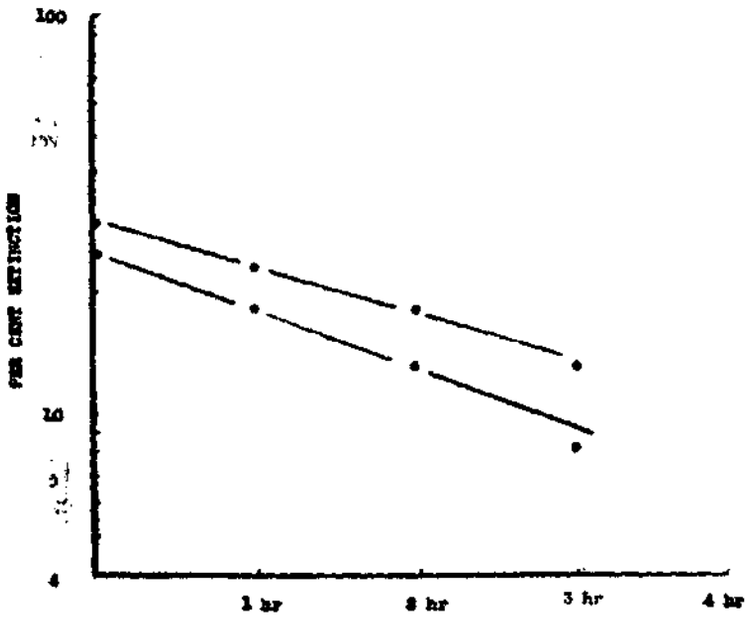

FIG. 1. Clearance of graphite particles with time. The percentage transmission of light has been used as an index for clearance.

The rate of filtration by animals of different sizes from 3·25 mg. (wet weight) to 73·25 mg. of tissue was determined to study the relationship with increase in size and growth. The results are presented in Table II. The total amount of water filtered was about 14·03 c.c./hr. for animals weighing 3·25 mg. and it gradually increased with increasing body weight (73·25 mg.) when it was 43·48 c.c./hr. widely varying values have been obtained in animals of the same sizes suggesting that the two animals were transporting water at different rates during the experiments. No linear

relationship was noted between the wet weight and the total filtration when all the sizes studied were considered. However, when the values were plotted on a semilogarithmic graph (Fig. 2) it was noticed that the total filtration seemed to show a steady increase with increasing body size up to about 30 mg. wet weight, beyond which this was not maintained.

TABLE II

The rate of filtration of Martesia fragilis *in relation to body weight*

No.	Wet weight mg.	Total filtration c.c./hr.	Rate of water filtration c.c./mg/hr.
1	3·25	14·03	4·316
2	7·75	26·08	3·365
3	8·25	23·87	2·891
4	10·25	19·56	1·908
5	11·25	17·35	1·542
6	13·00	15·41	1·185
7	13·75	14·68	1·068
8	15·50	26·08	1·683
9	17·00	23·87	1·405
10	17·25	19·56	1·134
11	17·50	29·70	1·348
12	18·25	19·56	1·072
13	23·25	25·80	1·414
14	23·25	55·59	2·391
15	23·25	32·86	1·110
16	24·00	40·57	1·690
17	24·25	25·39	1·047
18	28·00	43·10	1·539
19	34·50	26·59	0·770
20	48·50	27·45	0·566
21	59·00	24·86	0·421
22	73·25	43·48	0·594

The regression line gave the value of the slope as 0·44202. The data was analysed statistically using the formula

$$r = \frac{\sigma x^2 + \sigma y^2 - \sigma x - y^2}{2\sigma xy}$$

and that t value obtained was

$2{\cdot}065 < 2{\cdot}12$ at 16 degrees of freedom

at 5% level of significance.

It will be clear from the above data that the linear relationship is significant up to this weight group.

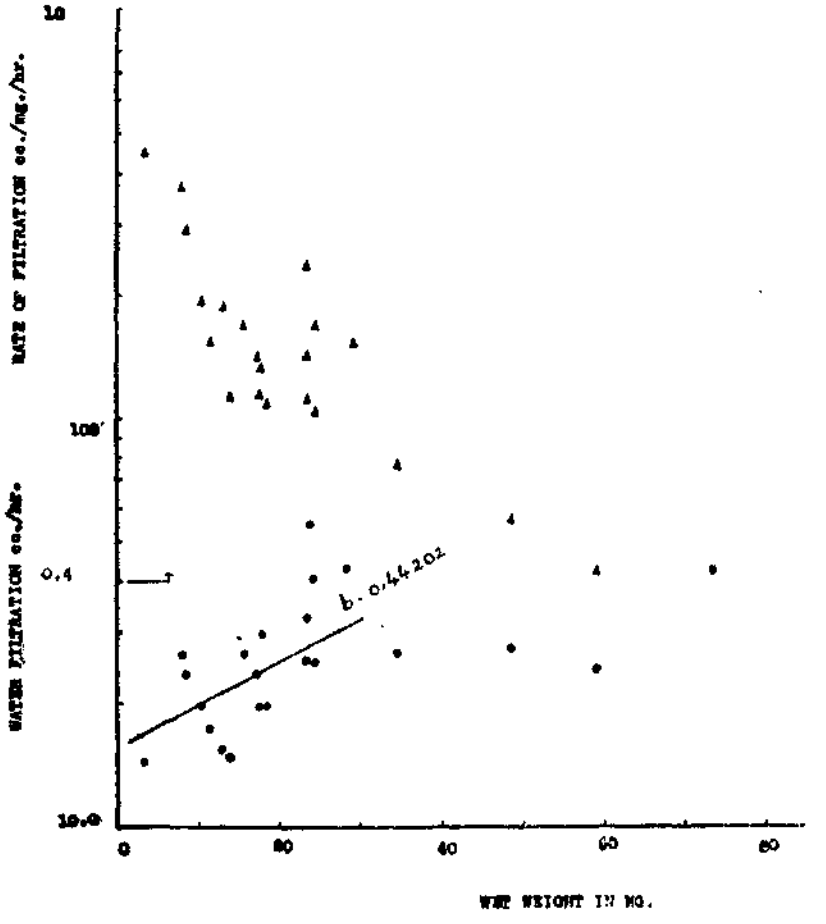

FIG. 2. Total water filtration in relation to body weight (●) and per unit of body weight (▲) plotted in semilog graph. Up to 30 mg. level the water filtration increases by a factor $b = 0{\cdot}44202$.

Similarly the rate of filtration per mg. weight (Table II, Fig. 2) shows a sharp fall up to this size (*i.e.*, 30 mg. with increasing body weight) although there is considerable fluctuation. Animals weighing 3·25 mg. filtered at the rate of 4·316 c.c./mg./hr. which decreased to 1·539 c.c./mg./hr. in animals of 28·00 mg. wet weight. Beyond this size there was no significant decrease in filtration rate. These observations suggest that the smaller animals filtered at a higher rate and as they grow, initially, there was a sharp decline in the rate of filtration, but it remained unchanged later.

Filtration rate and oxygen consumption

While studying the rate of oxygen consumption of *M. fragilis*, Srinivasan (1965) indicated that with increase in size increasing oxygen uptake was observed ($b = 0{\cdot}55346$), but beyond about 30 mg. wet weight the oxygen consumption indicated a fall ($b = 0{\cdot}30950$). Similarly, the total water filtration increases by a factor ($b = 0{\cdot}44202$) only up to 30 mg. level. A correlation of filtering rates with the rate of oxygen uptake shows that the filtering rates increase with increasing oxygen uptake

up to about 20 mg. level. This suggests that the smallest *Martesia* studied filters more and respires more intensely than larger ones.

When the filtration rate is expressed as litres of water transported for each c.c. of oxygen consumed in comparable weights, the rates varied from 1·28 to 4·465 (for each c.c. of oxygen taken up) as could be observed from Table III. Thus the ratio between oxygen uptake and filtration rates varies in different size groups and in large animals (34·5 mg.) it is 1 : 1·39. These values are comparable to those in tiny *Lasaea rubra* (Ballentine and Morton, 1956) whose filtration/O_2 ratio is 1–4. Higher rates of filtration relative to oxygen consumption were found in the young active forms, and they gradually decrease with growth. As pointed out by Jørgensen (1952) this is probably because young growing forms utilise much more nutrients and consequently higher rates of water transport corresponding to oxygen consumption might be expected in young than in adults.

TABLE III

Relationship between rate of water transport and oxygen consumption in Martesia fragilis

No.	Wet weight mg.	Filtration rate l/c.c. O_2 consumption
1	7·20	4·440
2	8·50	4·465
3	11·25	1·510
4	12·00	1·673
5	17·20	1·060
6	18·25	1·280
7	19·00	1·400
8	21·00	2·180
9	34·50	1·390

DISCUSSION

Filtration in relation to size

Martesia fragilis which has been used in the present investigations, is the smallest of the lamellibranchs hitherto investigated, ranging from 3·25 to 73·25 mg. by wet weight. Previous work (Zeuthen, 1947, 1953) has shown that the body size plays an important role in any activity of the animal and an increased metabolism and activity is noted in the smaller individuals. It was thought of interest to use the rate of water filtration as an indication of activity in *Martesia*.

Martesia fragilis was observed to filter at the rate of 4·316 c.c./mg./hr. (for 3·25 mg. weight animal) while an animal of 73·25 mg. weight filtered at the rate of 0·594 c.c./mg./hr. However, there were fluctuations. The total filtration ranged from 14·03 c.c./hr. to 55·59 c.c./hr. for an increase of weight from 3·25 to 73·25 mg. A wide scatter was noticeable when both the total and unit values were plotted on a double logarithmic grid. However, when plotted on a semilogarithmic scale it was noticed that the total filtration seemed to show a linear relationship with size up to 27 mg. wet weight group beyond which this was not maintained. The rate of water filtration showed a sharp fall up to this weight group and the rate was found to be not significantly proportional to decrease as before.

The filtration rate of *Martesia* when compared with those of the other lamellibranchs reported by earlier workers seems to be high (Table IV). In *Martesia* the average rate of water transport of different sizes during short duration experiments was 1·44 lit./gm./hr., the range was from 4·316 lit./gm./hr. to 0·421 lit./gm./hr. (The results are expressed as lit./gm./hr. for comparison). Jørgensen (1960) found that *Mytilus* filtered 1·48 lit./gm./hr. which according to him corresponds closely to normal daily average filtration rates of submerged animals.

TABLE IV

Rate of water filtration in various lamellibranchs

		Wet weight gm.	lit./gm./hr.	
Mytilus edulis	..	1·0	1·48	Jørgensen (1960)
Pecten irradians	..	3·3	0·99	Chipman and Hopkins (1954)
		20·6	0·71	
Crassostrea virginica	..	12–14·0	1·00	Loosanoff and Nomejko (1946)
Mytilus californianus	..	1·5	0·17	Rao (1953)
Lasaea rubra	..	..	53 c.c./mg. N/hr.	Ballentine and Morton (1956)
Martesia fragilis	..	0·0032	4·316	Srinivasan (present study)
		0·0732	0·594	
Average rate	..	..	1·44	lit./gm./hr. or
	..		55·3	c.c./mg. N/hr.

In most of the above cases it has been shown that the rate of water propulsion increases with decreasing size. Further, taking individuals of comparable weight there is a tendency for the rates to be higher in smaller individuals and lower in larger individuals. The smallest individuals studied in all these cases are around 1 gm. But *M. fragilis* is a very much smaller species and most of the individuals are only 1/100-th to 1/15-th the weight of the smaller individuals of *Mytilus edulis* that have been studied (1 gm.—Jørgensen, 1960). Studies on *Crassostrea virginica* using both the direct and indirect method (Loosanoff and Nomejko, 1946; Loosanoff, 1958; Galtsoff *et al.*, 1947; Jørgensen, 1952) show that they filter a little less than 1 lit./gm./hr. and the bay scallop *Pecten irradians* (Chipman and Hopkins, 1954) also filtered at rates (0·99 lit./gm./hr. wet weight) similar to the oyster. For 1·5 gm. *Mytilus californianus*, Rao (1953) showed that it filters at the rate of 0·17 lit./gm./hr. A similar rate was obtained for *Mytilus edulis* by Willemsen (1952) on animals of comparable weights. When comparing curves given for *Mytilus californianus* (Rao, 1953, Fox *et al.*, 1937; Segal *et al.*, 1953) it will be noted that *Martesia* has higher filtration rates than *Mytilus californianus*. The filtering rate of *Lasaea rubra* (Ballentine and Morton, 1956) has been observed to be 53 c.c./mg./N/hr.

From the above data it will be found that the filtration rates of *Martesia fragilis* are higher than in *Mytilus californianus*, *Pecten irradians*, and *Crossostrea virginica*. The average filtration rates of *Martesia* are closely similar to those of *Lasaea rubra* of comparable sizes.

The ratio between O_2 uptake and rate of filtration in *Martesia* is comparable to that found in *Lasaea rubra* (Ballentine and Morton, 1956). Jørgensen (1966) gives a table showing this relationship in different lamellibranchs. The values of filtering rates per c.c. of oxygen consumed by *Martesia* could not be compared with other forms, since, these animals are smaller in size. In *Martesia*, in the smaller forms, higher rates of filtration was found, which decreased with increase in size, indicating that as the animal grows the water transport relative to oxygen consumption decreases gradually. This is probably because young and fast growing animals utilize more of the nutrients for growth and combustion than adults as suggested by Jørgensen (1952).

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SOME DATA ON THE BIOLOGY AND GROWTH OF *MYTILUS GRAYANUS* DUNKER IN EXPERIMENTAL CAGES IN THE PETER THE GREAT BAY

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ABSTRACT

The determination of the rate of growth and the duration of life of *Mytilus grayanus* is of atmost importance for the cultivation of commercial fisheries. The most wide-spread method of determining the growth and the age of the mollusc is based on the measurement and computation of annual growthrings which are formed on the shell.

However, in waters of middle latitudes growth of the mollusc can continue the year around so that it becomes difficult to determine the annual increment. The above is typical of the *Mytilus grayanus* of the Peter the Great Bay. The study of the growth of this mollusc should therefore primarily be based on experimental observations.

In August 1964 in the north-eastern part of the gulf in the vicinity of the Putjatin Island cages were submerged to the depths of 10, 15 and 18 metres. The cages made of iron bars had 30 centimetre props and were mounted on slabs of reinforced concrete. The cages were filled with tagged mussels by divers equipped with diving-lungs. For tagging small incisionss of a depth of not more than 1 mm. were made in two or three places on the front edge of the shell. The mussels were collected in the direct proximity of the cages and were put into them in natural groups together with small stones to which they were fixed. Overgrowth star-fish were removed from the shells. About 400 mussels were put into the cages. The maximum and minimum sizes of these were 185 and 30 mm respectively. Besides these several large groups of tagged mussels and single tagged mussels were placed on the concrete foundations of the cages.

After a year, in August 1965 the mussels were removed from the cages and new ones put in. The mortality of the mussels in the cages was found to be comparatively small—about 5%. The cages were in a good condition and could be used for further experimenting.

The results of the analysis of the data received show a very slow growth in length of *Mytilus grayanus*. Young individuals 30–70 mm long were found to have the maximum of annual increment equal to 13–15 mm a year.

After that the rate of growth falls abruptly so that the increment of mussels of 120–140 mm length is equal to 0–3 mm a year. In individuals of a size larger than the above-mentioned—the increase was less than 1 mm a year. Of all the mussels left unattached on the concrete slab only 25 individuals 120–150 mm in length survived. These were attached anew to the slab. Their increment is of the same order as of the individuals grown in cages.

Obviously *Mytilus grayanus* attains its maximum size at the age of 55 years and more.

At the present time, considering the steady increase in the catch of molluscs the world over and considering the extensive prospect of artificial cultivation of some species, the study of vital activities of commercial species relating to their growth and development is especially important.

Mytilus grayanus Dunker is one of the chief objects of commercial fisheries of invertebrates in the Peter the Great Bay of the Japan Sea. However up to now the biology of this species has not been sufficiently investigated.

In 1934 Razin determined the principal sites of accumulation of mussels in the bay. In a paper by Markovskaya (1952) there are some data on the terms of reproduction of *Mytilus grayanus* and

on its attaining sexual maturity; there are also data on the growth of mussels obtained by counting rings on shells of specimens not over 100–120 mm in length.

In the Peter the Great Bay *Mytilus grayanus* inhabits the sublittoral zone at the maximal depth of 50 m on rocks, silt or sand. According to inhabiting conditions in the district investigated (Putjatin Island) three groups of mussels can be defined, each having a morphologically different form of shell. Mussels taken from silt at the depth of 20 m have a delicate light shell with a black periostracum and a pearly layer weakly developed. Mussels from rocky ground have thick abruptly curved valves and a well-developed dark blue or violet mother-of-pearl layer. On silty-rocky ground at the depth of 4 meters mussels with brown periostracum and fine concentric rings on valves were found. Ecological groups of the same kind are observed in mussels of other species—*Mytilus galloprovincialis* and *Mytilus edulis* (Vorobieff, 1939; Palenichko, 1947; Matveyeva, 1948; Lubinscky, 1958).

Mussels can form considerable accumulations. However most often their colonies consist of small compact groups (so-called druses) from 2–3 to 15–20 specimens attached in small hollows of large boulders or on small stones. On the surface of druse only the largest specimens are found; young mussels ranging from 1·0 to 3·0 mm can be found only in byssus threads of the adult mussel in the middle of the druse. It has been observed that the smaller the specimen the further away it is from the surface. Mussels of 15–16 mm have shells covered by thick hair-like growths which cause the young specimens to be "tangled" in the byssus of the older specimens.

Young specimens of 30–40 mm attach these hard bristle growths firmly on the valves of grown mussels independently of their byssus. Analysis of the structure of 25 druses of mussels collected at various depths and on various kinds of bottom as to the size of the specimens showed that more than 50% of all mussels are young specimens of 5·0 to 15·0 mm; about 40% are young specimens of 0·9 to 5·0 mm. A small percentage (4–7%) are mussels of older age groups, 110–150 mm in length, and 3–6% are mussels of 50–90 mm. An almost total absence of mussels of 50–70 mm is characteristic of the majority of druses. The penetration of larvae of mussels into byssus threads of the adult specimens may take place first of all as a result of deposition of the larvae of dissoconch directly on the shell and its active movement along the valve and *secondly* passively as a result of being caught by mussel during filtration. It is known that the larvae of bivalve mussels during all stages of development passing through the intestine and mantle cavity of adult Lamellibranchia retain their vitality, and, after getting rid of slime, continue their normal development (Goikher, 1949). Therefore the inclusion of young specimens into the druse in this way is not excluded.

In druse the young specimens at first find favourable conditions for their nutrition. They are protected from predators and other unfavourable external conditions. Mortality in young specimens of mussels in druse is insignificant. However, after reaching a definite size young specimens cannot develop normally and try to move to the surface of the druse. According to observations (Cahn, 1951) not only young specimens, but adults as well can move actively along the substratum. Thus, the insignificant number of mussels of median sizes in the druse can possibly be explained by their gradual settling along the substratum. As in the case of most molluscs the shell of *Mytilus grayanus* is covered by concentric rings more or less sharply defined. These rings are formed as a result of periodical stops or delays in growth under unfavourable conditions and are annual. However in some of the forms—*Mytilus californianus* (Coe and Fox, 1943), *Pecten maximus*, predatory Gastropoda (Haskin, 1954)—the rings on the shell are formed twice a year. Therefore it must be proved experimentally for each of the species whether it is rightful to consider the rings as annual marks. Besides this, in mussels exceeding 100–120 mm in size the rings loose their sharp contours, overlap and are unsuitable for analysis of growth.

All these reasons called forth the necessity of determining the growth of *Mytilus grayanus* and the periods of ring formation with the aid of stationary submerged cages.

In 1964, 12 cages were set at three sites near the Putjatin Island at 10–12 and 20 metre depths. The mussels were collected directly near the cages and after the fouling matter and starfish had

been carefully removed were put into the cages by druses together with their natural ground. In order to study the annual increment, 2–3 transverse incisions were made on the posterior growing margin of the mussel with the aid of a file. 763 specimens ranging from 30 mm to 185 mm in size were put into the cages. All under-water operations were performed by divers provided with diving lungs. In contradistinction to the cages used previously for the determination of growth of *Mytilus californianus* and *Mytilus galloprovincialis* (Coe and Fox, 1943; Ivanov, 1967) which were checked every month, our cages were designed to remain submerged for a long time. They were constructed of iron bars set on props which were fixed to slabs of reinforced concrete. The cages were 80×50×30 cm, the cells of the walls and upper cover 4·0×4·0 cm. The area of the concrete slab corresponded to the area of the bottom of the cage.

In a year, in 1965, part of the material was removed from the cages and new mussels put in and in 1967 all the mussels were taken out for investigation purposes. Cages which had been submerged for one year were in a good state; the cells of the cages which had been submerged 2 and 3 years were almost clean of fouling. However, they could not be used any more because the props supporting the cage on the slab were brittle.

Besides the mussels, several sea-urchins were found in the cages and in one of the cages there was a large starfish. Mortality of the mussels remaining in cages for a period of one year was 5–6%; for a period of three years 14%. Investigation of the mussels showed that incision made on the growing margin cause a perceptible delay in growth which becomes regular only at the beginning of a new period of intensive growth. For this reason the material of the first year (150 specimens) was excluded from the analysis of the rate of growth. As a result 460 specimens of mussels were studied.

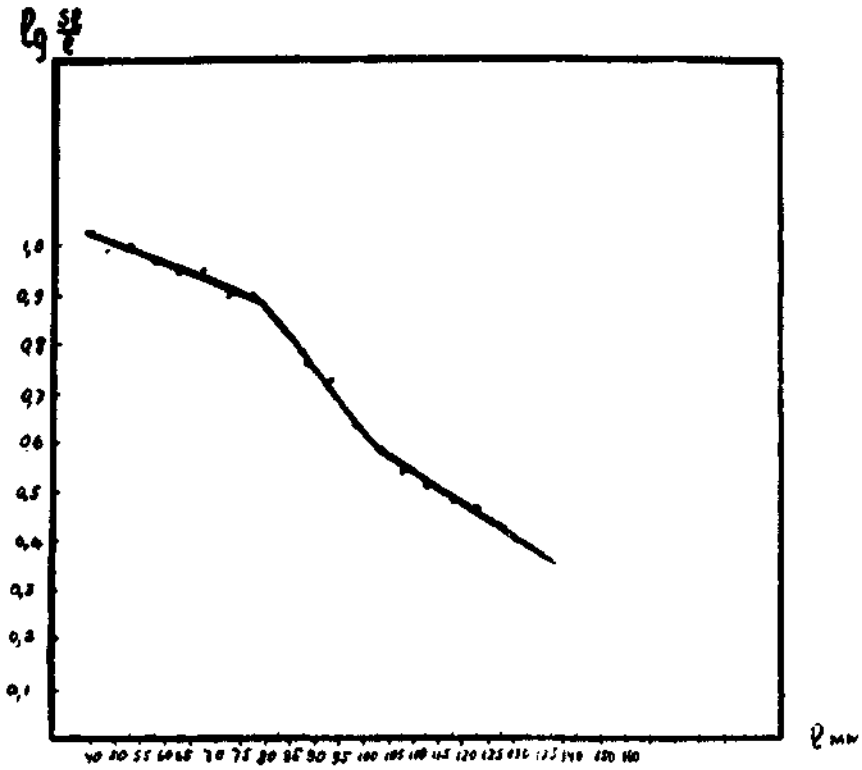

FIG. 1

Mussels, remaining in cages for one year had on the newly formed layer one clearly distinguished concentrical ring besides a shallow additional spawning ring. Those remaining for 2 years —2 rings, for 3 years—3 rings. This confirms the fact that *Mytilus grayanus* has annual check marks of the stoppage of growth connected with the seasonal fluctuation of temperature (the difference between summer and winter temperatures in Peter the Great Bay is equal to 22°).

The most significant annual increment in cages is found in mussels having a length of 30 mm (minimal size in our experiment) to 75–80 mm, median increments of the above-mentioned size classes are accordingly 12 mm and 8 mm a year, then increment decreases so that for mussels of 120–125 mm it averages 3·0 mm and for mussels 140–145 mm in size only 2·0–2·5 mm a year.

Mussels grow during their whole lifetime but as they near their size limit (180–190 mm) growth in length becomes very small and often the newly formed layer of periostracum during one year hardly reaches the margin of the valve. In the majority of cases mussels of 150 mm and more seldom have a new layer of more than 1·0 mm. In Fig. 1 where the dependence of increment on the size of the mussel is represented logarithmically two principal sections with different rates of growth of mussels can be defined.

When the size of 80–85 mm is reached the rate of growth decreases noticeably and the mussel grows more and more slowly. In large specimens it can be said that the rate of growth is levelled but that takes place as a result of a very small yearly increment in these forms. On the basis of growth data obtained and assuming the age of the smallest mussels in the cages (30 mm) to be 2 years (calculations were made with the aid of check marks) a formula of the relationship between the length of the mussel and its age has been obtained.

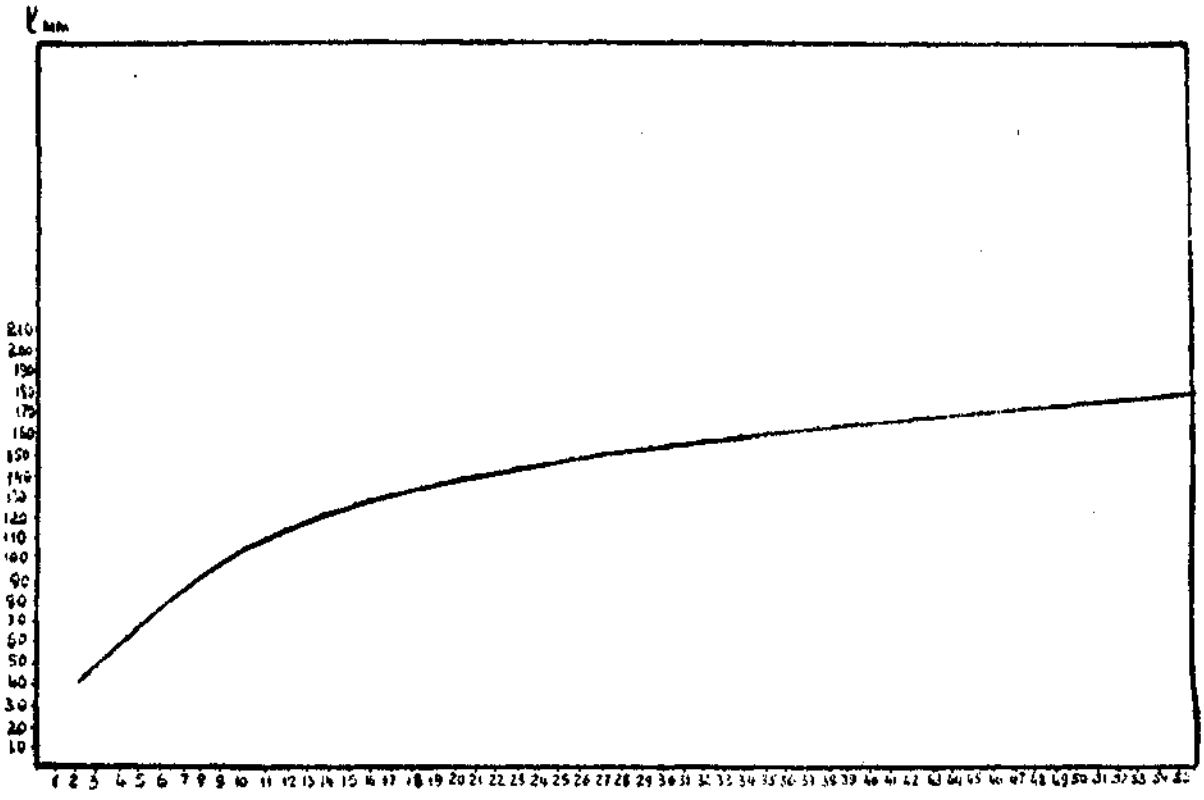

FIG. 2

$$L = L_0 + 1/\beta \log [(T - T_0)\, 1/K + 1]$$

where

$$K = \frac{1^{-a+\beta \cdot 04343\, t_0}}{0{\cdot}4343\, \beta}$$

L, L_0—length of mussels.

T-T_0—age of mussels.

a and β—coefficients calculated for each section of the logarithmic curve.

Growth curve calculated by means of this formula is shown on Fig. 2. Deceleration of the rate of growth which begins after 8–9 years is seen. According to our data mussels reach the length of 160–165 mm (the usual size of large mussels) at the age of 40–45 years. However the largest and oldest specimens (185–190 mm) which have insignificant yearly increment can reach the age of 55 years and more.

It has been recorded in literature that long life duration is characteristic of oysters. *Crassostrea virginica* is known to live upto 40 years (Loosanoff, 1965).

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A NOTE ON OYSTER DIMENSIONS

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ABSTRACT

Although shell shape in oysters is highly variable it becomes much less so when the specimens are grown in uniform conditions with ample space. Under such conditions an underlying distinctness of species dimensions becomes apparent.

INTRODUCTION

As pointed out by Yonge (1960) and others the dimensions of oyster shells are highly variable. Nevertheless two basic types were distinguished by Orton (1928), a subcircular shell typical of the genus *Ostrea* sensu strictu and the elongate shell typical of the genus *Crassostrea* (*Monoeciostrea* and *Dioeciostrea* of Orton). The genus *Pycnodonte* possibly presents a third type, being rather elongate but sub-globular, the lower shell being massive in the adult (Thomson, 1954).

Nelson (1938) pointed out the great variability within a species and gave an instance of shells of *C. virginica* which complied with the descriptions of other species of the genus, and reported one shell so extreme that it was identified as a *Spondylus* by a person familiar with the group. The variability is doubtless in response to variables in the environment. The present study was undertaken to test whether there would be distinguishable differences in the dimensions of different species grown under identical conditions.

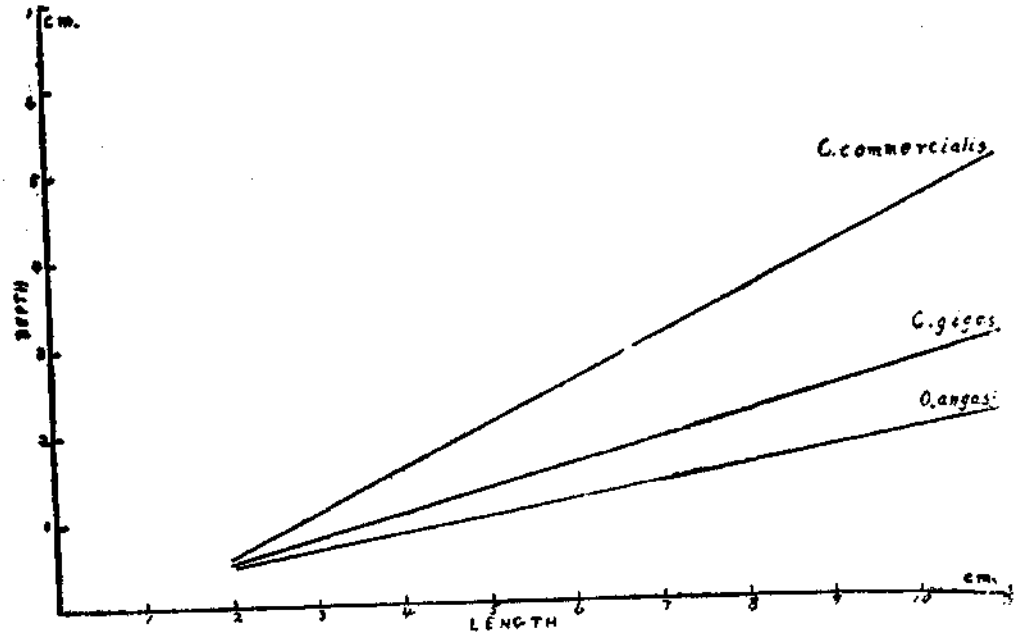

FIG. 1. Regression of depth on length for three species of oysters.

Material and Methods

Specimens of *Ostrea angasi* Sowerby, *Crassostrea commercialis* (Iredale and Roughley), and *Crassostrea gigas* (Thunberg), known to be less than 12 months old were set out in adjacent compartments on wire trays in Pittwater, Tasmania (*See* Thomson, 1952). They were measured at irregular intervals over a period of three years. Vernier calipers were used for measuring the shells. Weights were determined by a simple beam balance.

The "length" as used in this paper signifies the greatest distance from the hinge end to the lip end which is the usage of Loosanoff (1947) and others, but not of Orton (1926) who on anatomical grounds (the hinge is dorsal in the larva and antero-dorsal in the adult) termed this axis the height or depth.

"Width" is taken to be the greatest dimension at right angles to the length measured roughly in the plane of the upper (right) valve. This again is the usage of Loosanoff and Nomejko (1949), but corresponds to the "length" of Orton (1926).

"Depth" was uniformly estimated as the distance between the outer surfaces of the two valves at the point where the axes of the other two dimensions crossed. This is the "breadth" or "width" of Orton (1926). This dimension is termed the "section" by some conchologists (*e.g.*, Cotton and Godfrey, 1938).

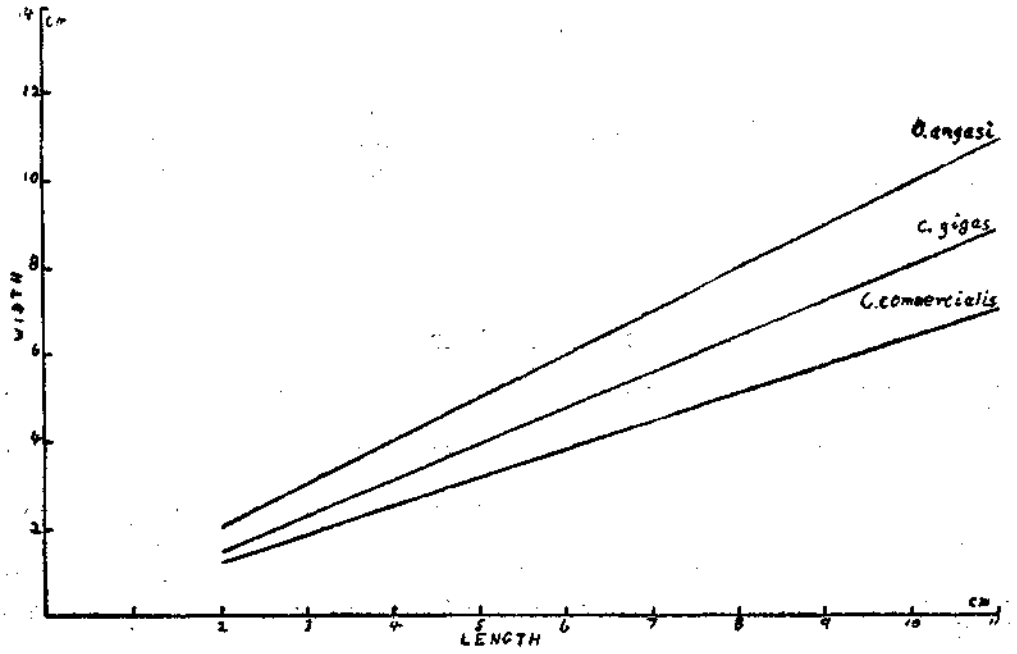

Fig. 2. Regression of width on length for three species of oysters.

Results

The regression lines of depth on length, and width on length are shown in Figs. 1 and 2. There was considerable scatter about each regression line, but although the scatter bands for each species overlapped to some extent that of the other species, a central tendency is evidenced for each. The regression equations for the lines are given in Table I.

TABLE I

Regression equations for depth on length, and width on length for each of three species of oyster

	Depth on length	Width on length
O. angasi	.. $Y = 0{\cdot}45 + 0{\cdot}18X$	$Y = X$
C. gigas	.. $Y = 0{\cdot}10 + 0{\cdot}25X$	$Y = 0{\cdot}96X - 1{\cdot}01$
C. commercialis	.. $Y = 0{\cdot}44X - 0{\cdot}11$	$Y = 0{\cdot}65X - 0{\cdot}10$

A higher proportion of the weight of *O. angasi* is contained in the shell than in the *Crassostrea* species. All species showed a decline in the relative contribution of fluid to the weight with age (Table II).

TABLE II

Proportions by weight (a) at one year; (b) at three years

Species		Shell	Meat	Fluid
O. angasi	(*a*) ..	81·8%	14·0	4·2
	(*b*) ..	81·0	16·7	2·3
	(*a*) ..	73·5	15·5	11·0
C. gigas	(*b*) ..	74·2	17·9	7·9
	(*a*) ..	75·6	12·9	11·5
C. commercialis	(*b*)	75·9	15·8	8·3

The relatively low proportion of fluid in *Ostrea angasi* is notable.

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DIFFERENT RADIOSENSITIVITY OF MALE AND FEMALE GERM CELLS OF *PHYSA ACUTA* DRAPARNAUD (GASTROPODA, BASOMMATOPHORA)[1]

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ABSTRACT

X-radiation decreases the fertility of *Physa acuta*, a freshwater snail. Histological evidence, as well as results of cross-breeding experiments, show that the male germ cells are more radiosensitive than the female germ cells.

INVESTIGATIONS carried out in our laboratory have shown that the reproductive capacity of *Physa acuta*, a freshwater snail, is reduced by exposure to acute doses of X-rays (Ravera, 1966, 1967; Ravera and Mariani, 1966).

The present report deals with histological observations made on the germ cells of irradiated *Physa*. Adults collected from the littoral zone of Lake Maggiore (Angera) were exposed to 10 KR[2] (250 KV, 10 mA) of X-rays at a dose rate of 200 R/min. Such a dose, which reduces to zero the viability of eggs spawned during the first 50 days after irradiation of the parents, has no significant effect on adult mortality (Ravera and Mariani, 1966). One group of animals was sacrificed 105 min. after irradiation and the other at the 19th day. They were fixed in Bouin's fluid. To avoid differences due to histological techniques each irrdiated individual was embedded with an unirradiated control in the same block of paraffin and then cut in serial sections (15 μ), which were then stained with aqueous haematoxylin and alcoholic eosin.

Examination of the slides (Fig. 1) reveals that: (1) male and female germ cells were present at different stages of development in the same acinus of unirradiated individuals; (2) there were no differences between the germ cells of irradiated individuals sacrificed 105 min. after irradiation and those of the controls ; (3) 19 days after irradiation, oocytes appeared morphologically normal at various stages of development; (4) in the same ovotestis, however, no male germ cells were found whereas immature male germ cells were always present in the controls, even during the resting period of sexual activity; (5) cellular detritus, probably the degenerated male cells, and some phagocytes were observed in the lumen of the acinus of the irradiated individuals sacrificed at the 19th day [it is known that the germ cells of molluscs are ingested by phagocytes after degeneration (Bacci, 1951]; (6) the *receptaculum seminis* of irradiated individuals, as well as that of unirradiated ones, was filled with spermatozoa. This fact may be due to the radioprotective action of seminal fluid as has been reported for starfish, urchins, and polychaete worms (Rugh, 1953; Henshaw, 1940; Evans *et al.*, 1942).

From these observations it seems that the male germ cells of *Physa* are more radiosensitive than the female ones, and that the reduction of viability of eggs spawned by irradiated adults is due mainly to the damage produced to the male cells. This hypothesis is supported by results on the viability of zygotes resulting from the fusion of gametes produced by two *Physa acuta*, one of which was

[1] This publication is contribution No. 295 of the Euratom Biology Division.

[2] KR = Kiloroentgen (10^3 roentgen).

irradiated and the other not. The viability of zygotes produced by normal spermatozoa and X-irradiated ova was higher than that of zygotes produced by irradiated spermatozoa and normal ova at the same dose (Table I). Greater sensitivity of male germ cells, at least the youngest stages (spermatogonium), has been observed also in *Arion rufus*, L. (Laviolette et Cuir, 1959).

TABLE I

Fecundity, fertility, and viability calculated on 7180 *embryos of* Physa acuta *during a period of* 60 *days after exposure to* 10 *KR (X-rays). Fecundity = number of eggs that would be produced by* 1000 *adults in one day; fertility = number of hatchlings produced by* 1000 *adults in one day; viability = percentage of hatchlings per* 100 *eggs.*

Germ cells male		female	Fecundity	Fertility	Viability	Total eggs
0	×	0	7346	6744	91·8	4628
0	×	10 KR	5907	3287	55·6	616
10 KR	×	0	6844	1378	20·1	638
10 KR	×	10 KR	4554	365	8·0	1298

ACKNOWLEDGEMENTS

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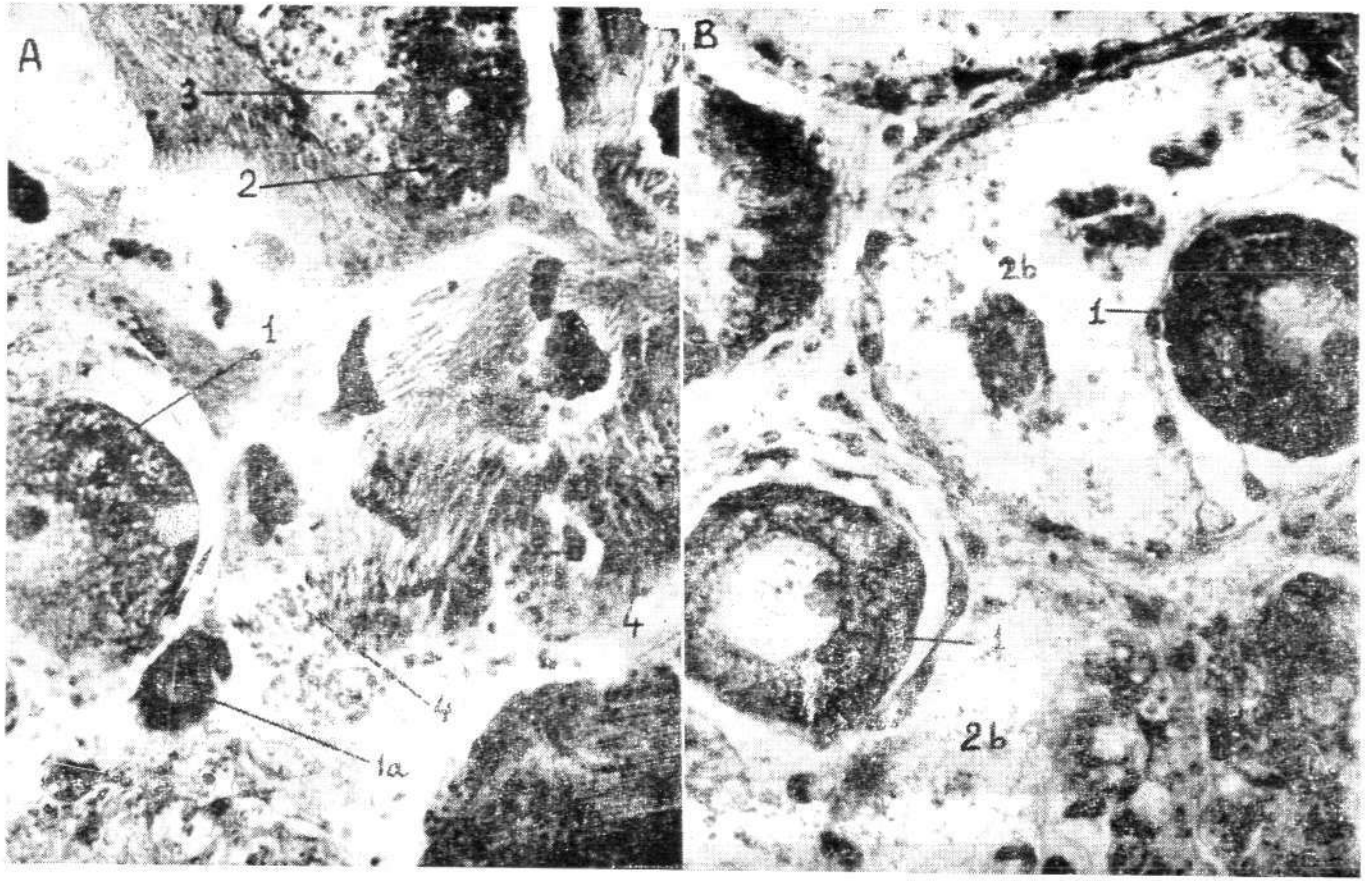

FIG. 1. Histological sections of ovotestis of *Physa acuta*. A. unirradiated; B. exposed to 10 KR and sacrified at 19th day after irradiation. 1. mature oocytes; 2. primary spermatocytes; 3. secondary spermatocytes; 4. spermatozoa; 1 *a*. young oocyte; 2 *b*. cellular detritus, (× 337).

FALLOUT ^{54}MANGANESE AND STABLE MANGANESE IN SOME FRESHWATER LAMELLIBRANCHS[1]

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ABSTRACT

The appearance of radioactive substances in the biosphere due to nuclear weapons testing and to the increasing number of reactor installations, has focused the attention of scientists on the importance of detecting these substances in organisms and their environment.

Certain organisms have proven to be excellent indicators for particular radionuclides and their stable chemical elements. The freshwater mussel *Unio mancus* var. *elongatulus* (Pfeiffer) from lakes of Northern Italy is an excellent biological indicator for fallout ^{54}Mn. This bivalve is able to concentrate both radioactive and stable manganese thousands of times over that found in the environment. Data are presented on stable and radiomanganese (fallout ^{54}Mn) in some freshwater lamellibranchs and the results discussed in the light of the animal and its environment.

FRESHWATER molluscs of the Class Pelecypoda (Lamellibranchiata), Family Unionidae, are found in most bodies of permanent water, such as streams, ponds, and lakes, all over the world. *Unio mancus* variety *elongatulus* (Pfeiffer) is one of the dominant macroinvertebrates of the littoral zone of the subalpine lakes, Lake Maggiore (Lago Maggiore) and the Lake of Varese (Lago di Varese), in Northern Italy (Fig. 1). *Anodonta cygnea* L. is also found in large numbers, particularly where the sediment is rich in organic matter. It is not as common as *Unio* which survives on sandy, rocky, or muddy lake bottoms. Like most freshwater molluscs, these bivalves spend their juvenile and adulthood in a restricted area and hence, reflect their immediate environment of water and sediment. If one adds to this sedentary existence the ability to accumulate and retain certain stable elements in relatively high concentrations, one has, for all intents and purposes, an ideal biological indicator for the radioisotopes of these same elements. It is implicitly understood, of course, that the physical-chemical state of the radioelement is the same as that of the stable element so as to render it available to the animals.

Fallout ^{54}Mn is formed by neutron activation of iron (^{54}Fe) and stable manganese (^{55}Mn) in the shells of detonated nuclear weapons. After the detonation of the Castle series of nuclear devices in 1954 in the Pacific Proving Ground, high levels of activity due to non-fission products were observed. Gamma spectra studies revealed, among other radioisotopes, the presence of ^{54}Mn in the kidney of the clam from Belle Island, and in other organisms (Lowman *et al.*, 1957). Subsequently, Lowman (1960) reported ^{54}Mn in the marine biota at the Eniwetok Test Site and the first report from Europe was in 1961 by Ravera and Vido. Since then many other reports on fallout radiomanganese have appeared in the literature (Ravera and Gaglione, 1962; Folsom *et al.*, 1963; Malvicini *et al.*, 1963; Scheffer and Ludweig, 1963; Ravera, 1964; Cavalloro and Ravera, 1965; Merlini, 1966; Gabay *et al.*, 1966).

In 1960 Ravera and Vido (1961) collected *Unio* from four different stations in Lago Maggiore and measured the radioactivity by gamma spectrometry. They found the soft tissues of these molluscs more active than the shells; and a comparison of the activity (on a unit weight basis) showed

[1] This publication is contribution No. 369 of the Euratom Biology Division.

that it varied according to collection site. The activity was due to ^{54}Mn, despite the fact that this radioelement could not be detected in the lake water, plants, or other organisms at that time.

Anodonta cygnea, collected in the fall of 1961 by this author from a river bed in Bologna (Italy), the Lago di Varese, and the Lago Maggiore, gave the following results:

Collection Site/Date	^{54}Mn, pCi/g dry weight Shell	Soft Tissues
River bed, Bologna, Nov. 13, 1961	not detected	1·12
Lago di Varese, Oct. 23, 1961 ..	0·11	24·0
Lago Maggiore, Sept. 28, 1961 ..	2·2	36·1

The results on *Anodonta* coincided with those found for *Unio*, that is, the soft tissues were more active than the shell, and the activity changed with the site of collection. However, when ^{54}Mn was determined in the different organs and tissues of *Anodonta*, only the gills had sufficient activity to be revealed under the conditions employed. Later, Ravera and Gaglione (1962) studied the organs and tissues of *Unio* for their ^{54}Mn content and also found the gills one of the most active parts. But unlike *Anodonta*, the mantle had measurable amounts of fallout radiomanganese. In addition, they noted that the amount of ^{54}Mn increased with the size or age of the animal. By December, 1963, Gaglione and Ravera (1964) were able to detect ^{54}Mn in all parts of *Unio mancus* of Lago Maggiore and the distribution was as follows: gills and mantle with the highest concentrations, visceral sac, foot, and lastly, the extrapalleal liquid. They had also examined *Anodonta cygnea* but found these bivalves had accumulated about one-third that found in *Unio*. When these authors expressed their data as per cent of the ^{54}Mn content in the whole animal, the results were similar to that obtained by Merlini (1962) for stable manganese in the same parts:

	% Stable Mn	% Fallout ^{54}Mn
SHELL ..	34·4	35·0
SOFT TISSUES ..	65·6	65·0
Gills ..	23·6	18·0
Mantle ..	25·4	38·0
Visceral sac ..	16·6	8·7

A comparison was made between *Unio mancus* of Lago Maggiore and the same species from Lago di Varese for their stable and radio-manganese content and distribution by Merlini (1966). Animals of different size classes were collected in 1964 and analyzed. The following conclusions were drawn:

(1) *Unio mancus* from an environment richer in stable manganese (Lago di Varese) than the other environment (Lago Maggiore), incorporated more of the element in its shells and soft tissues. This was true for each of the four size classes studied.

(2) The opposite was true for fallout ^{54}Mn: *Unio* of Lago Maggiore concentrated more radiomanganese than those from Lago di Varese. The higher number of stable manganese atoms dilutes the number of radioactive atoms of ^{54}Mn accumulated by the animal, and

this results in lower specific activities expressed as atoms of ^{54}Mn to atoms of stable manganese in *Unio* of the Lago di Varese.

(3) In both populations of this species the soft tissues had more manganese than the shells, and the rank order was as follows: gills, mantle, visceral sac, adductor muscles, and shell. The rank order for ^{54}Mn enrichment in the different parts of *Unio* followed that of stable manganese except for the adductor muscles which had no demonstrable activity under the conditions employed.

(4) This bivalve is an excellent indicator of contamination of an environment by fallout ^{54}Mn since the shells of the animals of the smallest size group had approximately one atom of ^{54}Mn for every 10^{19} atoms of stable Mn.

It has been sufficiently demonstrated that there is a correlation between the stable element content of a tissue or organ and its fallout ^{54}Mn content in *Unio*. When the data on stable Mn in *Anodonta* were analyzed, it was found that the distribution of the element in this mollusc was quite different from that in *Unio* (Merlini *et al.*, 1965). Only the gills have an increased quantity of stable manganese; the mantle was found to have about one-third the amount. This accounts for the fact that ^{54}Mn was demonstrable only in the gills when determined in 1961.

The difference in distribution of stable manganese in *Unio mancus* and *Anodonta cygnea* of Lago Maggiore collected from the same site, can be seen in the following table. The percentages reported for each organ are based on the total amount of stable Mn found in the soft tissues alone (Merlini, unpublished data):

Animal	Gills + Palps	Mantle	Visceral Sac	Adductor Muscle
Unio mancus ..	37·2%	36·2%	24·3%	2·2%
Anodonta cygnea ..	84·5%	10·0%	5·3%	0·2%

Although a direct comparison between *Anodonta* and *Unio*, based on the size or age of the animal, is not possible, it is still interesting to compare stable and fallout ^{54}Mn in certain organs of these bivalves for verification of what has already been discussed. Both species were collected from the Lago di Varese in May, 1964. The results are given for dry weights (Merlini, unpublished data for *Anodonta*; Merlini, 1966 for *Unio* results):

Length	Gills		Visceral Sac	
cm.	Mn, ppm	^{54}Mn, pCi/g	Mn, ppm	^{54}Mn, pCi/g
		Anodonta cygnea		
7·0–7·6	35,506	289	906	14
7·8–8·2	39,740	232	2167	21
8·5–9·0	39,204	211	2297	21
		Unio mancus		
5·8–6·0	14,185	103	3571	32
6·7–6·9	18,257	100	4308	33
7·2–7·5	20,737	108	5034	32

Environmental radioactivity due to fallout had begun to diminish in 1965 (deBortoli *et al.*, 1966). Manganese-54 was detected in the water of the Lago di Varese (0·05 pCi/l) in December, 1965, but it was not detected in the water from Lago Maggiore collected at intervals during the entire year. In 1966 fallout ^{54}Mn was not measureable in water from either lake (deBortoli *et al.*, 1967). *Unio mancus*, collected in September, 1966, from Lago Maggiore showed 4·6 pCi/g soft tissues dry weight; and 0·38 pCi/g of shell (Merlini *et al.*, 1968). From this it can be deduced that radioactive manganese in *Unio* has a slow turnover, lasting long after the activity in the water is not detectable. In fact, from recent work, stable manganese appears to be firmly bound to some large molecule since it is not electrolytically dialyzed out of soft tissue homogenates (Merlini, 1966).

The process of uptake and concentration of polyvalent ions in bivalves was discussed by Korringa (1952), reviewed by Brooks and Rumsby (1965) and Merlini (1966). The general scheme is based on the entrance into the animal of ions with food or organic molecules brought in with the water. Divalent manganese is trapped by the mucus on the gills or passed into the digestive tract from where it is transported to all parts of the body, concentrating in particular organs and tissues. Recent studies by this author have shown that the pericardium and the tissue at the base of the gills concentrate stable manganese more than any other part of the soft tissues on a unit dry weight basis. The same had been observed by Bowen in other freshwater bivalves (1948); and it was also noted for zinc in *Anodonta californiensis* from the Columbia River (Pauley and Nakatani, 1967). The mantle, which is responsible for the formation of the shell, may utilize the manganese as a carbonate or as an ion since the ionic radius of Mn is known to fit into the calcium carbonate lattice. In addition, the shell, with its lower metabolic rate and turnover of incorporated stable and radioactive trace elements, makes the bivalves *Unio mancus* and *Anodonta cygnea* exceptional biological indicators of fallout ^{54}Mn in an aquatic environment.

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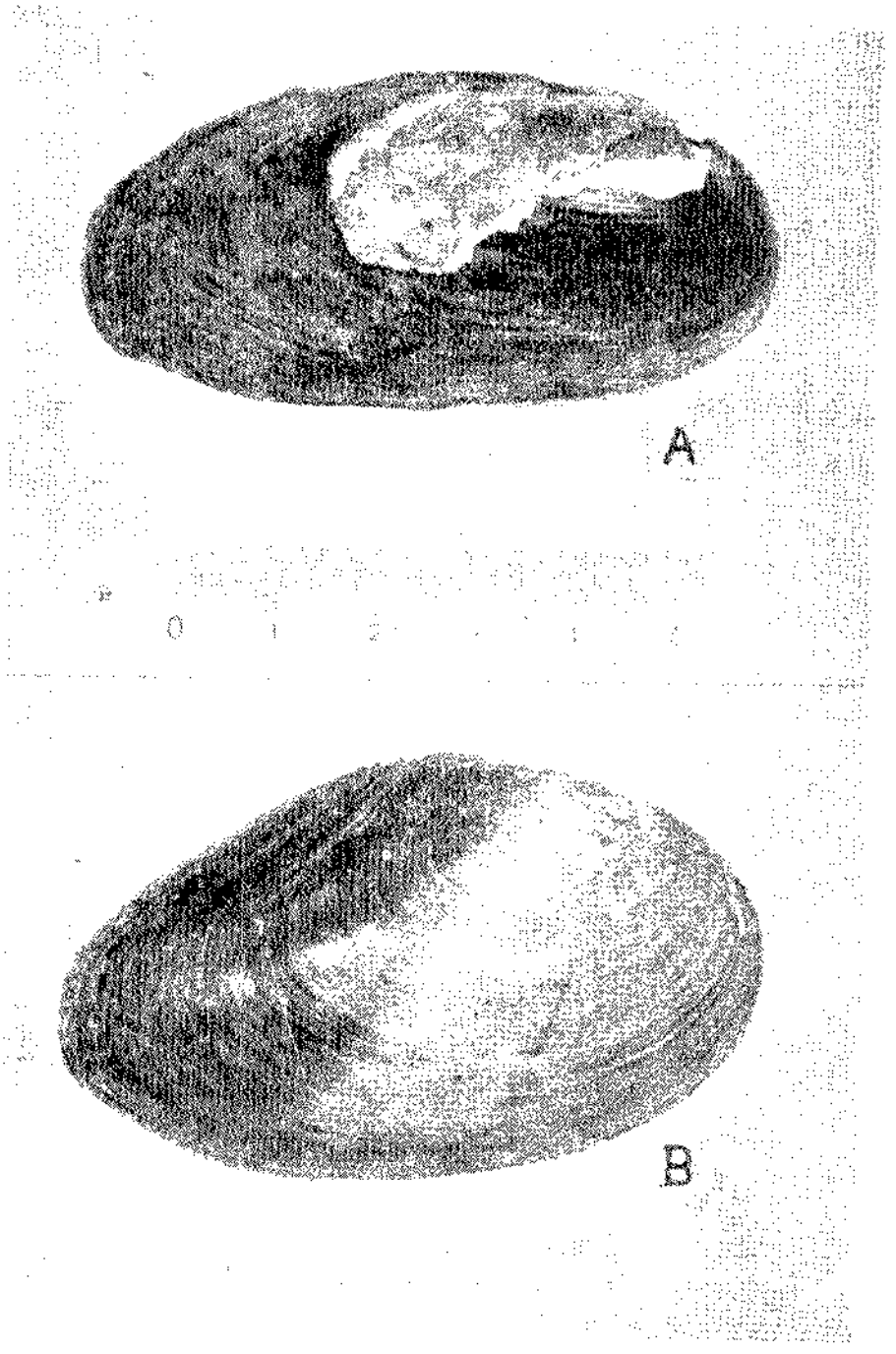

FIG. 1. — Two species of Unionidae from Lake Maggiore (Lago Maggiore) and the Lake of Varese (Lago di Varese) in Northern Italy. (A) *Unio tumidus* variety *elongatulus* (Pfeiffer). (B) *Anodonta cygnea*, L.

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CONCEPT OF ACUTE AND CHRONIC TISSUE CONCENTRATION OF ELEMENTS IN RADIOECOLOGY*

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ABSTRACT

The paper discusses the uptake-elimination patterns and concentration of elements and their radionuclides by aquatic organisms. The concentration and concentration factors of elements are further classified on the basis of respective metabolic roles. To differentiate two types of concentration and concentration factors encountered in radioecological studies, *viz.*, one obtained under natural environmental conditions and other under controlled laboratory conditions, the use of terms 'Chronic Concentration (CC) and Chronic Concentration Factor (CCf)', and 'Acute Concentration (AC) and Acute Concentration Factor (ACf)', respectively are suggested.

RADIOACTIVE NUCLIDES disposed in the hydrosphere are diluted, transported and concentrated by physical, chemical and biological processes. This has led ecologists to study the interaction between radionuclides and aquatic biota creating a new discipline of 'Radioecology,' which is a bridge between basic sciences (ecology, biogeochemistry and radiation biology) and applied sciences (nuclear technology, health physics and environmental engineering) (Odum, 1963). Polikarpov (1966) in his recent treatise on 'Radioecology of aquatic organisms' has further discussed the frontiers of this new discipline in detail.

Aquatic organisms extract and concentrate elements from the environment in which they grow. When effecting concentration often selectively, they can only discriminate the chemical species of the same element but have no ability to discriminate the radioisotopes of the elements absorbed. To study the pathway leading to concentration of radionuclides in biological systems from the different matrices of the environment under natural conditions, the concentration and concentration factors (Cfs) of the inactive and radioisotopes of elements—especially of those having conveniently long half lives—in tissues are being followed by number of workers (Vinogradov, 1953; Goldberg, 1957; Gong *et al.*, 1957; Ichikawa, 1961; Templeton, 1959, 1962; Brooks and Rumsby, 1965; and Mauchline and Templeton, 1966). Radioecologists in parametric studies, however, have devoted much of their attention to obtain Cfs of radionuclides by different organisms under laboratory conditions (Boroughs *et al.*, 1957; Rice and Smith, 1958; Townsley *et al.*, 1959; Chipman, 1960, 1966; Bryan, 1961, 1963, 1964; Bryan and Ward, 1962, 1965; Morgan, 1964; Keckes *et al.*, 1966; Patel *et al.*, 1966 *a*, *b*; and Polikarpov, 1966). Two sets of often widely different tissue concentrations and Cfs for elements have thus been obtained, *viz.*, one under natural environmental conditions and the other under controlled laboratory conditions. In this communication an attempt has been made to recognise these differences by proposing the term—'Acute Concentration (AC) and/or Acute Concentration Factor (ACf)'; and 'Chronic Concentration (CC) and/or Chronic Concentration Factor (CCf)'*. An approach has been suggested to interpret ACs and CCs of elements in the light of their metabolic significance.

It is desirable that the new terminology brings the types of exposure encountered in radioecological investigations in line with similar terms widely employed in radiobiological studies of external

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and internal irradiations (*cf.* Krumholz *et al.*, 1957). The acute effects are considered to be obtained under conditions (say laboratory conditions), where the organisms are exposed for short periods compared to the life span and the age of the organisms used in the experiments. The chronic effects are obtained under conditions including those in natural environment, where the organisms have been exposed for a duration comparable to their life span. To further elucidate the terminology the Cfs of some of the elements and their radioisotopes in marine organisms are given in Tables I–V.

Pillai and Ganguly (1967) in 'Systematics of acceptable radioactive contamination levels in the environment' suggested a classification of elements into three groups on the basis of respective metabolic roles. These groups are referred to in the present paper as group I, II and III. We recognise that elements belonging to these groups can in addition be species specific.

It is interesting to note from the data compiled in Tables I and II, that for metabolically significant trace elements (group I), *e.g.*, zinc, manganese, iron, cobalt, etc., which form parts of enzyme system, the CCfs are higher than the ACfs in the marine organisms studied. Table II demonstrates that CC is much greater than AC in tissues of shell-fish. It should be observed, for example in the lobster, that although in Bryan's (1964) experiments where the concentration of zinc is higher in an acute case by factor of about 20, the tissue concentrations do not differ markedly. However, Cfs have been observed to be consistently higher in chronic case than in acute (Table II). Further, tissue concentrations of these elements have been observed to be dependant to a small extent on the concentration of the elements in the growth medium (Bryan *et al.*, 1966). In accumulation of major elements of group I, *viz.*, Potassium, Sodium, Calcium, etc., the biological system regulates the tissue concentrations at somewhat constant level under different ambient conditions [*cf.* Bryan and Ward (1962) for potassium, Townsley *et al.* (1959) for sodium; and Townsley (1963) for calcium].

TABLE I

*Acute and Chronic concentration factors of induced radionuclides by marine organisms**

	Zinc		Iron		Cobalt	
	ACf	CCf	ACf	CCf	ACf	CCf
Green Algae ..	—	—	—	—	120–380†	15–440
Brown algae ..	190†	420–1400	1100†	1200–12500	45–420†	27–740
Red algae ..	840†	80	100–1650†	2000–4000	—	—
Sea anemones ..	70†	300	—	2400	20	1100
Malacostraca ..	26–37†	9400–15000	110	—	7–3†	220–770
Muscles ..	40†	1900–4400	20	1000–4000	—	4000
Molluscs ..	—	1400–31000	66†	50000	—	1–9800
Shell ..	10–49†	—	13–55†	—	36–500	—
Body ..	0·25–630	3500	25–100†	—	18–186†	—
Muscles ..	10†	2600–40000	30†	3000–17000	7†	52–200
Echinoderms (Viscera) ..	200†	—	30†	1000–10000	60†	—
Fishes ..	3·4	280–15500	5·8	—	2·5–5†	28–560
Muscles ..	1·4†	540–4400	0·05	400–3000	0·5	14–84

* Adopted from Polikarpov (1966). † Equilibrium not attained.

TABLE II

Levels of CC and AC of manganese and zinc in tissues of marine shell-fish

	Manganese		Zinc						Source
	CC (μgm/g) wet wt.	CCf	AC (mμCi/g) wet wt.	ACf	CC (μgm/g) wet wt.	CCf	AC (μCi/g) μgm/g*	ACf	
LAMELLIBRANCHS									
Anadara granosa									
Gills ..	16·23	4070	1·28	6·4	72·49	2030			Patel *et al.* (1966 *a*)
Viscera & foot	6·67	1667	0·56	2·8	46·68	1355			
Mantle folds ..	4·88	1220	2·30	11·5	91·47	2562	53·0	106	
Corpuscles ..	5·20	1250	1·00	5·0	44·50	1246			
Plasma ..	2·85	712	0·20	1·0	12·85	360			
Pecten irradians									
Soft tissues ..	—	—	—	—	35000·00	3500	200	20	Boroughs *et al.* (1957)
DECAPODA									
Homarus vulgaris									
Whole blood ..	2·4	686*	19·50	1·42	6·5	1300	10·0†	100	Bryan and Ward (1965)
Abdominal muscle ..	0·8	228*	2·1	0·15	17·7	3540	13·3†	133	
Hepatopancreas	4·8	1371*	31·5	2·30	23·3	4660	51·9†	519	Bryan (1964)
Gills ..	20·8	6000*	29·3	2·14	13·2	2640	37·5†	375	
Shell ..	225·0	64286*	19·4	1·42	0·9	180	9·3†	93	
Excretory organs	3·7	1060*	26·2	1·91	21·4	4280	27·8†	278	

* Calculated from data given by Bryan and Ward, taking 3·5 μgm/l manganese concentration in the medium.

† AC and ACf in *H. vulgaris* are for stable zinc uptake under laboratory experiments, hence AC is in μgm/g wet weight.

For the elements that have chemical properties similar to elements of group I, but whose metabolic functions/importance are not known (group II), *viz.*, Cesium, Strontium, etc., the ACfs and CCfs is soft tissues are observed to be often the same (Table III), the rate of uptake depending on the relative concentration of the chemically similar elements in the medium (Templeton, 1962; Bryan, 1963; Bryan *et al.*, 1966; Beninson *et al.*, 1966; and Polikarpov, 1966). Further, Townsley *et al.* (1959) found that *Tilapia mossambica* exposed to water concentrations of radiostrontium in multiples of one, ten and hundred does not accumulate corresponding multiples of radiostrontium in their tissues. Similarly Patel *et al.* (1966 *a*) observed that ACs and ACfs are practically the same in soft tissues of a clam *Katelysia marmorata* when exposed to two concentrations of cesium-137 in the medium. This may well suggest that these elements in such organisms are not required for the syntheses of biological complexes of the soft tissues. CCfs of strontium are higher than ACfs in skeletons of invertebrates and fish (Table III). This could possibly be understood as the result of fixation of minerals in the non-metabolic zone of the skeletal system.

For the elements which are metabolically not significant (group III), *viz.*, Chromium, Uranium, Plutonium, etc., the ACs and CCs depend on the concentration of the element in the medium (*cf.* Chipman, 1966). Under acute exposure conditions biological system either eliminates the element as soon as it is absorbed, or it accumulates and possibly gets poisoned. Thus in a clam *Tapes decussatus* the tissue concentration of chromium is 0·3 μgm/gm at 0·1 μgm/l under the natural environmental conditions, whereas under laboratory conditions at 10 μgm/l level only about 0·3 μgm/gm is reached after 20 days of exposure (Chipman, 1966). Olson and Foster (1956) observed inhibition of growth in juvenile salmon and trout when exposed for seven months to 20 μgm/l chromium concentration and significantly high mortality at medium concentration of 80 μgm/l. The concentration of uranium in soft tissues of bivalve *Anadara granosa* is $3 \cdot 3 \times 10^{-2}$ μgm/gm at 2·56

TABLE III

CCfs and ACfs of cesium and strontium in marine organisms

Organism	CCf (Sr)	CCf (90_{Sr})	ACf ($89/90_{Sr}$)	CCf (Cs)	CCf (137_{Cs})	ACf ($134/137_{Cs}$)	Source
SEA-WEEDS							
Ulva rigida	8·2	11·1	2	—	—	—	
	8·5	14·8	—	—	—	—	
Ulva lactua	2	—	1·2	240	—	7	
Fucus serratus	12	20	35	—	32	—	
Fucus vesiculosus	—	—	—	42	74	30	
Padina pavonia	—	257	40–49	—	—	—	
	148,258	223,265	—	—	—	—	
Laminaria digitata	16	—	14	—	—	—	
Cystoseria barbata	46,62	61,67	40–49	—	—	—	
	50,59	48,45	63	—	—	—	
Corralinea officinalis	—	264	4	—	—	—	
	209,210	317–332	—	—	—	—	
Laurencia obtusa	—	31	1	—	—	—	
	14·8, 15·7	29·3, 32·9	—	—	—	—	Barinov (1955),
Zostera marina	5	30·2	3	—	—	—	Bachurin *et al.*
	32·8	33·9	3	—	—	—	(1967), Bryan
Porphyra umbilicalis	0·8	0·2,0·9	—	18	18	5	*et al.* (1966),
BIVALVES							Kulebakina
Mytilus galloprovincialis							(1966),
Shell	130	315	6	—	—	—	Kulebakina and
Soft-parts	1·6	5	0·6	—	—	—	Polikarpov (1967),
Mytilus edulis							Parchevsky *et al.*
Shell	156	—	—	24	9	9	(1965),
Soft-parts	10	8	—	—	—	—	Polikarpov (1960,
CRUSTACEA							1966 *b*), and
Leander squilla	64	70	8	—	—	—	Polikarpov
Homarus vulgaris							*et al.* (1967)
Muscles	1	—	1	22	29	15	
Shell	180	—	100	—	—	—	
Cancer pagurus							
Muscles	—	—	—	26	30	11	
FISH							
Whole	4–5	—	1–5	—	—	—	
Muscles	0·1–1·5	—	0·03–0·15	5–23	—	5–35	
Bones	200	—	0·5–3	—	—	3·5	
Raia clavata							
Muscles	0·5	0·6	—	—	—	—	
Cartilage	16	12	—	—	—	—	
Pleuronectes platessa							
Muscles	0·6	0·3	—	26	24–27	—	
Bones	8·5	—	—	—	—	—	

μgm/l medium concentration under the natural environmental conditions. Under acute exposure of about 7 days to 2 mgm/l medium concentration, the concentration in tissues reaches about 2 μgm/gm. The same concentration level is reached after 4 days of exposure when the ambient concentration is increased 2·5 times. Further exposures at this level, however, have been found to poison the system (Patel *et al.*, 1966 *a*). Under chronic exposure the Cf of plutonium-239 in the flesh of fish *Sarda lineolata* is reported 3 at medium concentration of $4{\cdot}7 \times 10^{-4}$ $\mu\mu$Ci/l (Pillai *et al.*, 1964). Under acute exposure the flesh of *Homarus vulgaris* is also reported to obtain Cf 3 at medium

concentration of $6{\cdot}5 \times 10^{-3}$ μCi/l (Ward, 1966). Further work on chronic and acute exposure of the same species to the plutonium in the nutrient medium is warranted to arrive at a definite conclusion on the chronic and acute effects. It has been reported in the case of higher animals that equilibrium with plutonium is not attained in the lifetime. The rate of uptake and accumulation though slow is conditioned by the plutonium concentration (ICRP, 1959).

In general the CCfs are higher than ACfs for both chromium and uranium (Table IV *a* and *b*). An attempt has been made to understand these differences on the basis of the chemical form of the isotope employed (*cf.* Polikarpov, 1966). Chipman (1966) found that the chromium-51 in hexavalent form is readily accumulated and tightly bound in the tissues of *Tapes*, whereas the same element in trivalent form, and as EDTA chelate is not readily accumulated. With the hexavalent form the Cf reached after 20 days of exposure is only 22 in meat, whereas in the field population Cf is about 3,000. Chipman's observation that 'to reach such a value would require a very long time' could be understood as a recognition of the chronic and acute accumulations and effects. In our opinion, however, considering the asymptotic nature of the uptake curve the tissue concentration may not reach the CC level obtained in nature, alternatively the tissue Cf may reach such a level provided the ambient concentration is so low that Cf does't mean attainment of toxic level in the tissues of the living system.

TABLE IV (*a*)

Chronic and acute Cf s of chromium in marine organisms

Organism	CCfs	ACfs	Source
Tapes decussatus			
whole animal ..	..	13	Chipman (1966)
soft-parts ..	3,000	22	,,
shell ..	..	2·5	,,
Brown algae ..	100–500	60	Polikarpov (1966)
Red algae ..	(12,0000 ?)	80	,,

TABLE IV (*b*)

Chronic and acute concentration of uranium in tissues of ark-shell bivalve Anadara granosa (*Patel et al., 1966 a*)

Tissue	CC (ppm)	CCf	AC (ppm)	ACf
Soft-parts ..	$3{\cdot}281 \times 10^{-3}$	12·80	1·90	0·95
Corpuscles ..	$2{\cdot}713 \times 10^{-3}$	10·60	2·75	1·37
Plasma ..	$0{\cdot}425 \times 10^{-3}$	1·67	1·02	0·50
Shell ..	—	—	7·59	3·80

Organisms, in general, seem to concentrate significantly higher amounts of isotopes under chronic exposure conditions than under acute exposure. Prolonged exposure, covering the life span of an organism, to radioisotopes under natural conditions eventually results in Cfs for radioisotopes of same value as CCfs obtained with the stable isotope in the environment. Observations of Naidu (1963) and Seymour (1966) on the uptake of radioactive and stable zinc by oyster *Crassostrea gigas* from Willapa Bay, which receives radionuclides from the Columbia River, substantiate the above anticipation. The same pattern of Cfs was observed also, when they transplanted a population

TABLE V

Levels of chronic concentrations and CCf's of stable and radioactive zinc in Oyster Crassostrea gigas *from Willapa Bay and in transplant from Puget Sound (from Naidu, 1963)*

		Zinc-65		Zinc	
		CC ($\mu\mu$Ci/g dry wt.)	CCf	CC (μg/g dry wt.)	CCf
Willapa Bay population	..	531	$9{\cdot}3\times10^{4}$*	515	$8{\cdot}76\times10^{4}$
Transplant from Puget sound	8 days† ..	28·9	$3{\cdot}1\times10^{3}$*	684	$9{\cdot}30\times10^{4}$
	16 days ..	48·3	$5{\cdot}2\times10^{3}$*		
	32 ,, ..	82·2	$8{\cdot}8\times10^{3}$*		
	64 ,, ..	173·0	$1{\cdot}9\times10^{4}$*		

* Equilibrium not attained. † Period after transplantation.

of *C. gigas*, with stable zinc concentration higher than obtained in local population, but no radioactive zinc, from Puget sound to Willapa Bay, and studied the zinc concentration over a prolonged period. It will be seen from Table V that the Cf for zinc-65 in transplant slowly increased towards the value obtained for Willapa Bay population. In establishing ACs and CCs of very many elements in the organisms in an environment, and in understanding the ACfs and CCfs, the concentration of the element in the medium should be kept in view. Futhermore, investigations on CCfs in the tissues of different organisms in an environment may also reveal the presence of some 'indicator organisms' which concentrate specific elements to a very great extent, and thus could be used as indicators for the presence of radioactive pollutant.

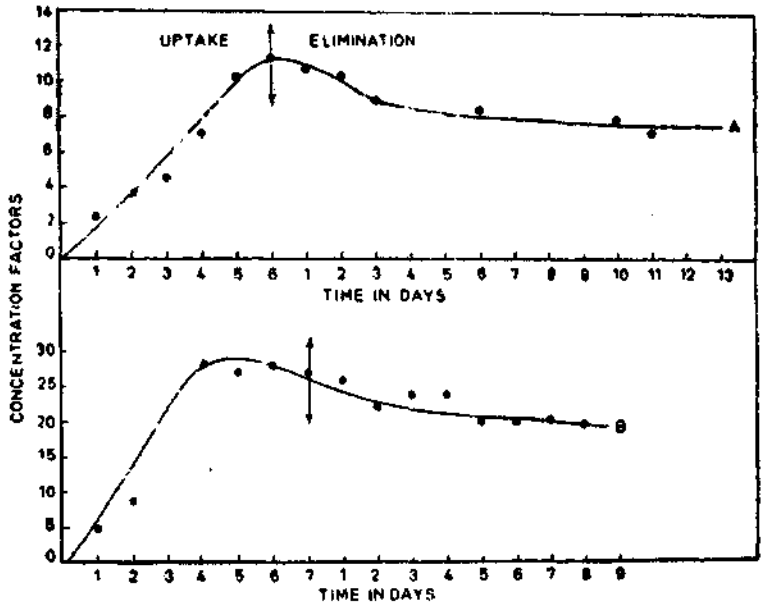

FIG. 1. Uptake-elimination of (A) Manganese-54, and (B) Zinc-65 by ark-shell *Anadara granosa* (after Patel *et al.*, 1966 *a*).

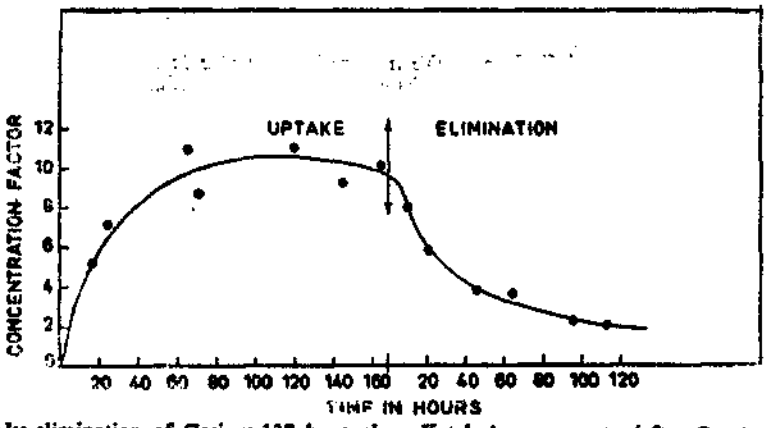

FIG. 2. Uptake-elimination of Cesium-137 by a clam *Katelysia marmorata* (after Patel *et al.*, 1966 *a*).

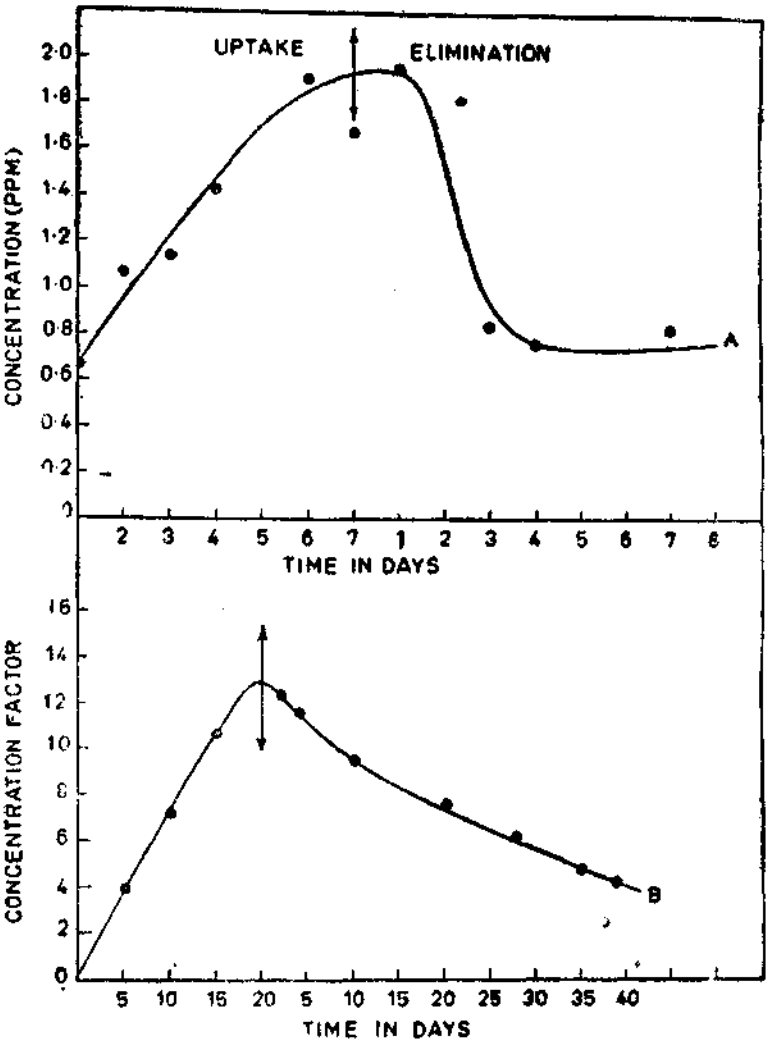

FIG. 3. Uptake-elimination of (A) Uranium by *Anadara granosa* (after Patel *et al.*, 1966 *a*) and (B) Chromium-51 by a Clam *Tapes decussatus* (after Chipman, 1966).

It may be worthwhile investigating the pattern of the rates of uptake-elimination* (RUE) of radionuclides by the soft parts under acute exposure conditions to see, if the classification of the elements on the basis of their metabolic roles is also reflected in RUE curves. In the RUE curves of manganese and zinc (group I) by shell-fish it has been observed that the uptake curves are always steeper than the initial loss rates (Fig. 1). Figure 2 illustrates the RUE of cesium (group II) by shell-fish. It could be observed that although the uptake rate is high as for group I elements, the initial elimination rate is also steep and the slopes are very close to one another. In the RUE curves for uranium and chromium (group III) the initial rates of absorption are lower than the initial rates of elimination (Fig. 3). The absolute values in these curves need not have significance when one considers particular elements since the chemical form of the elements in ambient would greatly determine these rates. These generalizations can only be taken as indicative since information available is inadequate and requires more detailed and better controlled studies.

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* Professor G. G. Polikarpov has drawn our attention to the following from his work:—

(i) The term 'Chronic concentration factor' used in this work is analogous to the term 'Limitary (maximum possible) concentration factor' used by Polikarpov and Zesenko (1965).

(ii) 'Uptake is the process of entry of radionuclides in organisms and accumulation is its result....Therefore, uptake (and accumulation) is the difference between intake and loss (elimination) (Polikarpov, 1966 *b*).

In the present work 'Uptake and accumulation' has been used interchangeably in this sense.

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MOLLUSCS IN RADIOBIOLOGY[1]

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ABSTRACT

Despite the fact that molluscs are very apt material for radiobiological research, they are generally neglected by radiobiologists. The reason for this and the advantages of knowing the effects of ionizing radiation on molluscs are discussed and some well chosen examples will be given.

This review concerns the most important studies carried out from 1914 onwards on marine, terrestrial and freshwater molluscs.

RADIOBIOLOGICAL research has been carried out on almost all the animal taxa, from Protozoa to Chordata, but most of the information concerns only a few species.

The organisms have been chosen, obviously, for the facility with which they can be reared and because their physiological and pathological characteristics are similar to those of man. For this reason, the animals most often used are the ones traditionally bred in the laboratory: mouse, rat, guinea-pig, and rabbit.

Aside from the scientific interest of knowing the effects of irradiation on a greatest number of species, it is useful to get a more complete information on those invertebrates that have a considerable importance in the biosphere and, then, indirectly to man.

I think that the radiobiological researches carried out with molluscs have the double advantage of giving information about one of the most important taxa among the invertebrates, not well-studied for this aspect, and provide a fit material for radiobiological research. The importance of molluscs in marine, terrestrial, and freshwater environment is well known. In some areas molluscs represent the greatest part of the total biomass of the biocoenosis and their role in the food chain is of great importance. Besides, the commercial value of some species must not be disregarded.

A large financial effort is made every year by governments and international organizations to destroy those molluscs which are intermediate hosts of parasites causing diseases in man and domestic animals. To kill these molluscs chemical products (molluscicides) are put in aquatic environment. Unfortunately, molluscicides are also toxic to the other species living in the same area, and in some environments (*i.e.*, flowing water) the rapid dilution of these chemical materials minimizes their effects.

The destruction of molluscs in their environment by ionizing radiation was suggested by Perlowagora-Szumlewicz (1964 *a*, *d*). This method has an advantage over the molluscicides in that it may be used in every type of environment, but it damages, as do the chemical products, other species as well. It should be of great help to kill harmful molluscs introducing in their populations lethal genes obtained by irradiating in the laboratory a part of the population and returning it to its habitat. Satisfactory results have already been obtained by applying this method in the fight against harmful insects. To attain this aim it is necessary, obviously, to get a lot of information on the effects of sublethal doses on the genetics of molluscs.

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The wide ecological, physiological, and biochemical differences existing among molluscs allow studies of comparative radiobiology in the same taxa. The study of the effect produced by irradiation on a wide range of sublethal doses is facilitated by the great radioresistance of molluscs. In addition, for some genera (*i.e.*, *Ampullaria*) the number of chromosomes is not high and their shape and size permit an accurate study.

The results of researches carried out on the interaction dose-temperature show that the effects produced by dose appear in a shorter time if the material is kept, after irradiation, at high temperatures. It is evident that a relatively high temperature, accelerating the metabolic rates, amplifies the effects of the molecular (or primary) lesions revealing, in a shorter time, the biochemical lesions and, consequently, the anatomical ones. The great resistances of several species of molluscs to a wide range of temperature allows accurate studies on the influence of temperature on irradiated material.

Several oviparous molluscs lay capsules perfectly transparent and, consequently, the embryonic development may be easily followed. Since the capsule frequently contains a high number of eggs, produced at the same time by the same individual, it is possible to have homogeneous material. The period of development, at least for oviparous species, is not so short as to make difficult the study of embryogenesis, but not so long as to prolong for more than 15 days the observations. It is clear that these characteristics facilitate the study of the irradiation effects on embryos. If the capsule has been cut in one or more parts the embryonic development is not affected. This permits the eggs contained in a part of a capsule to be irradiated while the other ones remain as controls. Besides, with a proper shield with one or more pin-holes, we may irradiate only a part of the eggs contained in a single capsule (Bonham, 1955).

Very important and interesting are the effects of ionizing radiation on the reproductive processes, from a biological as well as an ecological point of view. Molluscs present a great variety of reproductive characteristics: oviparous species, ovoviviparous, hermaphrodite, and gonochorist; some species with self-fecundation and other ones with heterofecundation. Some genera have secondary sexual characteristics so evident that it is possible to separate, without dissection, the males from the females. As a consequence, the effects of irradiation can be easily compared on both sexes without sacrifying the specimen. For the hermaphroditic species it is possible to observe the damage produced by irradiation on the male and female germ cells in the same individual; these cells, frequently, ripen at the same time and in the same biochemical environment.

The material used for researches on somatic and genetic effects caused by irradiation, must have the following characteristics: (1) easy to breed in laboratory; (2) facile adaptation to the standard conditions; (3) short period of embryonic development; (4) high fertility and fecundity; (5) individuals of small size to maintain a high number of them in a small space. It is not difficult to find molluscs, particularly among the freshwater species, which have these characteristics. I believe that some species are particularly suitable for the study of the effects of irradiation on the demography of experimental populations.

The field of research is very wide and promising but, until now, the data on the radiobiology of molluscs are so few that it is very easy to report the greatest part of the results obtained from studies carried out on this subject.

The marine species studied are the following: *Cumingia* sp. (Richards and Good, 1919); *Loligo pealii* (Rugh, 1950); *Thais* sp. (Bonham and Palumbo, 1951); *Ilyanassa obsoleta* (Cather, 1959); *Mercenaria mercenaria*, *Crassostrea virginica* (Price, 1965). For freshwater the species studied are: *Planorbis* sp. (Richards, 1914); *Lymnaea* (*Radix*) *japonica* (Sonehara, 1933; Bonham 1949; Bonham and Palumbo, 1951); *Helisoma subcrenatum* (Bonham, 1955); *Planorbarius metidjensis*, *Planorbarius corneus*, *Biomphalaria*[a] *pfeifferi*, *Melania tuberculata* (Fraga de Azevedo *et* Carvoo Gomes, 1956); *Lymnaea stagnalis* (Laviolette *et* Voulot, 1961); *Australorbis glabratus* (Fraga

[a] *Biomphalaria* is synonymous of *Australorbis*.

de Azevedo *et* Carvao Gomes, 1956; Perlowagora-Szumlewicz, 1964 *a*, *b*, *c*, *d*, *e*); *Physa acuta*, (Ravera, 1966 *a*, 1967; Ravera e Mariani, 1966). Among the terrestrial Pulmonata: *Helix pomatia*, *Arion empiricorum*, and *Helicella candidans* (Hug, 1958); *Arion rufus* (Laviolette *et* Cuir, 1959; Laviolette *et* Voulot, 1961).

Acute doses of X-rays were used in almost all the researches on molluscs; I have found only two studies with alpha particles (Hug, 1958, and Born, 1960), one with chronic gamma radiation (Fraga de Azevedo *et* Carvao Gomes, 1956) and one with gamma acute radiation (Price, 1965).

1. *Effect on the adult and on its offspring*

The reduction of longevity in molluscs, obtained by irradiation, is very small and of the same order as that calculated for arthropods (Bonham, 1949; Bonham and Palumbo, 1951; Fraga de Azevedo *et* Carvao Gomes, 1956; Laviolette *et* Voulot, 1961; Perlowagora-Szumlewicz, 1964 *a*; Price, 1965; Ravera, 1966 *a*, 1967). Molluscs are much more radioresistent than mammals; for instance, the length of the life of some gastropods (*i.e.*, *Physa acuta*, *Arion rufus*) is not reduced by 10 KR, while doses of 400-600 R are lethal for man. It is known that during mitosis the cell is more radiosensitive than in intermitosis and, consequently, the sensitivity of a tissue increases with the frequency of mitotic cells. The exceptional resistance of molluscs and arthropods may be explained, according to several radiobiologists, by the fact that the somatic tissues of the adults of these organisms have a very low frequency of cells in mitotic activity. This is supported by the fact that the germ and embryonic cells have a comparatively high radiosensitivity. Bacq and Alexander (1961) do not agree with this hypothesis and they have suggested the following one. Vertebrates maintain their osmotic equilibrium almost completely by mineral ions, while osmotic pressure of invertebrates is regulated by amino-acids and polypeptides. Since amino-acids have a certain radioprotective action, the higher resistance of Invertebrates may be due, at least in part, to this biochemical difference. Fraga de Azevedo and Carvao Gomes (1956) have advanced the hypothesis that the high radioresistance of some species (*i.e.*, *Planorbarius corneus*) is due to some radioprotective substances present in the body of the mollusc. On the other hand, this hypothesis has not yet been supported by experiments.

Independently from the causes responsible for the high radioresistance of molluscs, it is easy to predict that, if one environment is exposed to doses of some KR, its biocoenotic structure will alter in a short time and the molluscs will survive, with some other resistent taxa, if the radiosensitivity of their parasites and competitors is, comparatively, higher.

From the researches carried on different species (*Mercenaria mercenaria*, *Crassostrea virginica*, *Radix japonica*, *Physa acuta*, *Bithynia tentaculata*, *Viviparus contectus*) by different authors (Bonham and Palumbo, 1951; Fraga de Azevedo *et* Carvao Gomes, 1956; Laviolette *et* Voulot, 1961; Price, 1965; Ravera, 1966 *b*) on the influence of the dose on the reduction of the survival time (ST 50), it is evident that: (1) there are strong differences among the species; (2) all the species have a great radioresistance; (3) the effect is not directly proportional to the dose. This latter observation demonstrate that the death, caused by irradiation, will be due to the different radioresistance of the different tissues composing the organism. This hypothesis is supported by the fact that irradiation death in higher mammals is due to different causes: *i.e.*, death by blood cells, by the intestine, by the nervous system.

One of the most evident effects appearing soon after exposure to high doses is the more or less complete flaccid paralysis that affects gastropods. The organism remains immobile from a few hours to several weeks, in relation to the dose, species, and age. This was observed as for Pulmonata (*i.e.*, *Australorbis glabratus*, Perlowagora-Szumlewicz, 1964 *b* : *Physa acuta*, Ravera, 1967) as well for Prosobranchiata (*i.e.*, *Bithynia tentaculata* and *Viviparus contectus*, Ravera, unpublished data). During this period, the animal secretes abundant mucus, eats only a small amount of food and, consequently, produces a small amount of excrement.

Perlowagora-Szumlewicz (1964 *b*) has observed, among the immediate effects of irradiation on *Australorbis glabratus*, the temporary disappearance of the pigment in the young and in the

adults; and for the hatchlings, that the reddish pigment appears six weeks later. The author relates these effects to the alteration produced by X-rays on hemoglobin and cytochromes.

The researches carried out on the radiosensitivity of tentacles (Hug, 1958) and mantle cavity (Born, 1960) of some terrestrial and freshwater pulmonates may have a promising development. Since these organisms show a reaction if they are stimulated with ionizing rays, they may be considered as living dosimeters sensitive also to extremely low doses. From the results obtained, Hug found that ionizing radiations perfectly correspond to the definition of stimulus given by the neurologists, either they simulate the action of specific stimulus on some receptors of the molluscs, or have an aspecific action on the nervous system.

Perlowagora-Szumlewicz (1964 *b*) has observed that *Australorbis glabratus* grows more slowly if irradiated with doses ranging between 3 and 20 KR. This effect is more evident for the younger and smaller animals. This growth delay is followed by a period in which the irradiated mollusc grows more rapidly than the normal one. As a consequence, the size of the animal, irradiated at an adult age, is similar to that of the control but smaller, if the animal was irradiated at a very young age. It has been observed that the offspring of irradiated individuals never show growth anomalies. A certain growth inhibition has also been observed for *Arion rufus* and *Lymnaea stagnalis* irradiated at the age of 8 days (Laviolette *et* Voulot, 1961). For *Arion* there has also been noted a threshold (14 KR and 16 KR), under which growth does not differ significantly from that of the control. A dose of 2 KR seems to accelerate the growth, this stimulating effect is evident three months after irradiation.

Irradiation, besides slowing the growth, produces a delay of sexual maturity. For *Australorbis glabratus* this delay increases with decreasing age of the individual at the time of irradiation (Perlowagora-Szumlewicz, 1964 *c*).

Anomalies and reduction of the number of oviparous capsules and eggs laid by irradiated individuals have been noted for *Physa acuta* (Ravera, 1966 *a*) and *Australorbis glabratus* (Perlowagora-Szumlewicz, 1964 *c*). For *Physa* the frequency of capsules with a high number of eggs decreases soon after irradiation, but, later, this frequency does not differ from the control.

Physa (Ravera, 1967) and *Australorbis glabratus* (Perlowagora-Szumlewicz, 1964 *c*) embryos born from irradiated parents, show many anomalies. These anomalies have been observed also for the control and some of them are very similar to those noted by Raven (1958) for embryos of *Lymnaea* treated with chemical substances. We may conclude, then, that irradiation is not able to create new anomalies, but to increase the frequency of them; and they are proportional to the dose received by the parents.

Perlowagora-Szumlewicz (1964 *c*) has evaluated the effects produced on the fecundity (number of eggs produced daily by 1,000 individuals) of *Australorbis glabratus* irradiated with doses ranging from 3 to 9 KR. The author found that the damage increased with dose and decreased with age and size. For the hatchlings a dose of 9 KR reduced fecundity to zero, while for young and mature individuals she has noted only a temporary reduction. The damage produced in old animals increased with time. This effect may be the consequence of the interaction of the dose with the age (degeneration of germinal tissue). Ravera and Mariani (1966) have observed that the fecundity of adults of *Physa acuta*, irradiated with doses ranging from 2 KR to 10 KR, has an initial decrease followed by an increase. Higher doses (*i.e.*, from 28 KR to 110 KR) reduce the value and the period of the fecundity. The fecundity difference between irradiated and control animals was still evident 50 days after irradiation. The reduction of the fecundity is stronger if the adults are kept, after irradiation, at high temperature: 30° C (Ravera, 1967). For *Bithynia tentaculata* (Ravera, 1966 *b*) the reduction of the fecundity is more evident that for *Physa*; in fact, the dose of 110 KR, that is able to stop egg production for the first species, must be doubled (220 KR) to obtain the same effect on the second one. This conclusion concern experiments carried out at 20° C, but, if *Physa* is kept at 30 ° C, 110 KR are enough to abolish the production of eggs.

Since the experiment on *Bithynia* was carried out only at 20° C. we do not have data about the temperature effect on the fecundity of this species.

Relatively low doses may reduce fertility (number of hatchings produced daily by 1,000 individuals). For instance, 2–4 KR reduce the fertility of *Australorbis glabratus* (Perlowagora-Szumlewicz, 1964 *c*) and *Physa acuta* (Ravera and Mariani, 1966). To abolish fertility higher doses must be used: *i.e.*, for *Physa acuta* and *Bithynia tentaculata* 28 KR abolished completely the fertility and probably, also lower doses, but not lower than 10 KR. The fertility of adult *Physa* irradiated with doses ranging from 2 KR to 10 KR, showed a period of depression (this period is related to the dose) followed by a period of continuous increase until values very similar to that of the control were reached. The recovery of the fertility has been observed by Perlowagora-Szumlewicz (1964 *c*) for *Australorbis glabratus* exposed to doses ranging from 3 to 9 KR. For the same species it has been noted that the offspring of irradiated individuals have a higher fertility than that of their parents, but lower than that of the control (Perlowagora-Szumlewicz, 1964 *c*). This fact shows that the damage caused to the reproductive processes are present also in the following generations.

I think it is useful to report, besides the data on fertility, some informations on the viability of the eggs (percentage of hatchings from 100 eggs), because this value is independent from the number of eggs laid and, consequently, it is a more significant index to evaluate the capacity of the egg to complete its development. The viability for *Australorbis glabratus*, during the first two weeks after exposition to 4,980 R and 7,105 R, was equal to zero, at the seventh week, for the lower dose it was 49·4% and for the higher, 22·7%, while that of the control was about 55·7% (Perlowagora-Szumlewicz, 1964 *c*). The viability of *Physa acuta* (Ravera e Mariani, 1966) in the first 20 days after treatment, was reduced to 33% by 2 KR, to 7% by 4 KR, and to zero by 6, 8 and 10 KR, compared with the 96% of the controls. After 40 days the viability of eggs laid by parents irradiated with 2 and 4 KR was about 30%, while that for 6, 8 and 10 KR was 8%. The difference between the viability of the control and that of irradiated individuals was insignificant 120 days after treatment.

For molluscs (*Australorbis glabratus* and *Physa acuta*) irradiated with doses lower than 10 KR the reduction and the following increase of the reproductive capacity were explained by Perlowagora-Szumlewicz (1964 *c*), Ravera (1967) and Ravera e Mariani (1966) in the following manner. The gonocytes, irradiated during the advanced phase of their differentiation, are still able to produce zygotes, but these zygotes in many cases do not develop as normal embryos. The immature gonocytes, or other cells that are able to differentiate in gametes, are less sensitive and, consequently after their differentiation can fuse to form zygotes with a normal development. The time to reach normal values for fertility in irradiated animal is related to dose and to the length of the maturation period of the gametes. Histological examination of the gonad of *Physa*, exposed to 10 KR, has confirmed this hypothesis (Ravera, Gaddi and Giannoni, in press). The female germ cells appeared morphologically normal, while the male ones were destroyed, except for spermatogonia and the spermatozoa contained in *Receptaculum seminis*. This exception may be due in the first case, to the higher resistance of the undifferentiated cells, and in the second one, to the protective action of the seminal liquid, as has been noted for other species: *i.e.*, *Arbacia* and *Spisula* (Bacq and Alexander, 1961). The greater radio-resistance of the female germ cells is demonstrated by the high viability of zygotes of *Physa acuta* originated by the fusion of non-irradiated sperm with irradiated ova compared with that of zygotes produced by the fecundation of irradiated sperm with non-irradiated ova (Ravera, Gaddi and Giannoni, in press) The lower sensitivity of the female germ cell is also confirmed by the results obtained by Laviolette *et* Cuir (1959) for *Arion rufus* irradiated with X-rays.

2. *Effects on the embryo*

The results of researches conducted on *Radix japonica* (Bonham and Palumbo, 1951); *Australorbis glabratus* (Perlowagora-Szumlewicz, 1964 *a*, *d*) and *Physa acuta* (Ravera, 1966 *a*) have

clearly shown that the embryo of the gastropods, as well as for other taxa, is more radiosensitive than the adult.

Richards (1914) and Richards and Good (1919) have noted that irradiation at the beginning accelerated the cleavage of *Planorbis* and *Cumingia* eggs, but a delay followed in the successive divisions. Cather (1959) has observed a cleavage delay for irradiated eggs of *Ilyanassa obsoleta*. This delay increased with the dose from 1 to 10 KR. As to irradiation damage to nucleus and cytoplasm it seems that doses lower than 5 KR may damage only the former. For higher doses (*i.e.*, 8, 10 KR) a succession of cleavage was noted, but the nucleus division was abnormal and, consequently, the embryo development was blocked. The division delay was due principally to the prolongation of the prophase but also that of the interphase. It is interesting to observe that a prolongation of the prophase, as an effect of irradiation, has been noted also for other organisms [for example: *Arbacia* (Henshaw, 1940) and *Chortophaga* (Carlson, 1954)] but a prolongation of the interphase has been noted up to now, only for *Ilyanassa*. The delay of the prophase seemed to be due to the difficulty of division of the chromatin, while that of the interphase, was in coincidence with the critical period of DNA synthesis.

The embryo viability for *Physa acuta* (Ravera, 1966 *a*) and *Australorbis glabratus* (Perlowagora-Szumlewicz, 1964 *d*) exposed to different doses at the age of 3, 4, 5 and 7 days decreased with dose but increased with age at which it has been irradiated. This relation has also been observed for other taxa: *i.e.*, amphibians, fishes, insects, crustaceans and nemathelminths.

Bonham (1955) explained the correlation between radioresistance and age for *Helisoma subcrenatum* embryo with the higher sensitivity of the cells during mitosis compared to that of cells in resting phase. During the treatment a great number of cells of older embryos were in premitotic phase and, consequently, they suffered less damage. These cells could survive and generating other cells could substitute the damaged ones, that were those irradiated during the mitotic phase. As a consequence, the lethal dose (LD 50) calculated for the eggs of this mollusc irradiated during mitosis at the stage of 1–2 blastomers was of 100 R and of 300–400 R if the eggs were irradiated during resting phase.

This review demonstrates that molluscs can be a very useful material for radiobiological researches. In addition, a knowledge of the radiation effects on molluscan vectors of diseases (*i.e.*, schistosomiasis) may be of great practical importance.

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ACCUMULATION OF ZINC BY OYSTERS IN WILLAPA BAY, WASHINGTON[1]

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ABSTRACT

Oysters have a distinct capacity for the accumulation of zinc. Many studies based upon measurements of either total zinc in oysters from field collections or radioactive zinc (^{65}Zn) in oysters from laboratory experiments support this conclusion, although the estimates of accumulation as measured by the concentration factor are highly variable. To obtain additional information, zinc accumulation by oysters from Willapa Bay was determined by measurements of both ^{65}Zn and total zinc. Also, zinc accumulation by other components of the environment was determined to aid in the interpretation of the oyster data. Willapa Bay provides a unique opportunity for these studies since oysters, *Crassostrea gigas*, are grown commercially in this area and ^{65}Zn is present in unusual amounts.

On six occasions between February and June, 1963, samples of oysters, plankton from two nets, water and mud were collected. Analyses of these samples provided the following average values for oysters, plankton and filtered sea-water, respectively: *^{65}Zn* in terms of pCi/g dry for oysters and plankton and pCi/cc for sea-water—530, 170 and 0·0093; *total zinc* in terms of μg/g dry for oysters and plankton, and μg/cc for sea-water—520, 240, 0·0074; and *specific activity* in μCi of ^{65}Zn/g zinc—1·0, 0·73 and 1·3.

From these data, the concentration factors for oysters were calculated to be $1{\cdot}2\times10^{5}$ for total zinc and $9{\cdot}4\times10^{5}$ for ^{65}Zn. These values were based upon wet weights of oysters and the assumption that the ratio of wet weight to dry weight is 6·0. A comparison of ^{65}Zn and total zinc values for oysters and plankton indicates that the amounts in oysters were greater by factors of two to four.

INTRODUCTION

STUDIES of the distribution of zinc in Willapa Bay were undertaken in 1963.[3] The objectives of the experiment were as follows: (1) to determine the relationship of zinc in oysters to zinc in other components of the environment, namely, water, plankton and sediments; (2) to extend a previously established program to study the distribution of Hanford-produced radionuclides in the coastal waters of Washington and Oregon (Seymour and Lewis, 1964); and (3) to obtain estimates of zinc concentration by oysters for comparison with the estimates reported by other investigators. A significant feature of these experiments is the use of two methods to determine the zinc content of oysters growing in a natural environment: one by chemical analysis for total zinc, the other by radiological analysis for zinc-65 (^{65}Zn). From these analyses, two independent determinations of the zinc concentration factor for oysters were made.

Willapa Bay is well suited for these studies since oysters, *Crassostrea gigas*, are grown commercially in Willapa Bay and ^{65}Zn is present in unusual amounts. Zinc-65 produced in the cooling waters of the Hanford reactors is transported by the Columbia River to the Pacific Ocean and thence

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to Willapa Bay, which is 40 km. north of the mouth of the river and 600 km. from the Hanford reactors (Fig. 1).

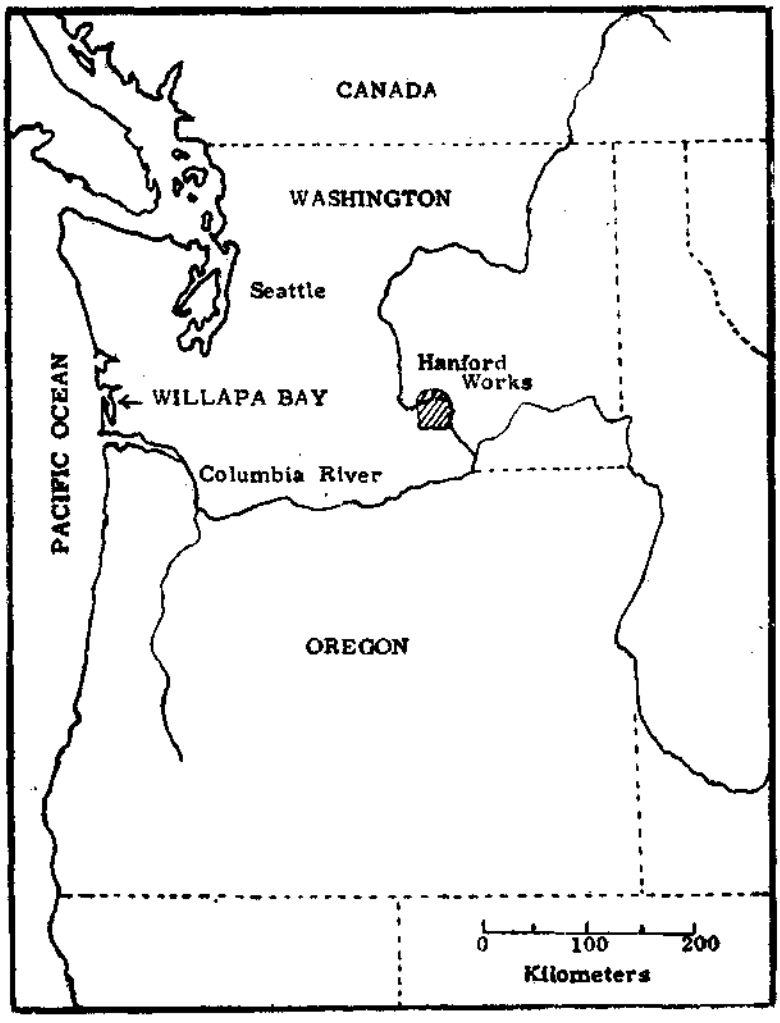

FIG. 1. Location of Willapa Bay.

ROLE OF ZINC IN OYSTERS

Zinc is recognized as a microelement essential to the growth of both plants and animals and is present in blood (Vinogradov, 1953), in respiratory pigments (Rice, 1963), in hormones (Polikarpov, 1966), and is a catalyst for at least eight enzyme systems of plants and animals (Heinz, 1964). Of the invertebrates, the Mollusca and the Crustacea accumulate the greatest amounts of zinc, and of the Mollusca, the Lamellibranchiata accumulate more zinc than the Cephalopoda and the majority of the Gastropoda (Vinogradov, 1953).

Galtsoff (1964) discusses the distribution of four metals, including zinc, in the body of adult oysters, *C. virginica*. The mean values of ten samples collected in early August, 1936, from Cape

Cod waters, in terms of mg/kg (dry weight), are as follows:

Body portion	Iron	Copper	Zinc	Manganese
Gills ..	382	178	4,480	39
Muscle ..	136	65	1,420	4
Ovary ..	151	63	1,710	60
Spermary ..	136	65	1,420	4
Residue ..	252	153	4,630	9

From these data it is evident that zinc is ten times more abundant than iron, which is more abundant than copper, which is more abundant than manganese. Galtsoff also reports a seasonal variation in zinc content of oysters, the summer values being approximately fourfold those of the winter. The values for the other metals also show the same seasonal pattern, hence those in the above table can be considered peak or near peak values.

Galtsoff (1964) also did some histological studies of the localization of zinc in the tissues of oysters and reports as follows:

> It appears probable that zinc is present in a soluble state and is more universally distributed through the tissues than iron, copper and manganese. Observations on the uptake and accumulation of radioactive zinc, Zn^{65}, confirm this view. Chipman, Rice and Price (1958) demonstrated that zinc in surrounding water is rapidly taken up in great amounts by the bodies of oysters, clams, and scallops. The gills of oysters were found to accumulate almost twice the concentration of radioactive zinc, as did the organs and tissues. The digestive diverticula and body mass contained a considerable amount of Zn^{65}.
>
> The role of heavy metals in the physiology of the oyster is not clear .... iron, copper and zinc may be stored in the tissues and in some blood cells as excess materials which are slowly eliminated. ....accumulations of iron, copper and zinc in the mantle and gills support this view.

These observations are supplemented by Polikarpov's (1966) comment that in marine molluscs stable zinc is concentrated mainly in the gills and gonads, and least of all in the muscle.

The zinc concentration factor for oysters, defined as the ratio of the amount of zinc in the living oyster to the amount in sea-water, is an index of the zinc capacity of oysters. Values for the amount of zinc in oysters are given in Table I and the concentration factors in Table II.

The large range of values in Table I can be attributed to (1) variability in analytical methods; (2) individual, species, and seasonal differences in the accumulation of zinc; and (3) locality differences in the zinc content of the water associated with runoff and industrial pollution. The values in Table I for zinc in oysters at Blackwater before and after operation of the nuclear power station are one example of the effect of an industrial operation upon the zinc content of oysters. There is good evidence that the source of the added zinc in oysters was the brass pipeline in the reactor cooling system (Preston, 1967).

The values for zinc in sea-water, as reported in twelve references, ranged from 0·6 to 24 parts per billion, for which a geometric mean of 6 ppb was calculated (Chipman *et al.*, 1958). Estimates in other references fall within this range, including the commonly used value of 0·01 mg/litre (approx. 10 ppb) reported by Goldberg (1963). The zinc content of Willapa Bay water for the period February-June, 1963, averaged 7·4 ppb and ranged from 3·6 to 12 ppb. The zinc content of sea-water at Beaufort, North Carolina, for all months of the year was similar, ranging from 2·8 to 14·6 ppb (Chipman *et al.*, 1958).

TABLE I

Zinc content of oysters by species

Zinc %wet weight Mean	Max.	Min.	No. of analyses	Species	Location		Reference
·428	—	—	—	*C. virginica*	..	USA	Vinogradov, 1953[a]
·170[b]	—	—	—	,,	Chesapeake Bay	,,	Alexander, 1966
·159	·317	·031	7	,,	Atlantic Coast	,,	Chipman, 1958
·077[b]	—	—	10	,, (residue)	,, ,,	,,	Galtsoff, 1964
·069[d]	·230	·003	134[c]	,,	,, ,,	,,	Vinogradov, 1953
·050	·060	·039	2	,,	Gulf of Mexico	,,	Chipman, 1958
·086	—	—	—	*C. angulata*	United Kingdom		Bryan, 1966
·060	—	—	1[c]	,,	—	—	Vinogradov, 1953[a]
·037	·042	·031	—	*C. edulis* prereactor	Blackwater, England		Preston, 1967
·083	·123	·038	—	*C. edulis* postreactor	,,		Preston, 1967
·024[d]	—	—	6	*O. edulis*	—	—	Vinogradov, 1953[a]
·006	—	—	8	*O. lurida*	—	—	Vinogradov, 1953[a]
·015[b]	·016	·013	2	*C. gigas*	Puget Sound	USA	Naidu, 1963
·009[b]	·011	·005	6	,,	Willapa Bay	,,	Naidu, 1963
·012[b]	—	—	—	,,	,,	,,	Alexander, 1966
·160	—	—	—	Not identified	—	,,	Heinz, 1964
·034[e]	·115	·010	—	Mixed	—	—	Preston, 1967

a. Compiled by Vinogradov; not original source.
b. Wet weight-dry weight ratio assumed to be 6·0.
c. Data with obvious errors not included.
d. Calculated by authors from data compiled by Vinogradov.
e. Geometric mean; compiled from eleven references.

TABLE II

Zinc concentration factors for oysters

Concentration factor[a]	No. of samples	Species	Locality	Reference
$7{\cdot}5\times10^5$	1	*C. virginica*	Gulf of Mexico, USA	Chipman, 1958
$1{\cdot}4\times10^5$	5	,,	Atlantic Coast, USA	Chipman, 1958
$3{\cdot}3\times10^5$	—	—	United Kingdom	Bryan, 1966
$2{\cdot}5\times10^5$	—	—	Blackwater, England	Preston, 1967
$6{\cdot}0\times10^{4b}$	—	—	—	Peston, 1967
$4{\cdot}0\times10^{4c}$	—	*O. edulis*	—	Ichikawa, 1961
$3{\cdot}1\times10^{4d}$	—	,,	—	Polikarpov, 1966
$2{\cdot}0\times10^{4d}$	—	,,	—	Polikarpov, 1966
$1{\cdot}2\times10^4$	6	*C. gigas*	Willapa Bay, USA	Naidu, 1963

a. Mean value for zinc in oysters ÷ mean value for zinc in sea-water.
b. Calculated by geometric means with data from eleven references.
c. Vinogradov's values for zinc in oysters and Goldberg's value for zinc in sea-water.
d. Mauchline's calculation from Vinogradov's data as given by Polikarpov, 1966.

The direct analysis of sea-water for elements in trace quantities is difficult. Recently, Joyner *et al.* (1967) have outlined methods of concentrating samples prior to analyzing them for zinc and other trace elements to improve precision and accuracy of the measurement. The extent to which preconcentration methods have been used in the determination of the values reported above is not known.

The concentration factor is a function of two variables: zinc in the oyster and zinc in the water, each with a considerable error. Therefore, concentration factors ranging from $1{\cdot}2\times10^4$ to $7{\cdot}5\times10^5$, as shown in Table II, were not unexpected and indicate that oysters have a distinct capacity for the accumulation of zinc. The subject of concentration factors will be given further consideration in the Discussion and Conclusions.

Methods of Sample Collection, Preparation and Analysis

Sample Collection

On six occasions between 11 February and 24 June, 1963, samples of oysters, plankton, sea-water and bottom sediments were obtained from Willapa Bay, Washington, for total zinc and ^{65}Zn analyses.

Oysters from two to four years of age were collected from the mud flats at low tide and then stored on ice for transportation to the home laboratory.

The plankton tows were made at high tide in the vicinity of the oyster collections. Two nets were used, one a 50 cm. net with 215 μ mesh (No. 6), the other, a 30 cm net with 73 μ mesh (No. 20). To obtain five grams of dry sample, five or six 10-minute tows were required. The plankton samples were preserved by the addition of 5 ml of 40 per cent formaldehyde.

The sea-water sample was collected at the time of the plankton tows. Ten gallons of water were dipped from the surface with plastic buckets and transferred to polyethylene containers. To prevent decomposition of microorganisms in the sample, 4 ml of chloroform were added to each gallon of water.

Efforts to collect bottom sediments with an Ekman grab and with an ooze sampler were unsuccessful because of the presence of oyster shells and other materials on the bottom that interfered with the normal operation of the samplers. Therefore, a plastic trowel was used to obtain surface sediments, which were collected at low tide along with the oysters. The samples were placed in thoroughly cleaned glass jars for transportation, on ice, to the home laboratory.

Sample Preparation

The oysters were processed within 24 hours from the time of collection. All of the oysters, after shucking, were washed with distilled water, followed by deionized water, drained and weighed. The oysters were dried to constant weight at 90° C., placed in a paper bag, pulverized, and stored in a plastic jar.

The plankton from the two nets were kept separate, as one of the objectives of the program was to relate ^{65}Zn in phytoplankton to ^{65}Zn in oysters. The catch in the net with the finest mesh (No. 20, 73 μ) was assumed to contain more phytoplankton than the No. 6 net, although it is recognized that many phytoplankters escape a net with a mesh even as fine as 73 μ. In the laboratory the samples were drained on filters, weighed, dried at 90° C., re-weighed and stored in plastic containers.

All sea-water samples were filtered through millipore filters of 0·45 μ porosity of known weight. The filters were dried at 60° C., reweighed to determine the dry weight of material in the filter and

then sealed in a plastic petri dish for later analysis. The filtered sea-water was stored in clean, polyethylene containers.

The bottom sediments, like the oyster samples, were processed immediately. The sample was weighed after drying at 90° C., thoroughly mixed and then stored in a plastic container.

Zinc Analyses

Zinc was determined colorimetrically after solvent-extraction with dithizone in carbon tetrachloride.

Various procedures were followed in the preparation of the samples for zinc determination. Oyster and plankton samples were wet-ashed, taken to dryness and redissolved in hydrochloric acid. After filtration to remove the residue, zinc was separated from other elements in the solution by the ion-exchange method of Kraus and Moore (1953), as modified by Joyner and Chakravarti (1960). The sample, reduced to the zinc fraction eluted from the ion-exchange resin, was then in proper chemical form for the quantitative determination of zinc by the solvent-extraction, colorimetric method developed by Sandell (1959).

The sediment samples were handled in the same manner as the oyster and plankton samples, except that the wet ashing step was preceded by treatment with concentrated nitric acid and by filtration to dissolve the organic matter and to remove siliceous material.

For sea-water, zinc was separated directly from a 250 ml sample by a solvent-extraction method with dithizone in carbon tetrachloride as described by Morrison and Freiser (1957). The remainder of the analysis, the colorimetric determination of zinc, was the same for sea-water as for the other samples.

Zinc-65 Analyses

The amounts of ^{65}Zn in all samples were determined by gamma-spectrum analysis. The principal components of the system were a 3-inch by 3-inch solid sodium iodide crystal and a multichannel pulse-height analyzer. Zinc-65 in oyster, plankton and sediment samples was determined simply by placing the dried sample on the detector, counting it for 100 to 800 minutes, and transferring the output data from the counting system to a computer for analysis. Details of the counting system and methods of analyses are discussed by Seymour and Lewis (1964).

Unfortunately, small amounts of ^{65}Zn in sea-water, such as those in our samples, cannot be measured reliably, if at all, by merely drying the samples. Therefore, zinc was separated from the sample by a chemical procedure described by Sood, Goldin and Velten (1960). To obtain a quantity of ^{65}Zn sufficient for reliable measurement a 25-litre sample was required. In essence the method was as follows: zinc was precipitated from the samples with hydrogen sulphide after the samples had been heated, acidified and made basic. The precipitate was collected on a filter, dried and then analyzed for ^{65}Zn by gamma spectrometry.

The step-by-step procedures for the zinc and ^{65}Zn analyses are described in detail by Naidu (1963).

Results

The results of analyses of oysters, plankton, sediments and sea-water for both zinc and ^{65}Zn are given in Table III. The values are arithmetic means, plus or minus one standard deviation, as calculated from the values for each of the six dates of collection. The dry weight of the sample was selected as the basis for reporting the observed values because there is greater confidence in a standard dry weight than in a standard wet weight for oysters, plankton and sediments.

Values for specific acitvity, defined as the number of microcuries of ^{65}Zn per gram of zinc, for the four types of samples, are given in Table IV. The zinc and ^{65}Zn concentration factors for oyster

and plankton also are presented in Table IV. The zinc concentration factor is defined as the ratio of zinc in the organism to zinc in sea-water. The ^{65}Zn concentration factor is similarly defined. Calculation of the concentration factor requires conversion of the oyster and plankton values for zinc and ^{65}Zn from dry to wet weights. For oysters, wet and dry weights for a limited number of samples were carefully determined and from these data the ratio was found to be 6·0. For plankton a ratio of 10 was arbitrarily chosen. Both the specific activities and the concentration factors were calculated from the data in Table III. The errors in Table IV were calculated from the following equation:

$$(A \pm a)/(B \pm b) = (A/B)\,[1 \pm (a^2/A^2 + b^2/B^2)^{\frac{1}{2}}]$$

The ratio for both zinc and ^{65}Zn between various components of the environment, other than the ratios that can be properly identified as concentration factors, is given in Table V. These values were calculated from the data in Table III and the errors were determined in the manner described above.

Discussion and Conclusions

The zinc content of *Crassostrea gigas* from Willapa Bay on the Pacific Coast of the United States is considerably less than that of *C. virginica* from the Atlantic Coast. The mean values are 0·010 and 0·18 per cent of wet weight. The zinc values for most other species of oysters from other areas are intermediate to these values (*see* Table I).

The maximum value for zinc in oysters noted in the literature is 0·4284 per cent of fresh matter for *C. virginica* as reported by Vinogradov (1953) from a publication by McHargue (1924). The next highest values are 0·3174 and 0·2933 per cent of fresh tissue for *C. virginica* from Milford, Conn., and upper Chesapeake Bay as reported by Chipman, Rice and Price (1958), who attribute these high values to pollution from nearby metal industries.

The minimum values for zinc in oysters are ·009 and ·012 per cent of the wet weight for *C. gigas* from Willapa Bay, as reported by Naidu (1963) and Alexander and Rowland (1966), with the two following exceptions: the values of ·006 per cent for *O. lurida* and ·003 per cent for *C. virginica* (Table I).

How much of the difference in the zinc content of Willapa Bay oysters and of those from the Atlantic Coast can be attributed to species and how much to locality cannot be answered directly as there are no reported zinc analyses of *C. virginica* and *C. gigas* grown in the same area. However, both locality and species differences probably are contributing factors for the following reasons: (1) as an example of locality differences, the influence of local conditions upon the zinc content of oysters has been reported by Chipman, Rice and Price (1958) and is illustrated by the observations of the increased zinc content of *O. edulis* after operation of the nuclear reactor at Bradwell, England (Table I), and (2), as an indication of species differences, the difference in the zinc content of Atlantic Coast sea-water, which ranged from 1·1 to 24 parts per billion, and Willapa Bay sea-water, which ranged from 3·6 to 12·0 parts per billion, does not explain the more than tenfold difference in zinc content of the oysters from the two areas.

The concentration factor is a function of the zinc content of both sea-water and the oyster, hence the rank of oysters by concentration factor in Table II would not necessarily be expected to follow the rank of oysters by zinc content as given in Table I.

The reported values for zinc in sea-water ranged from 0·6 to 24 parts per billion with evidence of large individual, seasonal and locality differences.

The concentration factors listed in Table II range from $1{\cdot}2\times10^4$ for *C. gigas* from Willapa Bay to $7{\cdot}5\times10^5$ for *C. virginica* from the Gulf of Mexico. There is evidence that the concentration

factor is inversely related to the amount of zinc in sea-water, *i.e.*, zinc concentration factors for oysters increase as the zinc content of sea-water decreases (Chipman *et al.*, 1958).

Calculation of specific activity values, of concentration factors and of oyster-plankton-sediment ratios is based upon the values for total zinc and ^{65}Zn given in Table III. The specific activity values, expressed in μCi of ^{65}Zn per gram of zinc, can be used as an index of the equilibrium condition between ^{65}Zn in sea-water and ^{65}Zn in other components of the environment, if all isotopes of zinc are equally available to the oyster and the stable zinc equilibrium has been established. For example, if the specific activity for oysters and sea-water is the same, then an equilibrium condition is assumed to exist; if the specific activity for oysters is less than that for sea-water, then it is assumed that the equilibrium condition has not yet been established; a specific activity for oysters significantly greater than that for sea-water should not occur, theoretically.

TABLE III

Zinc and zinc-65 values for Willapa Bay samples
Mean values ± standard deviation for six samples collected between February 11 and June 24, 1963

	Total zinc[a]		Zinc-65[b]	
Oysters	86 ± 21	10^{-6} g/g wet[c]	88 ± 11	10^{-12} Ci/g wet[c]
Oysters	515 ± 127	10^{-6} g/g dry	531 ± 64	10^{-12} Ci/g dry
Plankton, # 6	239 ± 174	10^{-6} g/g ,,	219 ± 159	10^{-12} Ci/g ,,
Plankton, # 20	232 ± 188	10^{-6} g/g ,,	126 ± 107	10^{-12} Ci/g ,,
Sediments	164 ± 52	10^{-6} g/g ,,	5·10 ± 1·68	10^{-12} Ci/g ,,
Filtered sea-water	7·37 ± 3·06	10^{-9} g/cc	9·32 ± 1·98	10^{-15} Ci/cc
Seston, etc.	8·47 ± 9·11	10^{-9} g/cc	Not detectable	

a. Grams of zinc per gram of oyster (or per cc of sea-water).

b. Curies of ^{65}Zn per gram of oyster (or per cc of sea-water).

c. Calculated on the assumption that wet weight-dry weight ratio is 6·0.

TABLE IV

Specific activity, zinc and zinc-65 concentration factors for Willapa Bay samples
(Values calculated from Table III data)

	Specific activity[a]	Concentration factors[b] Zinc	Zinc-65
Oysters ..	1·04 ± 0·28	11,700 ± 5,600	9,400 ± 2,300
Plankton, #6[c]	0·92 ± 0·96	3,200 ± 2,700[c]	2,400 ± 1,800
Plankton, #20[c]	0·54 ± 0·64	3,100 ± 2,900[c]	1,400 ± 1,200
Sediments ..	0·031 ± 0·014	—	—
Sea-water ..	1·26 ± 0·59	—	—

a. pCi of ^{65}Zn per gram of zinc.

b. Zinc in organism ÷ zinc in sea-water.
Zinc-65 in organism ÷ zinc in sea-water.

c. Calculated on the arbitrary assumption that wet weight-dry weight ratio is 10·0.

In Willapa Bay, where it is assumed that the stable zinc equilibrium has been established, the sources of zinc are run-off water, ocean water, Columbia River water *via* the ocean, and biological exchange processes. Although fallout ^{65}Zn may be present in all of these sources and Hanford-produced ^{65}Zn is present in Columbia River water, the amount of ^{65}Zn relative to the amount of stable zinc in Willapa Bay water is extremely small, the ratio of ^{65}Zn atoms to stable zinc atoms being one to a billion or more.

Inspection of the specific activity values in Table IV shows that (1) the values for oysters, plankton (No. 6), and sea-water are comparable; (2) the specific activity for plankton (No. 20) is somewhat less than that for sea-water, although the difference is not statistically significant; and (3) the specific activity for sediments is significantly less than that for sea-water. From these observations we conclude that ^{65}Zn in sediments has not reached equilibrium with ^{65}Zn in sea-water, but that ^{65}Zn in sea-water and oysters and in sea-water and plankton apparently is in equilibrium.

The concentration factors for oysters and plankton (Table IV), as determined either by zinc or ^{65}Zn analyses, are approximately the same when corrected for units of measurement. The concentration factor of $1{\cdot}2\times10^4$ for Willapa Bay oysters is less than that reported from other localities and for other species in Table II. Low concentration factors can result from either a small quantity of zinc in oysters or from a large quantity of zinc in sea-water. A comparison of the zinc values in oysters and sea-water from Willapa Bay and other areas led to the conclusion that the low zinc concentration factor for Willapa Bay oysters was a consequence of considerably less than the average amount of zinc in oysters and a near average amount of zinc in sea-water. The ^{65}Zn concentration factors confirm this conclusion and also indicate that ^{65}Zn and stable zinc are equally available to Willapa Bay oysters.

The exact chemical form of the ^{65}Zn in Willapa Bay as well as of that in the Columbia River effluent (Osterberg *et al.*, 1966) is not known; however, we know that this radionuclide is associated with filterable particulate matter and that it is transported with Columbia River sediments. At the mouth of the Columbia River, 70 per cent of the ^{65}Zn in the water was retained on a $0{\cdot}45\,\mu$ millipore filter (Seymour and Lewis, 1964). Columbia River sediment containing ^{65}Zn covers much of the shelf area near the river mouth and also is present in Willapa Bay, 40 km. north of the river mouth (Barnes and Gross, 1966).

Perkins *et al.* (1960) reported a ^{65}Zn concentration factor for Willapa Bay oysters of 2×10^5 a value greater by a factor of 20 than that reported in Table IV. The difference in the two estimates is primarily a matter of the difference in the estimates of the amount of ^{65}Zn in Willapa Bay water, since the values for ^{65}Zn in oysters used in both calculations were not greatly different. The values for ^{65}Zn in Willapa Bay water as reported in 1960 by the above authors (Perkins *et al.*, 1960) and given in Table III for the 1963 samples are $0{\cdot}2\times10^{-15}$ Ci/ml and $9{\cdot}3\times10^{-15}$ Ci/ml, respectively.

TABLE V

Zinc and zinc-65 ratios for Willapa Bay samples
(Ratios calculated from dry sample values in Table III)

Sample ratio	Zinc	Zinc-65
Oysters: plankton, #6 mesh net ..	2·2 ± 1·7	2·4 ± 1·8
Oysters: plankton, #20 mesh net ..	2·2 ± 1·9	4·2 ± 3·6
Oysters: sediments ..	3·1 ± 1·3	104 ± 37
Plankton, #6: plankton, #20 ..	1·0 ± 1·1	1·7 ± 1·9
Plankton, #6: sediments ..	1·5 ± 1·2	43 ± 34
Plankton, #20: sediments ..	1·4 ± 1·1	25 ± 23
Dry sediments: sea-water[a] ..	22,300±11,600	550 ± 210

a. Values per cc of sea-water.

The zinc ratio in oysters, or in plankton, to zinc in sea-water is identified as concentration factor and is given in Table IV. Other zinc ratios—oysters/plankton, oysters/sediments, etc.—are given in Table V. From these ratios the following conclusions can be made:

(1) The stable zinc and ^{65}Zn content of oysters was 2 to 4 times greater than that of plankton, which suggests a trophic level difference.

(2) There is no significant difference in the amount of stable zinc or ^{65}Zn in the plankton samples from the two nets, No. 6 and No. 20 mesh.

(3) The stable zinc ratios for oysters/sediments and for plankton/sediments on a dry weight basis ranged from 1 to 3; similar ratios for ^{65}Zn ranged from 25 to 100 because of the relatively low values for ^{65}Zn in sediments.

(4) The stable zinc ratios for sediments/sea-water and for oysters/sea-water are the same if it is assumed that the wet to dry ratio for sediments is about 2.

(5) Zinc-65 measurements can be used to make a reliable estimate of the zinc content of oysters, plankton and sea-water, but not of sediments.

The analyses reported in Table III were made of samples collected during a period when ^{65}Zn in Willapa Bay oysters was near an all-time high. For the years 1957 to 1960, the values were about 40 pCi/g (Watson *et al.*, 1961), and then increased during the period 1961–64 to about 85 pCi/g, after which they declined to 28 ± 7 pCi/g in 1966 (*U.S. AEC Report*, 1967). There are many reasons for the changes in ^{65}Zn content of Willapa Bay oysters, one being the operation of the Hanford reactors. The plutonium-producing reactors at Hanford began operation in 1944. Eight single-pass reactors in which Columbia River water was used as the primary coolant were in operation until 1965, when three reactors were shut down. The recent decline of ^{65}Zn in Willapa Bay oysters is attributed in part to the shutdown of the three reactors. The other source of ^{65}Zn is fallout, but this source has contributed only 1 pCi/g or less to the ^{65}Zn content of Willapa Bay oysters (Seymour and Lewis, 1964; Perkins *et al.*, 1960).

Although the amount of ^{65}Zn in Willapa Bay oysters is easily detectable by gamma spectrometry, the hazard from eating these oysters has been found to be negligible. For oysters with an average ^{65}Zn value of 50 pCi/g, Perkins *et al.* (1960) estimated that the added exposure which a person could receive from eating such oysters at a sustained rate of one pint per week would amount to less than 10 per cent of the radiation dose from natural sources. Another approach is to determine the maximum amount of ^{65}Zn in oysters with no observable effects to those who eat the oysters. Preston (1967) derived a value of 2,900 pCi/g as the permissible level of ^{65}Zn in oysters, based upon the allowable intake of ^{65}Zn by man recommended by the International Commission on Radiological Protection, and a maximum sustained consumption of oysters by man of 75 g per day, as determined by a survey near the Bradwell Power Station, England. By comparison, the present level of ^{65}Zn in Willapa Bay oysters is one per cent of the permissible level derived by Preston.

The concentration factor is defined as the ratio of zinc in oysters to zinc in sea-water but often data used in its calculation obscure the true meaning. Some of the reasons are as follows:

(1) *Relationship of zinc in oysters to zinc in sea-water.*—The value for zinc in oysters reflects the accumulation of zinc over a long period of time [biological half-life, 300 days (Seymour, 1966)], whereas the value for zinc in sea-water, commonly used in the calculation of the concentration factor, is the value for only the moment of the collection of a single sample. The use of a value for zinc in sea-water calculated by the integration of zinc values for many sea-water samples, collected during the period of zinc accumulation by oysters, would provide a more meaningful concentration factor.

(2) *Equilibrium.*—A true concentration factor can be calculated only if the zinc in the oyster and in the water is in equilibrium. Concentration factors calculated from data obtained before

equilibrium is established are less than the true concentration factor. If the concentration factor for the whole organism is being considered, it should be recognized that zinc is being taken up by several systems of the oyster, each at its own rate. Hence, the true concentration factor can be calculated only after equilibrium has been established for all systems.

(3) *Source of zinc.*—Since oysters obtain zinc both from sea-water and from their food, the concentration factor is not a measure of direct uptake of zinc from sea-water as is implied in its definition.

(4) *Wet weight of oysters.*—Values used in the calculation of concentration factors were obtained by multiplying the dry weight by a constant (6·0). The use of a constant introduces an error because the wet weight-dry weight ratio of oysters is not constant; however, the error is believed to be less than the error in the determination in a standard wet weight. The variability in wet weights results from methods of discarding the fluid retained in the mantle cavity and in the water-tubes and chambers of the gills and to injury incurred by the oyster in the shucking process. Puncturing the mantle and pericardium results in up to 50 per cent loss of body weight (Galtsoff, 1964).

Summary

The distribution of zinc in Willapa Bay (Washington) was investigated in 1963. It was determined that the average zinc content of *C. gigas* from Willapa Bay, 0·010 per cent of wet weight, is less than that of *C. virginica* from the Atlantic Coast by a factor greater than 10. Also, for this reason the concentration factor for Willapa Bay oysters, $1{\cdot}2\times10^4$, is less than that for Atlantic Coast oysters since the zinc content of sea-water (the other term in the calculation of the concentration factor) is similar for the two areas. Although the ratio of ^{65}Zn to zinc for oysters was less than that for sea-water the difference was not significant. Thus, it is assumed that zinc and ^{65}Zn are equally available to oysters, that ^{65}Zn in oysters is in equilibrium with ^{65}Zn in sea-water, and that the zinc concentration factor can be calculated from either stable zinc or ^{65}Zn values, although the ratio of stable zinc to ^{65}Zn is greater than a billion to one. The present amount of ^{65}Zn in Willapa Bay oysters, 28 pCi/g, is one per cent of a permissible level of 2,900 pCi/g derived by Preston (1967).

Note.—This report has been prepared by the junior author, mostly from information taken from the senior author's master's thesis (Naidu, 1963), "Stable and Radioactive Zinc in Willapa Bay". Unfortunately, the senior author was unable to prepare the report at this time. The junior author was research supervisor for Mr. Naidu's thesis.

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LARVAL DEVELOPMENT IN THE CLASS BIVALVIA[1]

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ABSTRACT

Larval development has not been described in over 97% of extant bivalve species. Nevertheless, the diversity of larval types exhibited by the Bivalvia exceeds that of any other molluscan class and it is possible to make some hypotheses about the evolutionary history of bivalve larval development.

There are five known basic types of bivalve larvae: (1) Protobranch, (2) Pandoracean, (3) Veliger, (4) Glochidium, and (5) Lasidium.

Protobranch larvae are oval and develop inside a ciliated epithelial test. Modifications of this test have evidently resulted in two evolutionary lines of development. In the Pandoracean-Veliger line the test has been modified to form a velum. In the Glochidium—Lasidium line it has been lost or reduced.

Pandoracean larvae resemble Protobranch larvae with a velum. Both types are lecithotrophic. The original shells of Veliger larvae are secreted by the shell gland. At this stage their development is probably homologous to that of Pandoracean larvae. Subsequent development is planktotrophic. Smaller egg size, longer pelagic period and development of an umbo are characteristic of the Veliger type of development.

Glochidia develop within the adult. At time of discharge they are characterized by relatively heavy shells that may be armed with spines. Further development is usually dependent on a parasitic stage with a fish or amphibian host. This parasitic stage may be optional or lacking in some species.

Lasidium larvae are highly modified fish parasites bearing little resemblance to other types of bivalve larvae. At the time of their discharge from the parent their most conspicuous feature is an extremely long thread or tentacle that is lost when the lasidium attaches to its host.

Two basic trends have influenced the evolution of bivalve larval development. The first appears to be an evolutionary tendency to increase dispersal of larvae. This has been accomplished in advanced types by lengthening the pelagic period (Veliger) or development of parasitic stages (Glochidium, Lasidium). A second evolutionary trend seems to be toward protection of larvae through internal or external incubation. Incubation may be for a few hours or up to the length of the entire larval period. External incubation can be in brood sacs or nests or in gelatinous egg strings or masses. Internal incubation occurs in brood pouches in the mantle cavity or gills. The influence of incubation on larval development has resulted in direct development or modification of the basic larval types to create several sub-types.

INTRODUCTION

OF the molluscan classes the Bivalvia display the greatest diversity in larval development. This is surprising since the Gastropoda display the greatest diversity in form, habitat, mode of locomotion, food and methods of feeding (Table I). The diversity of bivalve larval types is apparent even though our knowledge is fragmentary. Larvae of only about 3%, or less than 500, of the 15,000 extant bivalve species have been described. Larval development has never been described for any species in two orders, Palaeoconcha and Septibranchia. Virtually nothing is known of fossil larvae or larvae of extinct species. Nevertheless, the confusing array of bivalve larval types that are known has been a challenge to malacologists attempting to classify them. Miyazaki (1962) reviewed descriptions of the larvae of 200 species and described 20 types of bivalve larvae. His classification included 4 marine types—Protobranch, Standard, Incubatory, and Egg mass. Freshwater forms were listed as Glochidium, Lasidium, Sphaerium, and Corbicula types.

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Ockelmann (1965) classified larvae of marine bivalves as planktotrophic (feeding on plankton), lecithotrophic (needing only egg yolk), and those having direct development (no pelagic stage). He emphasized that these major types may be modified, especially when associated with brood protection.

In this report I would like to utilise some of the concepts expressed by Miyazaki and Ockelmann and formulate a classification scheme based on what appear to be evolutionary trends in the larval development of the Bivalvia. This will involve the introduction of a few new terms and the assignment of special meanings to some familiar terms.

TABLE I

Comparisons of Gastropoda and Bivalvia

	Gastropoda	*Bivalvia*
Form	No shell Univalve shell Bivalve shells	Bivalve shells Three shells
Habitat	Marine Freshwater Terrestrial	Marine Freshwater
Food and Feeding	Filter feeding Detritus feeding Devouring { Herbivores, Carnivores, Omnivores } Parasitic	Filter feeding Detritus feeding Commensal algae
Larval development	Veliger { Planktonic, Egg case }	Veliger { Planktonic, Incubated, Egg case } Glochidium Lasidium Protobranch Pandoracean

BASIC LARVAL TYPES

Adult bivalves are relatively sedentary with species dispersal depending primarily on transport of the larvae. This has resulted in an evolutionary advantage in the development of mechanisms for increasing dispersal of larvae. Accordingly, several types of larvae, selected for dispersal under a variety of ecological conditioning, have evolved. Based on this assumption, there are five known basic types of bivalve larvae: Protobranch, Pandoracean, Veliger, Glochidium, and Lasidium (Fig 1).

The most primitive, or archeotype, bivalve larva is the Protobranch (Fig. 1 A). Such larvae develop from large eggs and are lecithotrophic. Development is within a ciliated test which also serves as a swimming organ. At metamorphosis the test ruptures posteriorly and is cast off anteriorly (Fig. 1 B). Larvae are not strong swimmers and the larval period is short.

From this archeotype larva two lines of development have evolved. In the marine environment bivalve larvae are distributed by water currents and have developed mechanisms that permit maximum dispersal by currents. In freshwater, a second line of development has evolved in which parasitic larvae are dispersed through the activity of the host.

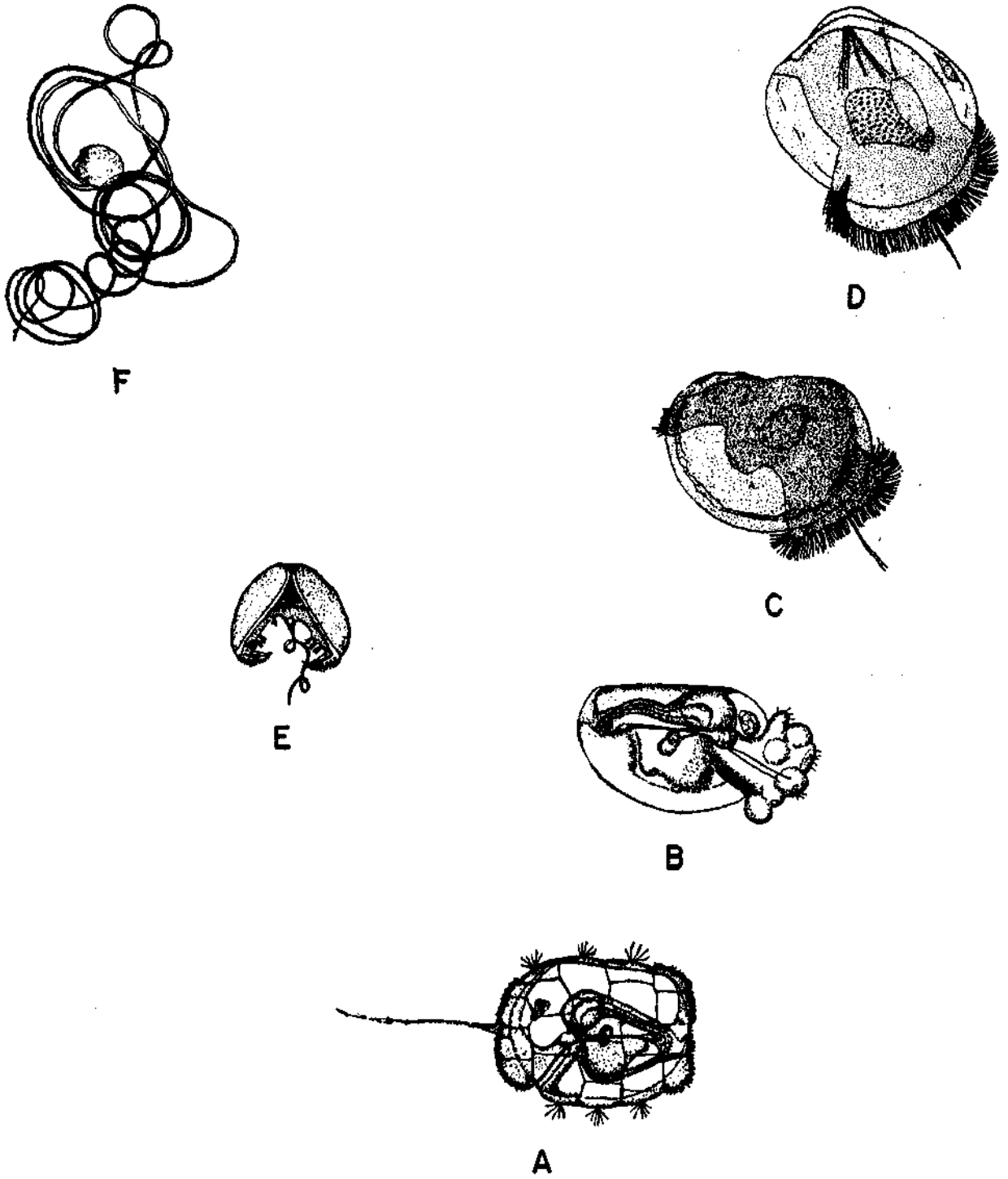

FIG. 1. The five basic types of bivalve larvae. A. Protobranch larva with ciliated test, *Yoldia limatula* Say, (from Drew, 1897, 1899 *b*). B. Protobranch larva in the process of casting test, *Yoldia limutula* Say, (from Drew, 1901). C. Pandoracean larva of *Lyonsia hyalina* (Conrad) (from Chanley and Castagna, 1966). D. Umbonate Veliger larva of *Tellina agilis* Stimpson. E. Glochidium larva of *Anodonta imbecillis* Say (from Pennak, 1953 after Tucker, 1927). F. Mature free living Lasidium larva of *Mutela bourguignati* (Ancey) Bourguignat (from Fryer, 1961).

In the marine environment the Protobranch ciliated test is modified to form a true velum (Drew, 1899 *a*; 1901). This is the extent of change in larvae of the superfamily Pandoracea (Fig. 2). Pandoracean larvae are still lecithotrophic, do not increase appreciably in size or change shape during larval development, and have a short pelagic period. The similarity between Protobranch larvae in the process of casting their test and Pandoracean larvae is striking (Figs. 1 B, 1 C).

The most advanced and the most common type of bivalve larva is the Veliger (Figs. 1 D and 3). Veliger larvae characteristically have a D-shaped, straight-hinge stage which is homologous to the Pandoracean larva, though much smaller. Development to the straight-hinge stage is lecithotrophic and is completed within the first or second day. Larvae then become planktotrophic. During this stage larval shape changes, as larvae develop an umbo and increase considerably in size.

The swimming activity of bivalve larvae probably does little more than keep them in a stratum where they can be distributed by water currents. Such distribution may be enhanced by vertical migration of larvae in response to environmental stimuli (Galtsoff, 1964; Thorson, 1964).

In most freshwater species, the protobranch test, instead of being modified and developed, is reduced or lost. Increased dispersal has been accomplished by assumption of a parasitic stage on either fish or amphibians. Two types of larvae have a parasitic development. The most common is the Glochidium (Figs. 1 D and 4). Glochidia are typically incubated within the adult. At their release they resemble straight-hinge Veliger larvae without a velum and are probably homologous to them. Typically they lie on the bottom until they come in contact with the host. The valves then close, generally on the fins or gills of a fish, and the Glochidium becomes encysted. Some species grow during encystment while others do not. After completion of development, a period lasting from a few days to several months, Glochidia leave the host as juvenile clams.

Lasidium larvae (Figs. 1 E and 5) differ considerably from all other types of bivalve larvae. At their release from the adult, Lasidium larvae have two small ciliated lobes anteriorly. These may be homologous to the velum of Veliger larvae. The most conspicuous feature of Lasidium larvae is the extremely long tentacle or thread at the posterior end (Figs. 1 E and 5 A). The tentacle becomes entangled in the substrate, trailing the larva in the water. This tentacle is discarded when larvae attach to a fish host by minute hooks on the body of the Lasidium (Fig. 5 B). The Lasidium is then firmly attached to the fish by mucus. It develops by elongation and formation of two root-like appendages, the haustoria. Haustoria have both a nutritive and an anchoring function. They unite in a single stalk which forms a bud at the distal end (Fig. 5 C). The bud eventually develops into a juvenile clam (Fig. 5 D) which breaks free and drops to the bottom to assume a free-living existence.

Secondary Larval Types

Conservation of larvae is sometimes of greater evolutionary significance than dispersal. For example, development might be so slow in cold water that few larvae could survive the longer pelagic period. In an abyssal environment the scarcity of food presumably precludes planktotrophic development. Small species with an adult length of less than 10 mm. may not be able to produce adequate quantities of eggs for sufficient numbers to survive a hazardous pelagic larval existence. Most species living under these conditions incubate their larvae either externally or internally (Thorson, 1946 *a*, 1946 *b*; Ockelmann, 1965; Sellmer, 1967). Larvae may be incubated externally in egg masses or mucous nests or they may be incubated internally in brood pouches of the gills or mantle. The incubation period may be for a few hours or for the entire larval period. Some representatives of all the five basic larval types (except possibly the Pandoracean) have larvae that are incubated (Fig. 6).

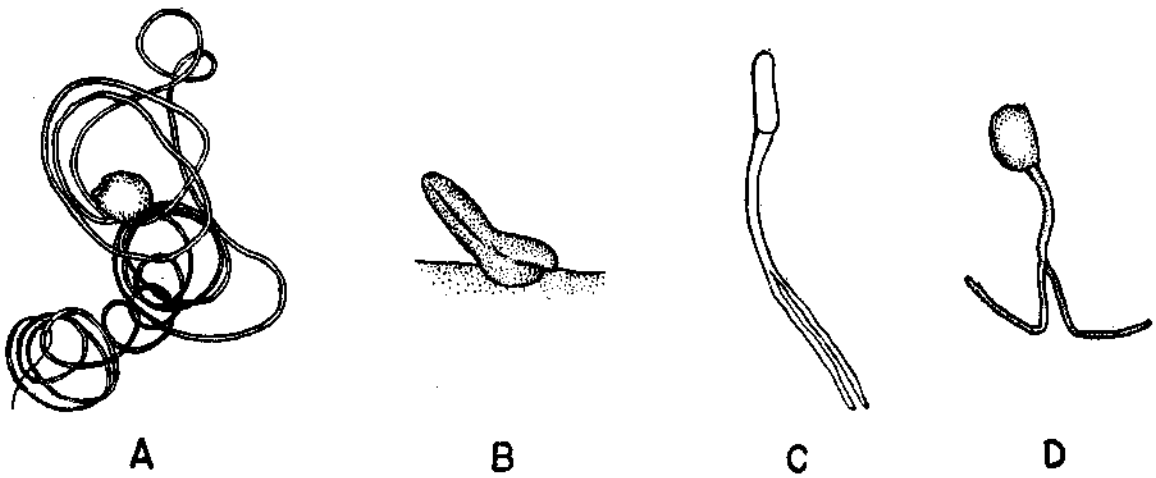

FIG. 5. Development of the lasidium larva of *Mutela bourguignati* (Ancey) Bourguignat. A. Free living mature lasidium. B. Early haustoria stage attached to fin ray of fish. C. Late haustoria stage showing haustoria, stalk, and bud. D. Young bivalve just prior to release (all from Fryer, 1961).

The modifications brought about by incubation permit further classification of larvae into "developmental" subtypes. I have categorized these types as follows.

Pelagic development—Fertilization external. Free-living throughout larval development. Planktotrophic during shelled stages.

Hypolarviparous development—Fertilization internal. Facultative incubation from only a few hours to two days. Released as young larvae. Primarily planktotrophic.

Larviparous development—Obligatory incubation through about half the larval period. Released as half-grown larvae. Planktotrophic after release.

Hyperlarviparous development—Incubated through almost entire larval period. Released as larvae just prior to, or during, metamorphosis. Probably primarily lecithotrophic.

Direct development—Incubated through entire larval period. Released as juveniles. Probably lecithotrophic.

Further classification into "morphological" types is possible on the basis of anatomical characteristics. Glochidia are of the hooked, hookless or axhead type (Baker, 1928). Veliger larvae can be short, moderate, or long hinge-line. If short hinge-line, they are further divisible into equivalve and inequivalve. Hinge structure can also be used to describe a "morphological" type (taxodont, irregular, toothless, etc.). "Family" types, which may or may not correspond to taxonomic families, are determined by major characteristics of shape, such as umbo, length-height relationship, color, and texture. Further classification would be to species and involve minor differences in shape and appearance.

As an example, the classification of the larvae of *Anadara transversa* would be:

		Characteristics
Basic type	Veliger	Planktotrophic with velum.
Developmental type	Pelagic	Free-swimming throughout larval development.

		Characteristics
Morphological type	Mytilacean-Arcacean	Equivalve, long hinge-line. Dentition: taxodont or absent.
Family type	Arcid	High length-height ratio, knobby umbo, distinctive brown color.
Species	*Anadara transversa*	Ventral margin nearly straight. Shoulders short; ends sharp.

Lack of information prevents a listing of morphological, family, and species types.

Discussion

In reviewing the major types of bivalve larvae it is apparent that some types of development are associated with certain ecological conditions. In freshwater the most successful or common larval development involves parasitism. Pelagic Veligers are found in only one small group. In salt-water the most common larval type is the pelagic Veliger. There are no known parasitic marine bivalve larvae.

The scarcity of pelagic larvae in freshwater can be readily explained. Horizontal currents are either weak and ineffective or unidirectional and would tend to carry larvae out of a satisfactory environment to sea (Sellmer, 1967). Thus dispersal by water currents is generally unsatisfactory in freshwater and species employing this mechanism would be at a selective disadvantage.

It is more difficult to explain the absence of parasitism among the larvae of marine bivalves. There are several possibilities.

(1) In freshwater, parasitism has a selective advantage over pelagic development since it is a more effective dispersal mechanism. In salt-water, dispersal by currents is as effective, or more effective than, dispersal by parasitism. Without selective pressure or advantage (selective breeding), perhaps the necessary modifications in the development of parasitic larvae have been submerged in the general population and have not had the opportunity to develop.

(2) Incubation is apparently a prerequisite to parasitism but has the evolutionary disadvantage of decreasing larval dispersal. Accordingly, incubation is scarce except under those special conditions where pelagic development is too hazardous (freshwater, cold water, abyss, etc.).

(3) A parasitic life-cycle necessarily involves the interaction of two species. The parasitic species is not only dependent on factors that influence it directly but also on factors that influence the host species. The hazards of this dependence are evident in some freshwater areas today where populations of healthy clams are disappearing because the fish that serve as host for the glochidia are no longer present.

Therefore, in the marine environment parasitism not only fails to offer a selective advantage over pelagic development but is a disadvantage since it relies on an extra variable—the host species.

Summary

The five basic types of bivalve larvae—Protobranch, Pandoracean, Veliger, Glochidium, and Lasidium—show evolutionary trends toward greater dispersal of larvae. In specialised habitats a secondary, counter trend toward greater protection of larvae is evident. This has resulted in "developmental" subtypes reffered to as pelagic, hypolarviparous, larviparous, hyperlarviparous, and direct development. Further subdivision into "morphological", "family", and

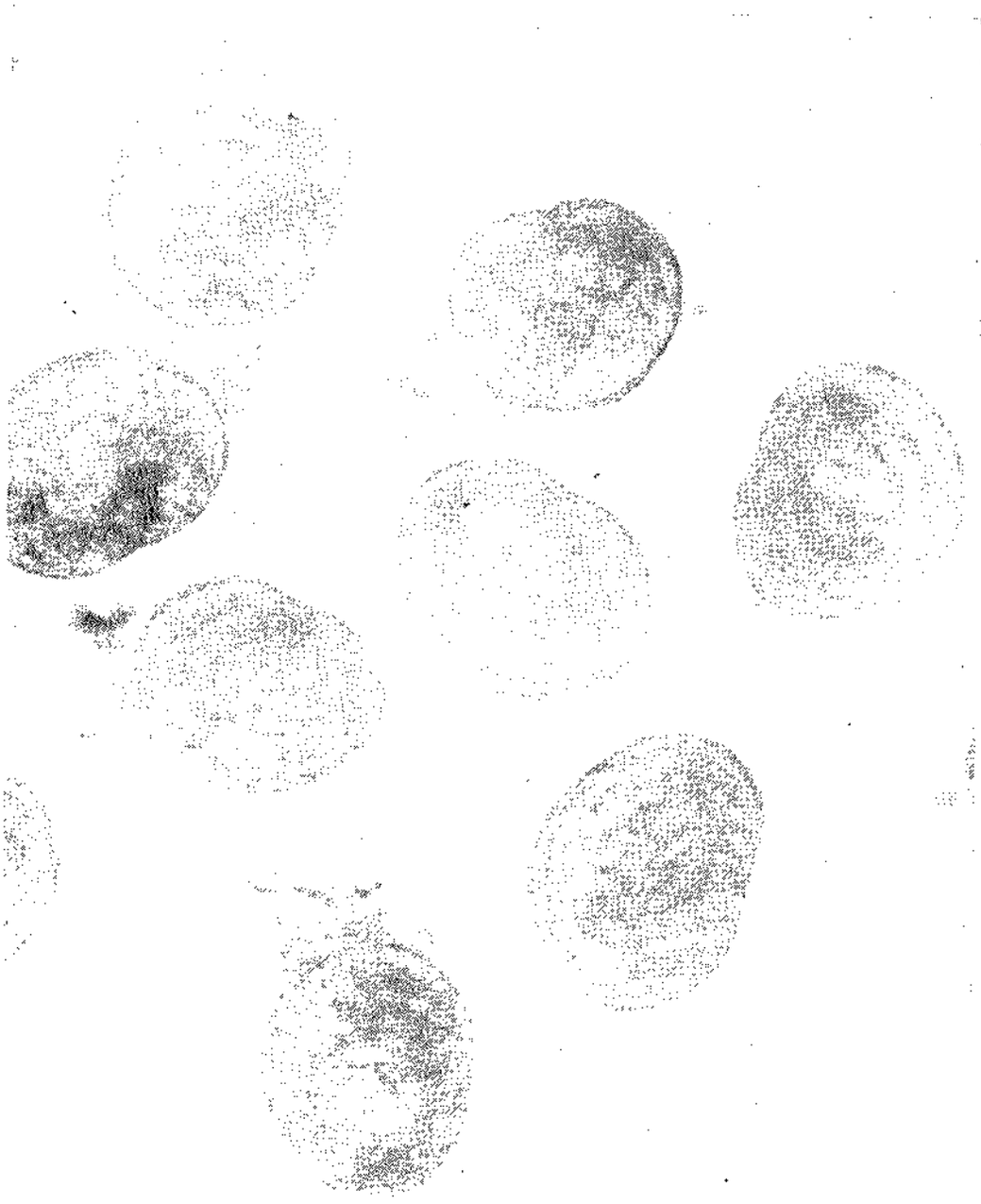

FIG. 1. Larvae of *Crassostrea hrabra* (Conrad). These larvae are typical of the Pandoracean type and do not have straight hinge or umbo stages (from Chanley and Costagna, 1966).

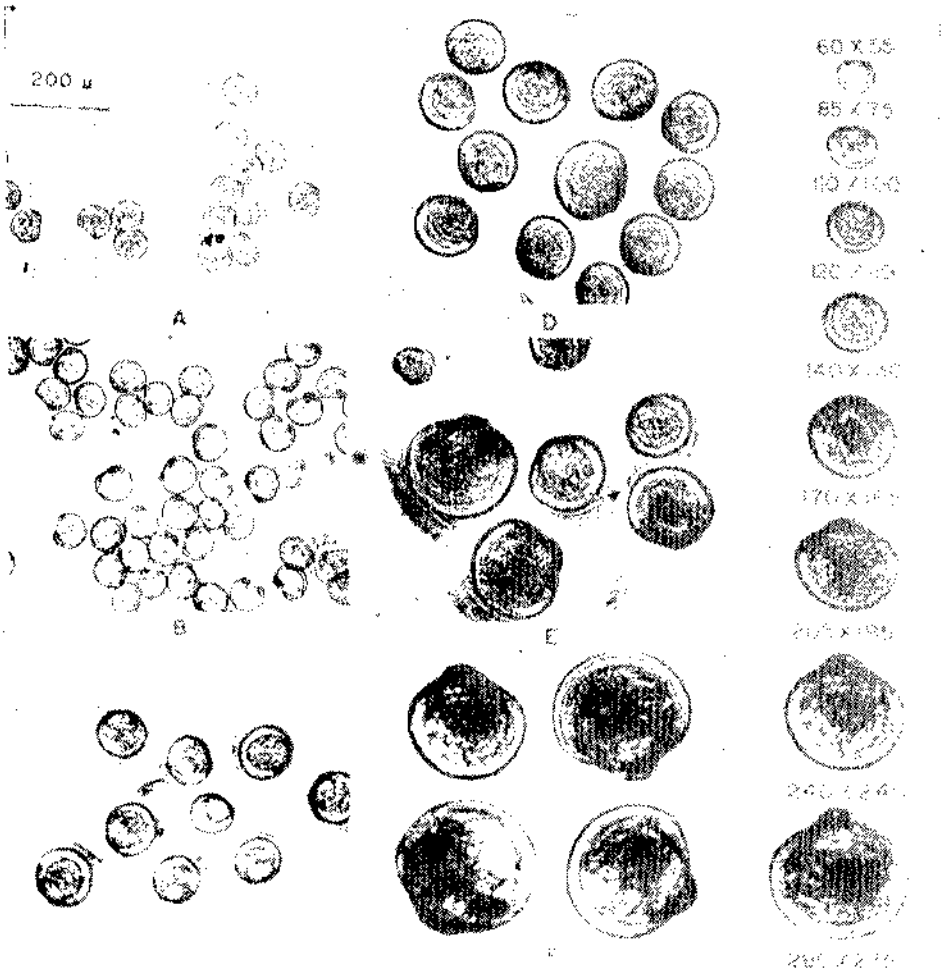

FIG. 3. Veliger larvae of *Barnea truncata* Say showing change in size and shape from early straight hinge stage (A) to late umbo (F). Velum is extended and visible in C and E (from Chanley, 1965).

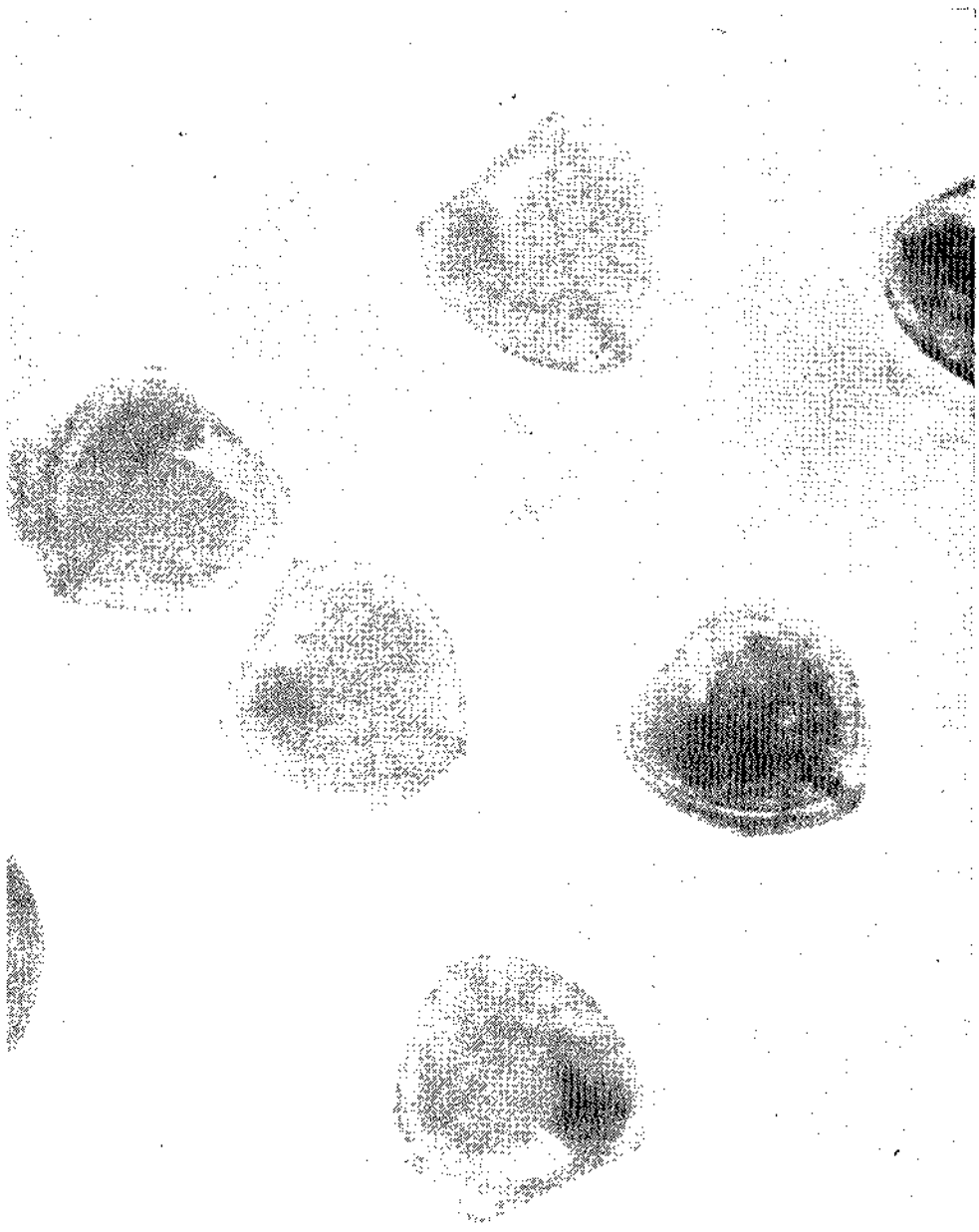

FIG. 3. Glochidia of *Anodonta grandis* Say that have been stripped from the gill chamber.

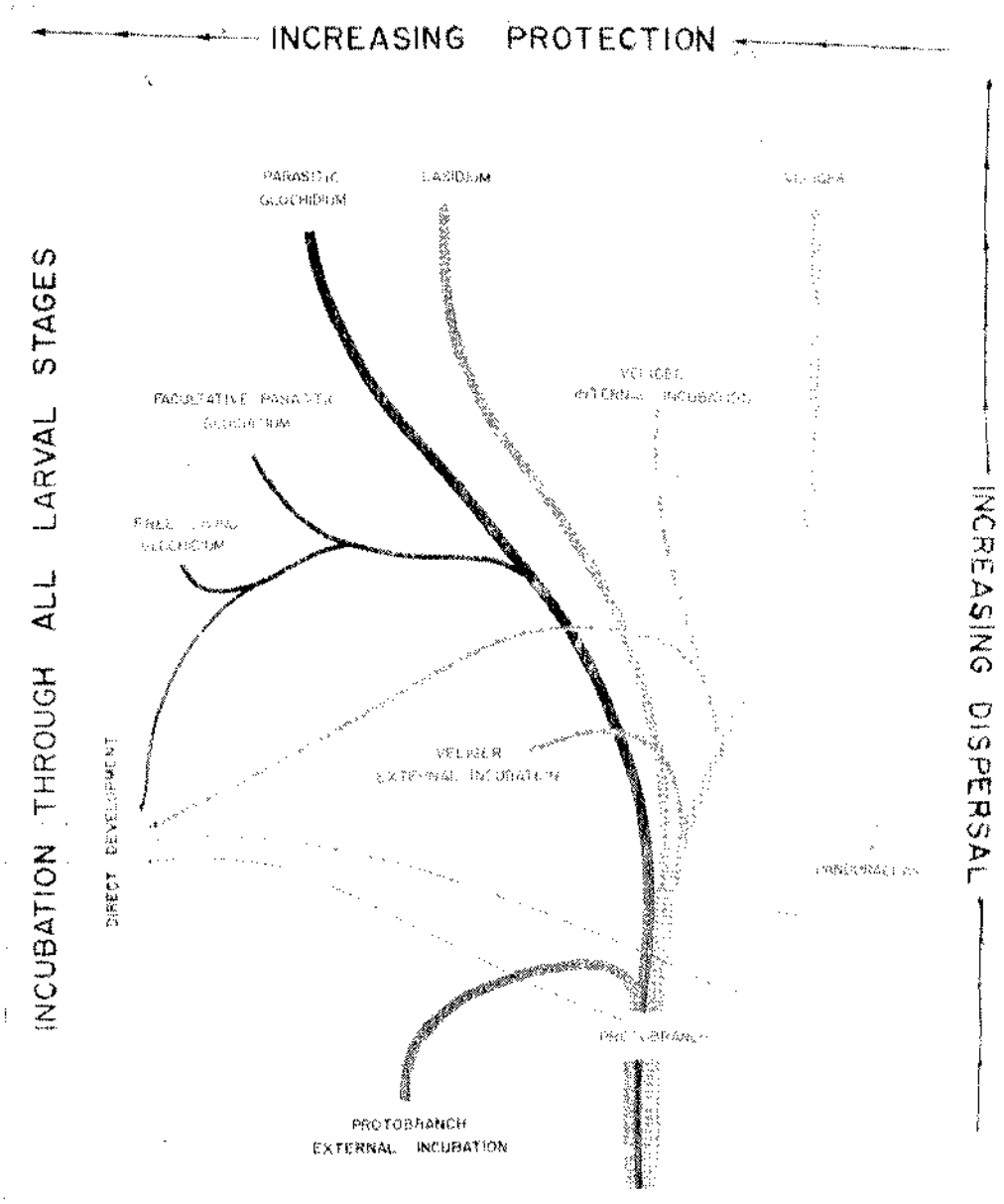

FIG. 6. Schematic representation of the modifications of bivalve larval types. The five basic types Protobranch, Pandoracean, Veliger, Glochidium and Lasidium, display an evolutionary trend toward greater larval dispersal. A counter trend, toward protection of larvae, through incubation, has resulted in modification of the basic types. Dotted lines represent hypothetical possibilities that have not, as yet, been observed

"species" types is also possible, though a complete listing of these is presently impossible because of lack of information.

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STUDIES ON THE OOGENESIS OF *PILA VIRENS* (LAMARCK) (PROSOBRANCHIA-GASTROPODA)

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ABSTRACT

The paper records observations on the oogenesis of *Pila virens* with special reference to the formation of the pronuclei and their fusion. The maturation divisions take place only after the eggs are laid. The actual entry of the sperm into the egg is accomplished at the time of second maturation division. The chromosome number is $2n = 28$.

INTRODUCTION

DETAILED studies of the oogenesis and fertilization in molluscs have been made mostly on the Opisthobranch and pulmonate species. The more important investigations are on *Helix aspersa* and *Arion empiricorum* (Garnault, 1888–1889); *Limax agrestis* (Byrnes, 1900); pulmonate gastropods (Linville, 1900); *Haminea solitaris* (Say) (Smallwood, 1904), and on *Lymnaea stagnalis* (Crabb, 1927). Recent studies are by Raven (1945, 1949) and Raven *et al.* (1958) on *Limnaea*.

Studies on the oogenesis and fertilization in Prosobranchs have been relatively few. Conklin (1902) studied the maturation, fertilization, and cleavage in *Crepidula*, Popoff (1907) and Ankel (1925) studied the development of the ova and fertilization in *Paludina*. The cytoplasmic inclusions in oogenesis in *Paludina* (Vivipara) have been investigated by Gatenby (1919) and in *Pila* by Bhattacharya and Mathur (1930). Cytological studies in relation to parthenogenesis have been made in *Potamopyrgus jenkinsi* by Rhein (1935) and Sanderson (1940); in *Campeloma rufum* by Mattox (1937); and in species of *Melanoides* by Jacob (1957).

The present study relates to the nuclear cycle during oogenesis in the common South Indian apple snail, *Pila virens* (Lamarck).

METHODS

Specimens were collected from paddy fields and maintained in the laboratory aquaria, where they laid eggs. Eggs deposited during the early hours of the day were found to be most suitable for a study of oogenesis, as many of them showed maturation stages.

The egg has a white calcareous outershell with two thin inner membranes which enclose a viscous albumen. The ovum has a yellowish tinge and can easily be distinguished from the enveloping albumen.

The eggs were opened with fine needles and after removing the shell and albumen the ova were transferred to the fixatives. Materials fixed in Bouin's fluid, picrosulphuric, Sanfelice's mixture, and Carnoy's 1 : 3 acetic alcohol gave good results. Embedding was in paraffin and the sections were 6 to 12 micra thick. Delafield's haematoxylin, Crystal violet, and the Schiff's reagent after Feulgen hydrolysis were used for staining. In some cases, to facilitate easy handling, the ova were first embedded in small rectangular blocks of rat liver, which were then subsequently embedded in

paraffin, sectioned at 10 to 16 micra thick and stained. Material fixed in Bouin's fluid and Picro-sulphuric and stained with Heidenhain's Azan gave best results. Acetocarmine squashes of the ova proved helpful for the determination of the chromosome number. The optical equipment used was a Zeiss Winkel Standard Research microscope for routine work and a Zeiss Opton microscope for critical work. The drawings were made with the aid of a Zeiss camera lucida and the photographs were taken with Zeiss photographic apparatus.

OBSERVATIONS

1. *Stages in the ovary*

During the breeding season the germinal epithelium actively proliferates large number of cubical cells with elliptic nuclei, which later transform into nurse cells and oogonia. The earliest oogonial nucleus measures 3 to 4 micra in diameter and contains irregular masses of very faintly staining chromatin and a small nucleolus. With continued proliferation of the germinal epithelium the first formed cells are gradually pushed to the central regions of the ovarian tubule. The oocytes which are surrounded by nurse cells gradually increase in size.

When the nucleus of the oogonium attains a diameter of about 12 to 15 micra, chromatin threads staining faintly with Feulgen's reaction become apparent. This represents the leptotene stage which is of short duration. At this stage a small nucleolus appears as a homogenous body

The zygotene stage is characterised by the bouquet orientation of the paired chromatin threads. In the pachytene, the pairing of the homologous chromosomes is more intimate and complete, and the bouquet orientation continues. The bivalent threads are now much thicker and also stain deeper. At this stage the nucleolus is not apparent. The changes in the nucleus are difficult to follow after pachytene since it appears as though the nucleus has reverted to a resting condition with faintly staining, irregular clumps of chromatin. The nucleus at this germinal vesicle stage measures about 70 micra and is eccentrically placed at the periphery of the ovum and has a conspicuous nucleolus. The fully grown ova show marked increase in size due to active accumulation of yolk and measure about 120 to 130 micra when they leave the ovary.

2. *Stages in the freshly laid egg*

The ovum in the freshly laid egg measures about 160 micra and its nucleus about 75 micra. The disappearance of the nuclear membrane and the nucleolus and the condensation of chromatin masses into chromosomes of definite shape mark the beginning of I metaphase stage in the ovum of freshly laid eggs. With the appearance of the spindle the chromosomes arrange themselves in the equator. The first maturation spindle is almost perpendicular to the surface of the ovum as shown in Fig. 1 measuring about 20 micra in the long axis and 12 micra near the equator. Though the centrospheres are clearly visible no centrioles could be seen during the first maturation division. The close arrangement of the chromosomes in the equatorial plate of the I division makes it difficult to count them. In squash preparations, fourteen bivalents could be made out. For comparison, the chromosomes in the I metaphase of the male were studied and the number of bivalents was found to be the same as in the female.

By the late anaphase the chromosomes form two daughter groups at opposite poles. At the end of the first division, the chromosomes at the outer pole of the spindle together with a small amount of cytoplasm constitute the first polar body, which later separates from the ovum as shown in Fig. 2. The chromosomes in the ovum immediately arrange themselves on the equator to form the II metaphase plate without entering into an interphase.

The first polar body is somewhat flattened and measures about 7 micra in width and about 3·5 micra in height. Figure 2 shows the I polar body extruded by the ovum but remaining attached to it by the persisting spindle fibres. Another interesting feature of this stage is the presence

of the so-called 'mid-body' situated on the egg surface, which seems to provide anchorage for the persisting spindle fibres. A similar feature has also been reported by Raven (1945, 1949) in *Limnaea*, where the mid-body appears after the two maturation divisions while in *Pila* it is found after the extrusion of the I polar body.

The first polar body in *Pila* soon after its formation gets detached. In *Paludina* (Tonniges, 1896) and *Crepidula* (Conklin, 1897) a similar phenomenon has been reported, but in *Melanoides* Jacob (1957) observed the polar bodies to remain attached to the egg for a long time and also to divide. In *Pila*, however, the first polar body does not divide. A similar observation has been reported in *Limnaea* (Raven, 1945, 1949) and *Bulinus* (Larambergue, 1933–cited by Raven, 1958).

The second maturation spindle is obliquely situated and measures about 12 micra from pole to pole and 10 micra near the equator. In a polar view of the II metaphase the full complement of 14 chromosomes is clearly discernible. The II metaphase is of longer duration than the first metaphase.

During telophase stage of the second division, the chromosomes of the outer pole constitute the II polar body while the inner group forms the female pronucleus. The second polar body is smaller in size than the first one and measures about 5 micra in width and about 3 micra in height. Unlike the first, however, the II polar body remains attached to the ovum for sometime.

The sperm head is curved, stains deeply, and measures about 2 to 3 micra in length. It lies in a vacuolated area inside the mature ovum. A similar condition has been observed in *Limnaea* (Raven, 1945, 1949) and *Limax* (Byrnes, 1900). The female pronucleus in this stage is spherical with irregular chromatin threads and measures 15 micra in diameter. No nucleolus could be seen. During the stages prior to fusion with the female pronucleus, the sperm head is transformed into male pronucleus, which is spherical, with irregular chromatin strands and measures 7 micra in diameter. Both the male and female pronuclei now come to lie near the egg surface and the former has increased in size measuring about 13 micra in diameter.

In the stage (Fig. 3) which immediately precedes fusion, both the male and female pronuclei lie close to each other. The male pronucleus by now has enlarged almost to the size of female pronucleus (16 micra in diameter). The two pronuclei appear very similar. It is possible, however, to distinguish the female pronucleus by its thicker and more deeply staining nuclear membrane.

Nucleoli appear in both pronuclei at the time of their fusion, five in the female pronucleus and three in the male pronucleus (Fig. 4), but disappear immediately. The fusing pronuclei continue to remain at the peripheral region of the ovum. A zygote nucleus, however, was not observed. The fusion is apparently an extremely rapid process.

The first cleavage spindle lies a little deeper in the egg. In the metaphase of first cleavage 28 chromosomes could be made out. Cleavage in *Pila* is holoblastic, unequal, determinate, and of the spiral type as in all gastropods.

Discussion

The differentiation of the oogonia and the nurse cells from indifferent cells of the germinal epithelium observed in *Pila* is similar to what has been reported so far in other prosobranchs. A bouquet orientation of the paired threads and their polarisation have been observed in *Pila* during the Zygotene stage. Jacob (1957) did not report polarisation of the chromosomal threads at any stage in *Melanoides*. In *Pila* the sperm head is visible in the ovum only after the second maturation division. A similar condition has been noticed in other molluscs such as *Mactra*, *Crepidula*, *Pleurophyllidea*, *Physa*, and *Bulinus* (cited by Raven, 1958). The time of actual entry of the sperm into the egg varies in different molluscs. In *Unio* (Lillie, 1901) and *Limnaea* (Raven, 1945) the sperm enters at prometaphase and at anaphase respectively of the first maturation division.

The formation of the male and the female pronuclei in *Pila* without passing through a Karyomere stage is similar to what has been reported in *Unio* (Lillie, 1901) and *Arion* (Lams, cited by Raven, 1958). Crabb (1927) and Raven (1945, 1949) observed that the definitive sperm nucleus in *Limnaea stagnalis* is reorganised by the fusion of the karyomeres, which come to be surrounded by a continuous membrane thus forming the pronucleus. The female pronucleus in *Limax* is also reported to be formed in more or less the same way (Raven *et al.*, 1958).

Though the two pronuclei in *Pila* are closely approximated, their complete fusion to form the zygote nucleus could not be observed. The next stage available is the I cleavage metaphase with a fully formed spindle. Apparently, the interval between these two stages is of very short duration. In other Prosobranchs investigated so far, the transition between the two stages is not abrupt.

The chromosome number in *Pila* is $2n = 28$ in both the male and the female. A count of the I cleavage metaphase chromosomes also confirmed this number. Patterson (1967) in a recent review of the chromosome numbers in streptoneuran snails has reported the haploid chromosome number in the three genera of Pilidae, *Pila ovata*, *Lanistes bolteni*, and *Marisa cornuarietis*. This is in agreement with the findings reported in the present paper for *Pila virens*.

Summary

1. The oogenesis and fertilization in the Prosobranch *Pila virens* (Lamarck) have been investigated.

2. The early stages are passed through when the oocytes are in the ovary, but the maturation divisions take place only after the eggs are laid.

3. The actual entry of the sperm into the ovum takes place only after the second maturation division.

4. The interval between the fusion of the male and female pronuclei and the formation of I cleavage spindle is of very short duration, unlike what has been observed so far in other Prosobranchs.

5. The chromosome number of *Pila virens* in both the female and the male is $2n = 28$.

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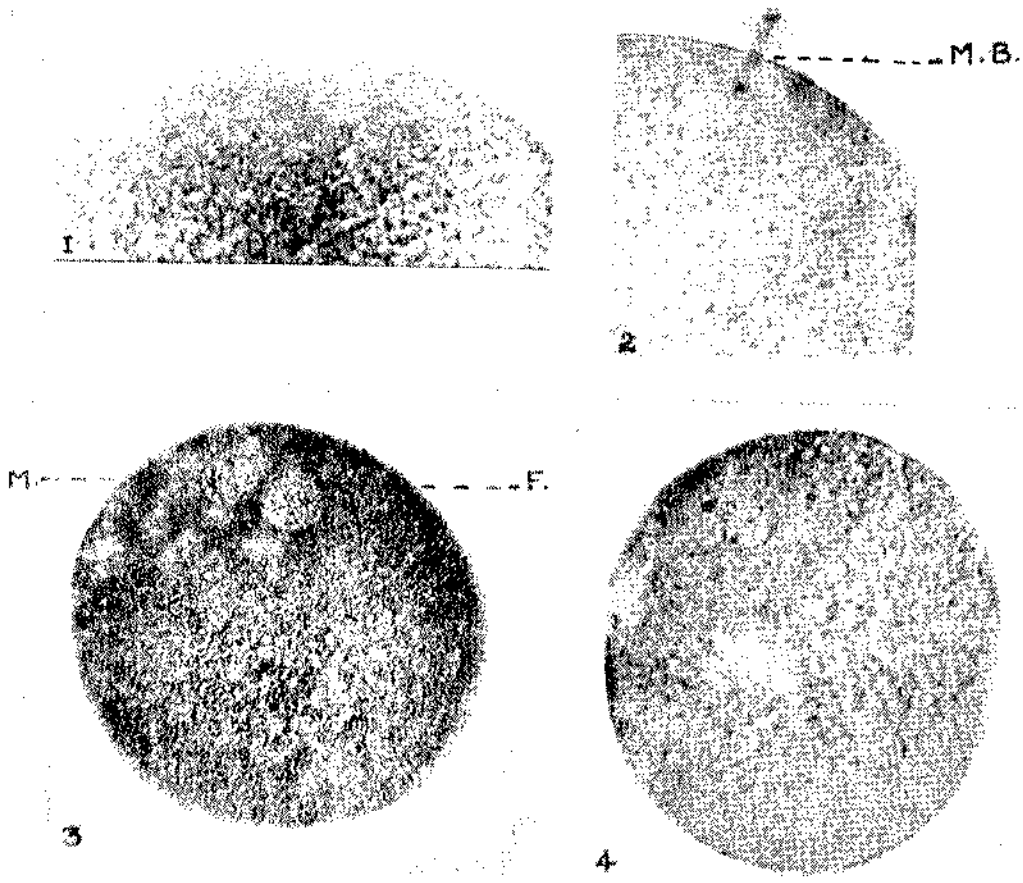

FIGS. 1–4. Fig. 1. Photomicrograph of an ovum showing I maturation metaphase (Bouin–Heidenhain's Azan section) showing the spindle recess (× *ca.* 400). Fig. 2. A magnified photomicrograph of an ovum showing the formation of I Polar body and the persisting spindle fibres and mid-body (M.B.) (× *ca.* 500). Fig. 3. Photomicrograph of mature ovum in which both the pronuclei are apposed to each other (M. Male pronucleus; F. Female pronucleus). (Bouin–Heidenhain's Azan section. *ca.* 300). Fig. 4. Photomicrograph of mature ovum showing the beginning of fusion (Picro-ripharic–Delafield's haematoxylin section, *ca.* 300).

DEFENSIVE ADAPTATIONS TO PREDATION IN THE MOLLUSCA*

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ABSTRACT

Especially among the herbivorous forms, the mollusca exhibit a multitude of adaptations which are essentially defensive in function. They may be broadly classified as passive, or active. Passive adaptations include morphological, physiological and behavioural characteristics, such as the possession of a protective exoskeleton, or toughened skin; the habits of burrowing, nestling, boring or nest-building; the possession of cryptic colouration; the habit of camouflaging the shell, or the display or warning colouration. Active adaptations imply the release of a specific response by the near presence of or contact with, a potential predator, and comprise mainly behavioural characteristics. This latter group are reviewed, and several hitherto undescribed examples of behavioural modifications presented.

Autotomy of parts of the body, when disturbed or seized by a predator, occurs in the Gastropoda, Bivalvia and Cephalopoda. The major instances are the autotomy of the mantle margin in the Doridacea (Gastropoda), of the cerata in the Aeolidacea (Gastropoda); of the posterior end of the foot in some Prosobranchia and Pulmonata (Gastropoda); of the siphons in the Tellinacea and Solenacea (Bivalvia); and of the arms in some Octopodacea and Argonautacea (Cephalopoda).

Locomotory adaptations occur in the Gastropoda, Bivalvia and Cephalopoda. A group of allied responses which include movements described in the literature as "mantle responses", "mushrooming", "flight responses" and "galloping", and which also include leaping movements occur in Zeugobranchia, Patellacea and Trochacea (Gastropoda). Similar movements, which occur as isolated examples from some other groups, include the classic leaping movements of members of the genus *Nassarius* which occur in response to contact with asteroid starfish. Leaping movements are also exhibited by members of the Cardiacea, Veneracea, Tellinacea, Solenacea, Mactracea and Trigoniacea (Bivalvia) in response to contact with asteroid starfish, or, in some cases, with naticid gastropods. Other responses include those of the Tridacnidae (Bivalvia) which show a directed spurting response to grazing fish, and of the gastropods *Natica* and *Trivia* in which lobes of the foot extend to cover the shell in response to contact with starfish.

The release of acid or otherwise distasteful mucus may be considered in some cases to be an active defensive adaptation although there is, in many cases, too little information to decide whether the cells concerned release their contents only in response to specific stimuli. Authenticated reports of such responses refer mainly to the Gastropoda. The release of ink into the water as a smoke screen, irritant, decoy, or sensory anaesthetic occurs in the Aplysidae (Gastropoda), and the Cephalopoda.

A synopsis of these active defensive responses in the mollusca is presented, and their ecological and evolutionary significance briefly discussed.

I. INTRODUCTION

AMONG the most basic interspecific relationships of animals within a community are those between predator and prey species. Knowledge of the action of such relationships is essential towards any understanding of community structure, or of the quantitative trophic structure of a community, yet many such relationships are little known and their quantitative significance in affecting predation patterns even less studied. The purpose of this review is to draw attention to those cases reported in a scattered literature and especially to a lack of knowledge in many cases, to encourage the recording of full details of new examples of such behavioural adaptations, and perhaps to stimulate research into the quantitative significance of such adaptations in the regulation of communities and especially in their effect on trophic interrelationships of species.

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† Since this manuscript was completed several further papers have appeared or come to my attention. As far as possible these have been included in the synopsis, but they are not referred to or discussed in the paper. Such references are marked with an asterisk in the reference list under 'Literature Cited'.

The mollusca play an important role in many communities on land, in freshwater and in the sea. Overwhelmingly they are herbivorous, and thus form the link in the food web of the community between the primary producers and the larger carnivores. This vulnerable position of the herbivorous molluscs is reflected in the development of a wealth of adaptations which aid the survival of their possessors in the face of predation pressure. Indeed, there are numerous references in the zoological literature to specific defensive adaptations, and the first task in reviewing such adaptations in the mollusca is one of classification and selection.

I have found it necessary therefore to limit the scope of the review and in so doing to reject from consideration those adaptations which are not primarily defensive or which may be termed merely passive. Thus, the possession of the shell by most molluscs confers some protection, as does the presence of the operculum in many gastropods or the leathery skin of some nudibranchs. Such habits as nestling in crevices in rocks, of burrowing beneath the surface of sand, mud or gravel, of nest-building, all confer on the molluscs in which they are exhibited a degree of protection from some potential predators. The possession of cryptic colouration or the habit of covering the shell with foreign objects as camouflage are protective in function, as may be also the display of warning colouration by species which thus proclaim their unpalatability. Other molluscs mimic such unpalatable species and thus they themselves obtain a degree of protection. Such adaptations serve to protect the animal by decreasing the chance that it will be found by a predator, or if found, by decreasing the chance that it will be attacked and killed. They thus confer defence passively, since while they may involve a rejection reaction by the predator to the prey species, they do not normally involve any reaction by the molluscan prey to the presence of a predator. Although this type of adaptation presents many interesting problems, I have considered them outside the scope of this paper, which I shall confine to a consideration of what may be termed active defensive adaptations.

I should perhaps define the characteristics of active defensive adaptations in more positive terms. One would expect firstly that there should be a specific response on the part of the mollusc to the near presence of, or contact with, a potential predator, and secondly, that the response should be of an appropriate form to significantly increase the possibility that the mollusc would escape capture, or of a form which would significantly reduce the injury caused by the predator. In particular, the speed of reaction may be expected to bear a close relationship to the speed of approach, or attack, of which the predator is capable. I have excluded from consideration here, one reaction which falls within this definition; that of complete or partial withdrawal of the soft parts into the shell. This reaction exhibited under certain circumstances by most shelled molluscs is essential if the shell is to perform a protective function.

In this review of active defensive adaptations I shall firstly describe with appropriate examples the main types of response exhibited by the mollusca to predators; secondly, present a synopsis of the occurrence of specific reactions in different molluscan groups; and thirdly, briefly consider as far as is possible from the information available, the ecological and evolutionary significance of these adaptations.

II. TYPES OF DEFENSIVE ADAPTATION IN THE MOLLUSCA

1. Autotomy

The ability of some molluscs to autotomise portions of the body has been known for many years. The occurrence of this phenomenon has recently been the subject of a most thorough review by Stasek (1967), who lists in a synopsis of autotomy in the mollusca 95 examples relating to 18 families of the gastropods, three families of the Bivalvia, four families of the Cephalopoda and one family of the Scaphapoda. Some of these examples, together with one not listed by Stasek, are of particular interest here.

Several gastropod species will autotomize the posterior part of the foot when attacked. Species which exhibit such behaviour include the marine prosobranchs *Harpa ventricosa* (Quoy and Gaimard, 1832; Reynard, 1834, in Stasek, 1967), *Gena varia* (Fishelson and Qidron-Lazar, 1966), and the pulmonates, *Prophysaon coerulium* (Raymond, 1890) and *Helicarion* (Semper, 1881). In *Harpa ventricosa*, according to Guoy and Gaimard (1832), autotomy of the posterior third of the foot is easily induced and is initiated by contractions of the foot. The rejected mass contracts independently for some moments. In *Gena varia*, that part of the foot which extends posteriorly beyond the shell is autotomized when the animal is touched, the autotomy following a well-marked course along a fine white line which traverses the foot. The animal does not attempt escape but remains attached firmly to a nearby stone, now completely covered by the shell (Fishelson and Qidron-Lazar, 1966). The amputated part of the foot continues to exhibit movements for two to six hours after autotomy. In *Helicarion*, Semper (1881) describes how the tail is shed a little behind the shell, by the animal whisking the tail up and down with extraordinary rapidity until it drops off. The animal escapes with a convulsive leap (Cooke, 1895) leaving the predator with the tail only.

Unfortunately, very little information is available in the literature on the predators of these forms. Semper (1881) suggested that autotomy in *Helicarion* acts as a defence against such predators as birds and lizards. For the marine species the predators are entirely unknown. Birds may again be a possibility, or perhaps fish. These gastropods are all species in which the soft tissues cannot be entirely housed within the shell, and it is perhaps significant that they are mainly representatives of groups in which other escape responses are well developed (*see* synopsis, p. 497).

Among the Bivalvia, species from two groups, the Tellinacea and the Solenacea, exhibit autotomy of the siphons. Stasek (1967) quotes five species of *Solen* from Great Britain and the United States in which parts of the siphon are cast off and he concludes that predatory fish comprised the ecological factor leading to the evolution of autotomous siphons in the Solenidae. Smith (in Atkins, 1937) observed autotomy of the siphons in *Solen marginatus* when a medium-sized plaice nibbled at their tips. Predation by fish is also the cause of autotomy in the Tellinacea, where autotomy has been recorded from *Solecurtus coarctacus* and other species (Forbes and Hanley, 1853; Rawitz, 1892; Faussek, 1897; Atkins, 1937; Morse, 1919) and for *Tellina tenuis* (Ansell and Trevallion, 1967). Portions of the siphons of *T. tenuis* are commonly found when digging on sandy shores where this form is abundant, and the siphons form an important part of the food of O-group plaice and other flat fish in some areas (Edwards, personnel communication).

The third major example of autotomy in the mollusca is that of autotomy of the mantle edge, which may be thrown off whole or in part in some species of Doridae, or of the cerata in the Aeolididae. In neither group is there much information available in the litereture on the predators which elicit such responses, but these reactions in the two groups of nudibranch in each case represent only one of a wide spectrum of defensive mechanisms (Thompson, 1960; Edmunds, 1966).

The ability to autotomize parts of the body can be effective only if certain other conditions apply. Firstly, the organ which is lost must be that which is most frequently attacked or most exposed to attack. This is true of all the examples discussed. Secondly, the animal, after autotomy has taken place should not be available for a second attack. The amputated organ should serve to delay the predator while the animal makes good its escape, and may thus act as a decoy in an analogous manner to the ink shadow produced by some other molluscs, especially the cephalopods (Hall, 1956). Autotomy is also thus often followed by rapid withdrawal. In the case of *Gena varia*, where the animal immediately clamps down the shell onto a nearby rock (Fishelson and Qidron-Lazar, 1966) the shell is presumably strong enough to resist further attack. In the land-snail *Helicarion*, the escape is apparently spectacular, the animal leaping from the foliage on which it lives to be lost among the leaf litter on the ground (Cooke, 1895). In *Solen* the animal withdraws deeper into the sand by the rapid burrowing movement typical of these forms.

Autotomy implies also the ability on the part of the animal to regenerate the lost organ. While it is clear that this ability is possessed by those molluscs which exhibit autotomy, the process has received little attention and such information as for example the time required for regeneration to occur is not recorded in most instances. The part lost may be regenerated by a distal proliferation of cells at the site of the wound, or may be replaced serially from a continuously active proximal zone of proliferation which forces older portions outward. Stasek (1967) has suggested that serial replacement has advantages over regeneration in that a new and functional element is immediately available, while in regeneration a variable and perhaps critical period elapses before a new element is produced. In populations which are subjected to heavy predation replacement of parts autotomized may compete with other growth processes, so that increase in body size or even gonadial proliferation may be restricted (Ansell and Trevallion, 1967).

2. Locomotory Adaptations

"Mushrooming" and related responses in prosobranch gastropods

A large group of responses by gastropods belonging to three superfamilies of the Prosobranchia have been described in a number of papers in recent years, following the discovery by Haderlie (1947) of distinct responses by certain limpets of the genus *Acmaea* to starfish. The superfamilies concerned are the Zeugobranchia, Patellacea and Trochacea, and the responses, while differing in kind, show clear relationships to each other yet still exhibiting clearly the modification of a basically similar response to suit the specific habits of differing animals.

In the Zeugobranchia, defensive responses have been described from the families Haliotidae and Fissurellidae. The Fissurellid *Diodora aspersa* exhibits a mantle response to contact with various starfish or to their close proximity without contact (Margolin, 1964). When an individual *D. aspersa* is touched by the starfish, the siphon is extended from the keyhole as much as 10 mm. above the shell surface with the siphon swollen and the aperture closed. Simultaneously, the mantle is protruded from beneath the shell, the outer mantle fold being extended about 10 mm, the inner becoming thin and scalloped and reflexed dorsally over the shell which is covered except for a small area around the keyhole. The shell is raised high above the substratum by great elongation of the columella muscle, while the outer mantle fold extends down to cover the foot. There is no movement of the animal away from the predator.

Responses by members of the Haliotidae have been described by Bennett (1927), MacGinitie and MacGinitie (1949), Bullock (1953), Clarke (1958), Feder (1963), Montgomery (1967) and Forster (personal communication). The responses of all species so far described are similar although they differ in details of reaction time and in degree of sensitivity. The responses described were provoked by contact with various starfish, or by diffused substances originating from starfish in the water. On stimulation, the cephalic tentacles are extended to their maximum length and sweep back and forth through the water with the tips vibrating. The epipodium is protruded beneath the shell, with the tentacles around its edge waving in the water and the lobes swelling and extending away from the body, or in some cases over the shell. The animal moves rapidly away from the point of stimulation at a speed several times greater than that of normal locomotion and with a gait, described by several authors as galloping, in which the foot is alternately flexed and extended in a manner reminiscent of a leech or measuring worm (Feder, 1963). As the animal moves away, the shell is lifted by elongation of the columellar muscle and is turned back and forth through an arc of 30° to 180°. This twisting movement may be repeated several times. In an aquarium, if the animal during flight encounters a vertical or sloping wall, it will climb out of the water. Flight may also be accompanied by the emission of quantities of mucus from the respiratory pores (Bennett, 1927; Montgomery, 1967).

In the Patellacea, similar responses have been reported from the two families Patellidae and Acmaeidae. Contact of the starfish, *Asterias rubens*, with the limpet, *Patella vulgata* elicits a well-marked response on the part of the gastropod. The cephalic and mantle tentacles are extended

and the shell is then raised well above the substratum by elongation of the columellar muscle, the shell being raised higher on the side of the animal receiving the stimulus. The shell may then be rotated violently back and forth, rocking as it does so, with the result that the shell edge is rubbed against the rock. Parts of the starfish arms may become trapped under the shell and damaged by this violent motion. Only very occasionally in my observations has the response been followed by movement of the limpet away from the starfish, although Conell (in Margolin, 1964) is reported as observing a flight response in this species.

The limpets *Patina pellucida*, *Cellana radians* and *C. ornata* exhibit flight, the former in response to contact with the starfish *Asterias rubens* (personal observation), the latter to contact with various carnivorous gastropods (Clarke, 1958). In *Patina pellucida*, the shell is lifted well clear of the substratum and the animal moves away from the stimulus at a speed some three times greater than that employed in normal locomotion.

Limpets of the family Acmaeidae show a similar flight response to starfish. The shell is first elevated above the substratum in a movement perfectly described by Bullock (1953) as "mushrooming." The cephalic tentacles are extended and wave about, the mantle and its sensory projections extend from around the shell and the animal then moves away, usually turning at an angle so that its early movement is directed away from the side which received the stimulus. Locomotion takes place initially at a rate some three times greater than normal, justifying the term "running" employed by Margolin (1964).

The third superfamily of prosobranchs to exhibit responses of this type are the Trochacea, for which there are many records in the literature all referring to the family Trochidae, and including responses to contact or close proximity of starfish or carnivorous gastropods. The typical response of the group involves extension and waving of the cephalic and mantle tentacles, elevation of the shell and a greatly increased rate of locomotion, directed away from the stimulus. The shell may be rotated 90° in either direction preceding or during locomotion, or may be swung back and forth through an arc of 180°, especially following continued stimulation such as occurs when the starfish maintains a grip on the shell with the tube feet. The posterior region of the foot, which in some trochids (*e.g.*, *Calliostoma zizyphinum*) is raised vertically in contact with the shell during normal locomotion, is placed in contact with the substratum during rapid locomotion following starfish contact and seems particularly sensitive to stimulation. In rock pools (Feder, 1963) or in an aquarium movements directed up sloping walls may result in the animals leaving the water.

The British top-shell, *Calliostoma zizyphinum* (Trochidae), shows a particularly spectacular response, hitherto undescribed, to contact with the starfish *Asterias rubens*. The initial response is that characteristic of the trochids described above, but is usually followed by violent twisting movement which causes the animal to roll over to rest on one side of the conical shell. The foot is fully extended and twisted to obtain a point of contact on the substratum, and further contractions then cause a rolling/leaping motion. This may be repeated up to seven or eight times resulting in a rapid irregular movement of the gastropod away from the starfish. These movements of *Calliostoma* recall those of members of the stenoglossan genus *Nassarius* which have received much attention and which will be described later (p. 493).

The responses described for these three superfamilies of prosobranchs have many features, in common, and must surely be regarded as expressions of a behaviour pattern of great antiquity in the stock which gave rise to the three groups. The features which are exhibited wholly or partly in all of these responses may be summarised as follows:

(*a*) Extension and waving of the cephalic and mantle tentacles.

(*b*) Extension of the mantle lobes, in some cases over the shell—"Mantle response".

(*c*) Elongation of the columellar muscle—"mushrooming".

(*d*) Twisting of the shell back and forth through an arc of up to 180°.

(*e*) Turning of the foot to direct subsequent locomotion away from the stimulus.

(*f*) Acceleration of the pedal locomotory wave—"running".

(*g*) Change of gait—"galloping".

(*h*) Inversion and violent movement—"leaping".

(*i*) Movement upwards on vertical surfaces or out of pools.

It is impossible to generalise at present about the degree of expression of these characteristics of the response to predators and the normal habits of the animals concerned. It is, however, of interest to consider these defensive reactions in relation to what is known of the normal behaviour of the species in which they are exhibited, and especially in relation to the "homing" behaviour. Gastropods of the family Trochidae, are common inhabitants of rocky or stable boulder shores, which are not unduly exposed, or of the shallow sublittoral. They may retire into crevices, under stones or into rock pools at low water but wander freely on the shore without a "home" territory. Their responses invariably involve flight, either by an increased rate of pedal locomotion or by leaping. In contrast to these freely moving gastropods the limpets and ormers of the Patellacea and Zeugobranchia exhibit varying degrees of homing behaviour. In *Patella vulgata*, individuals return from their feeding excursions before low water to a definite position on the rocks where they remain stationary during the period of exposure. The home site is occupied intermittently for long periods and the shell fits closely the underlying irregularities of the rock. Homing behaviour is also a feature of the biology of *Diodora*. It is notable therefore that both these forms exhibit in their response to predators a reluctance to leave the home site relying instead on a mantle or twisting response. The position in the Acmaeidae is more complex. Some species exhibit strong homing tendencies while in others this behaviour appears to be absent. At least two species of *Acmaea* from the Pacific coast of North America which exhibit strong homing behaviour, *A. scabra* and *A. digitalis*, fail to respond in any way either to the presence of, or close contact with a starfish.

With the exception of reports that quantities of mucus are produced by a retreating *Haliotis* (Bennett, 1927; Montgomery, 1967) there are no suggestions in the literature that these gastropods might secrete repellent substances as an added response which could effectively increase the efficiency of the escape mechanism. Yet several of the groups concerned possess exceptionally well-developed groups of glands in the epithelium of the foot or mantle which correspond in their structure to glands whose known function is to produce acid or otherwise repellent secretions which are forcibly ejected by contraction of a muscular sheath when the animal is disturbed. In *Acmaea* (= *Patelloidea*) *virginea* (Fretter and Graham, 1954) the mantle edge contains a complex of three types of gland cells of which one, the most conspicuous and the commonest, produces threads of white secretion when the muscles surrounding them contract. The glands are surrounded by a great plexus of nerves connected to the main pallial nerve which also involve groups of sensory cilia set on the mantle edge, and the whole system appears to be organized as a defensive system. Similar glands to which repugnatorial qualities have been suggested also occur in the lateral glandular streak of *Patella* and other genera (Fretter and Graham, 1962), and in *Acteon*, *Siphonaria*, *Onchidella* (Fretter and Graham, 1954), *Calyptraea* and *Crepidula* (Graham, 1954). Fretter (in Fretter and Graham, 1954) reports that the secretion is rapidly toxic to small animals. Discharge of these glands appears to occur only after rather drastic mechanical stimulation, a point which was noted also for the repugnatorial glands of the marine pulmonate *Onchidium floridianum* (Arey and Crozier, 1921). It would perhaps be worthwhile investigating the possibility that the sensory threshold for discharge might be lowered in the presence of predators which also elicit the muscular responses of many of these types.

Other responses of gastropods

Isolated instances of responses similar to those described for the Zeugobranchia, Patellacea and Trochacea occur in other prosobranch groups. Feder (1963) has described how the winkle

Littorina scutulata responds to the near presence or contact of the starfish *Pisaster* by moving away "as fast as its small foot will carry it". If the starfish is placed in a tide pool with a number of these small winkles the gastropods usually move out of the water. The cypraeid, *Trivia arctica*, in British waters, will extend the mantle lobes up over the shell in response to contact with starfish, but exhibits no locomotory response, and a similar covering reaction occurs in *Natica millepunctata* (Schiemenz, 1896) and *Natica catena* (Thorson, 1953) in which the propodial and metapodial lobes of the foot extend upwards to cover the shell. I have since been able to demonstrate this reaction in *Natica alderi*.

Kohn and Waters (1966) have recently described the interesting responses of two species of the family Strombiidae, *Strombus canarum* and *Lambus lambis*, to the predatory gastropod *Conus textile* which feeds exclusively on other gastropods. Members of the Strombacea are remarkable in exhibiting a mode of locomotion in which the foot is first extended and the shell then thrown forward vigorously while the operculum at the posterior end of the foot is thrust into the substratum. This leaping mode of progression has been described by Parker (1922). Kohn and Waters (1966) have shown that the near presence of *Conus textile* results in an increase in the absolute rate of locomotion of *Strombus* and *Lambis* to some three times the rate normally employed, the movement being directed away from the predator. The authors interpret the advantage of the leaping mode of locomotion to be the striking increase in agility that it confers, and the almost instantaneous increase in distance from the predator that a single leap provides.

I have already described the leaping response of the trochid *Calliostoma zizyphinum*. The classic and best studied case of a leaping response in the Gastropoda, however, is undoubtedly that of members of the genus *Nassarius* (= *Nassa*). When the tube foot or skin of an asteroid starfish, or of certain echinoids touches the posterior surface of the foot of *Nassarius reticulata*, *N. mutabilia*, or *N. fossata*, the shell is thrown forward and the animal inverted. The foot extends and twists to obtain anchorage on the substratum and the columella muscle then contracts to lever the shell backward in a rolling, leaping movement which is repeated several times resulting in a zigzag retreat of the gastropod from the starfish (Bauer, 1913; Weber, 1924; Hoffman, 1930; Bullock, 1953). *Nassarius* is a scavenger and is attracted to damaged, dead or even putrefying organisms (*see* review in Kohn, 1961), a habit which is shared to some extent by many starfish. The feeding behaviour of *Nassarius* may, therefore, bring it into close proximity to these potential predators and the survival value of the leaping response under these circumstances must be great. An exactly similar, although slower and ponderous, response is exhibited by the whelk, *Buccinum undatum*, when contacted by, or in the proximity of, the starfish, *Marthasterias glacialis* (Feder and Arvidsson, 1967). Similar leaping responses also occur in the freshwater pulmonates *Physa fontinalis* and *Lymnaea*, the stimulus arising from contact with leeches of the genus *Glossosiphonia* (Degner, 1921; Herter, 1929).

Leaping responses in bivalves

A second major group of locomotory responses to predators is found in the Bivalvia, and again comprises a leaping movement. The impressive leaping ability of members of the Cardiidae has been known for many years (Gosse, 1877; Cooke, 1895), and has recently been redescribed for several species (Ray, 1959; Thorson, 1964; Margolin, 1964; Ansell, 1967 *a*). Contact of a starfish with the mantle margin results in gaping of the shell, protrusion of the siphons to the full extent, and extension of the L-shaped foot which twists into a short spiral with the tip on the surface of the substratum under the shell. Rapid straightening of the foot throws the shell upward and backwards at the same time rotating it through 180° on an antero-posterior axis. The cycle of recovery of the foot into the spiral form and the active straightening movement is repeated for a variable number of times, depending on the species, resulting in a rapid movement over the surface. The movements may be initiated from the buried position when one leap is sufficient to uncover the bivalve and the movements are often sufficiently violent to overturn the attacking starfish and hence render it immobile for a short period.

An apparently parallel evolution of this behaviour pattern has occurred in the genus *Trigonia* (a primitive eulamellibranch found only in Australia) which shows a striking superficial similarity of form to the Cardiidae especially in the shape of the foot, and which also exhibits spectacular leaps. Cooke (1895) quotes the case of the first living specimen of *Trigonia* ever obtained which when placed after capture on the thwart of the small boat "baffled.... efforts to discover its generic position by suddenly leaping into the sea". Apparently, *Trigonia* shares with the Cardiidae the ability to leap and the habit of doing so when removed temporarily from the water, but it is not recorded whether this leaping response is provoked by predators other than man.

I have recently shown that leaping behaviour in the Bivalvia is not restricted to the Cardiidae and Trigoniidae, but occurs also in the Asaphidae (Ansell, 1967 *b*), the Solenidae, the Veneridae and the Mactridae (Ansell, 1969) and probably in representatives of all families with a shallow burrowing habit. Leaping responses similar to those of the Cardiidae occur in *Gari tellinella*, in *Venus ovata* and *Venerupis rhomboides* and in *Spisula elliptica*, *S. subtruncata* and *Mactra coralline*, several of these species moving much more effectively by this method since a flattened streamlined shell results in a planing action through the water, contrasting with the somewhat cumbersome movements of the tumid cardiids. In *Gari tellinella*, following contact of a starfish tube foot with the mantle margin, the foot is protruded and twisted so that the tip and part of the side come to lie on the surface of the sand adjacent to the ventral shell margin. The foot then moves forward relative to the shell and simultaneously the twist of the foot is straightened so that, since the tip remains pressed against the sand, a backward movement of the shell results. The straightening of the twisted foot also causes a rotation of the shell through 180° about an antero-posterior axis. The cycle may be repeated up to five times, each leap occupying a period of about 2·5 seconds, during which the active straightening movement of the foot is completed in less than one second. I have no doubt that such responses in the bivalves are of much more common occurrence than has been considered hitherto and that observation of contrived contacts between bivalves and starfish would reveal the true frequency of this behaviour.

The occurrence of leaping behaviour in the Solenidae has been recorded, but its significance not fully appreciated. Morton (1964) has described how the foot is extended, looped back under the shell and then straightened to heave the animal several centimeters a number of times in succession propelling the animal backwards through the water in a manner essentially similar to that of leaping in other bivalves. The significance of this movement is made clear in a paper by Turner (1955) who describes an encounter between the bivalve *Ensis directus* and the naticid gastropod, *Polynices duplicata*. Turner describes finding a 97 mm razor clam on the surface of the sand with a specimen of *Polynices* attached at the posterior end, and continues: "The clam was extending and retracting its foot in writhing motions as if making an attempt to escape. The clam finally managed to work its foot down into the sand, pull itself into an erect position and begin to burrow. As soon as the clam had erected itself, the snail quickly crawled down the body and burrowed into the sand alongside of its prey. The razor clam continued to dig in until 2/3 of its length was buried and then stopped. After a two-minute period the razor clam suddenly forced its way out of the sand with violent thrusts of the foot. The snail was attached to the lower third of the body but the final thrust detached it. The razor clam immediately extended its foot and began to burrow, touching the snail as it did so. The snail then dug in alongside of the clam which again stopped as soon as 2/3 of its length was buried. The clam made one or two feeble attempts at digging in deeper. The posterior end was just level with the surface when it suddenly forced its way out again with the snail attached to the lower portion. The last motions detached the snail and thrust it a few centimetres away from the clam." It is clear from this account that the leaping movements of members of the Solenidae, responsible here for the violent exit of the clam from the sand, may be regarded as a defensive response to predation by naticid gastropods, the significant point being that this response occurs only to a predator which attacks below the surface of the sand and hence approaches the exposed mantle margins anteriorly rather than the siphons.

Burrowing responses in bivalves

Members of the Solenidae exhibit a different response to predators approaching on the surface of the sand, for example, starfish, or bottom-feeding fish which touch the siphons in feeding. Such

mechanical stimulation, or chemical stimulation by extracts of starfish injected into the inhalant current initiate the rapid burrowing movements, typical of this group (Fraenkel, 1927; Trueman, 1967). There are indications too that the presence of starfish will also initiate burrowing movements in other bivalves. Thus Pratt and Campbell (1956) recorded a greater mean depth of burial of *Venus mercenaria* from experimental boxes from which the starfish *Asterias forbesi* was also recorded than from those which did not contain this predator and suggested that the greater depth indicated that the clams were "somehow apprised (tactually, or possibly chemically ?) of the presence of the starfish and were driven to greater depths." I have noticed although I have made no controlled experiments, that the introduction of a starfish into an aquarium containing sand with bivalves which have come to rest often causes renewed burrowing activity on the part of the bivalves, for example in *Cardium edule*.

Swimming in the Bivalvia

Perhaps the best documented adaptation involving accelerated or directed locomotion as a protective device in the molluscs is that of the swimming behaviour in the superfamily Pectinacea of the Bivalvia. Three families, the Pectinidae, Amussidae and Limidae, include members which swim. In the Pectinidae and Limidae swimming is employed both as a natural method of locomotion and as an escape response and the same is probably true of the Amussidae, but little is known of the habits of members of this group. Swimming in *Pecten* has been described by Vies (1906), Dakin (1910), Buddenbrock (1911), Bauer (1912) and Yonge (1936). Normal swimming in *Pecten* is accomplished by clapping movements of the shell valves, the water taken into the mantle cavity when the shell valves open being prevented from escaping ventrally by the greatly enlarged inner folds of the mantle edge, the so-called "pallial curtains" (Nelson, 1938). The water passes out on either side of the hinge at the base of the auricles, in some species equally on each side, and in others alternately (MacGinitie and MacGinitie, 1949) so that a straight or zigzag movement results. Since the mantle of the left (upper) valve passes down over that of the right valve some water is ejected downward imparting a certain amount of lift to the animal at each stroke. The escape responses differ from those of normal swimming in taking place in a direction determined by the point of the mantle edge which is stimulated by the predator this result being achieved by the withdrawal of the pallial curtain at the point touched by the predator so that when the shell valves close the water is forcibly expelled through this opening. The animal thus retreats by a sudden, hinge-foremost darting movement. Several further adductions may take place subsequently, the water being expelled from the mantle cavity on either side of the hinge line as in the normal swimming movements.

In the Limidae the swimming movements correspond essentially to the normal swimming movements of *Pecten* except that the animal swims with the shell valves vertically instead of horizontally disposed. The direction of water ejection is controlled as in the Pectinidae by the action of the mantle margin. In *Lima hians* the swimming movements are aided and modified by rowing movements performed by the numerous mantle tentacles. The normal habit of *Lima* is one of nest-building, and the movements used in swimming are also those employed in burrowing into the substratum during the process of nest construction. If the animal is disturbed outside the nest, these movements result in free swimming whilst if the animal is disturbed within the nest they result in the animal burrowing further into the substratum. Both nest-building, or burrowing, and swimming appear to be essentially defensive. *Lima hians* can also autotomize entire pallial tentacles, or parts of tentacles which then secrete a viscous mucus, distasteful to potential predators. This series of defensive adaptations in the Limidae has recently been described for the species *Lima hians* from Millport in a series of papers by Gilmour (1963, 1967).

Directed spurting in the Tridacnidae

Stasek (1965) has recently described an interesting adaptation of the sessile giant clams of coral reefs, the Tridacnidae. In *Tridacna maxima*, the siphonal apertures may be directed, probably

by visual stimuli, towards grazing fish, and spurts of water directed from the siphons by adduction of the shell valves. The sudden spurting by the bivalve causes panic flight in the fish, so that the exposed mantle margins with their contained zooxanthellae are protected from grazing. Stasek has suggested a similar function for the spurting of clams in temperate waters, but there is as yet no evidence that this is the case.

3. Reactions of Cephalopods

The most complex responses to predators exhibited by any molluscs appear, as may be expected, in the Cephalopoda. These responses have been extensively studied only in certain inshore species for which their characteristics are described by Holmes (1940: *Sepia officinalis*), Packard (1960) and Wells (1962; *Octopus vulgaris*) and Boycott (1965: *Sepioteuthis sepiola* and *Doryteuthis plei*).

Hall (1956) gives an account of the escape response of an unidentified squid, summarised as follows:

(1) Assumption of dark colouration immediately before,

(2) Discharge of a quantity of ink sufficient only to discolour water to the size of the animal, the ink functioning mainly as a decoy, but also masking,

(3) The rapid change to lighter colour,

(4) Rapid departure from scene of attack.

This short account summarises three of the elements of the Cephalopod response, which comprises (*a*) colour change by the action of the chromatophores, (*b*) discharge of ink, and (*c*) flight.

Colour changes during the response can be very rapid and apparently act in a variety of ways. Some colour patterns may produce a disruptive effect or blend with the background so that the animal seems to disappear, while others may serve to induce 'fright' in the predator by the sudden appearance of startling patterns—the so-called dymantic response. As Holmes (1940) has remarked the responses of cephalopods must be sophisticated if they are to be effective against the senses of predators of which the most important are probably mammals, including whales, dolphins and porpoises. In deeper waters the use of changing colour patterns *may* be accentuated or replaced by changes in pattern of light emission from photophores, while the discharge of ink may be accompanied or replaced by discharge of a luminescent cloud from glands embedded in the ink sac.

The flight response of cephalopods is produced by forcible ejection of water from the mantle cavity through the funnel which is directed in the opposite direction from that used in normal swimming. Fins, normally used as stabilisers (Boycott, 1956) are folded flat against the body as part of a general streamlining which helps in the production of maximum speed. The flight response is mediated by impulses in the giant fibre system (Wells, 1962). In the Onycoteuthidae, triangular fins provide a broad horizontal vane which enables the animal to emerge from the water to escape schooling carnivorous fish—"the only essay in the animal kingdom into jet flying" (Morton, 1964).

4. Discharge of Secretions

I have suggested that the mushrooming and related responses of the Zeugobranchia, Patellacea and Trochacea, might be accompanied by the release of secretions from the repugnatorial glands which have been described in some species (Fretter and Graham, 1954). Glands of similar structure in other groups of molluscs are under sensory control and release their secretion by muscular action, although there is little information available on what constitutes the natural stimulus causing this release. The marine pulmonate, *Onchidium floridanum* which lives on the shore, in nests, emerging at low water to crawl on the rocks and feed, possesses repugnatorial glands

around the mantle margin dorsally, which on stimulation become turgid and point inward, releasing their contents by contraction of the surrounding muscles. The secretion is a viscid fluid, milk-white, non-miscible with sea-water and acid in reaction. Under water it forms threads, but in air breaks up into a fine spray which may travel a distance of 12–15 cm, or ten times the length of the animal (Crozier and Arey, 1919). Semper (1881) considered that the release of this secretion in *Onchidium* species was caused by shading of the dorsal eyes, perhaps at the approach of the blenny, *Periophthalmus*, but Arey and Crozier (1921) and Arey (1937) criticise this theory and suggest that the discharge is used to repel shore animals such as crabs which might dislodge the *Onchidium* from the rocks. Arey and Crozier (1921) noted that while the discharge seemed to be produced only by severe mechanical stimulation, most natural contacts between *Onchidium* and a diverse selection of other shore animals resulted in discharge, indicating perhaps that the natural stimulus is more subtle than gross mechanical disturbance.

Defensive acid secretion from glands which release their secretion only following stimulation has been recorded from five other marine gastropods from three families, the Lamellaridae, Pleurobranchidae and Philinidae (Thompson and Slinn, 1959; Thompson, 1960 *a*). Fluids toxic to small animals are also secreted from the repugnatorial glands of *Acteon*, *Oncidella*, *Siphonaria* (Fretter and Graham, 1954), *Calyptraea* and *Crepidula* (Graham, 1954). For *Siphonaria alternata*, Yonge (1952) notes that the dorsal surface of the head, the side of the foot and the dorsal surface of the siphon all possess numerous opaque white glandular areas which discharge copious supplies of a very viscid mucous secretion following mechanical stimulus. Yonge suggests that the secretion probably serves to entangle foreign matter and then hardens so that it is left behind when the limpet moves on, or else is expelled by contractions of the shell muscles. It would be interesting to see whether this secretion also plays some part in defence, and indeed to determine whether any locomotory responses are developed in the Siphonaridae, which, although pulmonates, have assumed the limpet form, and play the same part in the ecology of rocky shores in the Indian Ocean and Western Pacific as do the archaeogastropod limpets on shores in the northern hemisphere.

Several gastropods discharge a purple secretion from the hypobranchial gland. This occurs in whelks such as *Murex* in which, however, its function appears to be not primarily defensive, and in several groups of opisthobranch gastropods including the Acteonidae, Aceridae, Philinidae and Aplysiidae. The significance of these secretions in the life of the animal is not fully understood. Eales (1921) considered that the purple secretion of *Aplysia* formed a screen behind which the animal retreated from predators. Some gastropods when disturbed may discharge in similar fashion a luminous cloud from epithelial glands and this discharge *may* also have some protective function (Harvey, 1952; Bowden, 1950). The effectiveness of the ink discharge of cephalopods as a decoy during the escape response of this group has already been described. It has also been suggested that the ink (for example of *Octopus*) may serve as an olfactory anaesthetic, temporarily destroying the olfactory sense of predators such as the moray eel (*Gymnothorax mordax*) which rely on this sense for prey recognition (MacGinitie and MacGinitie, 1949).

III. A SYNOPSIS OF ACTIVE DEFENSIVE ADAPTATIONS IN THE MOLLUSCA

The classification used in the synopsis, which is presented here as an appendix is that of Theile (1955) with only minor modifications.

IV. DISCUSSION

The examples of defensive adaptations in the mollusca reviewed here come predominantly from the marine environment. This may reflect the interests of the reviewer, since the literature on marine molluscs is more familiar and accessible to me, but probably also reflects the greater ease with which encounters between molluscs and their known or potential predators can be observed,

or can be engineered in marine aquaria. The majority of records for the marine environment involve as predators either asteroid starfish or carnivorous gastropods. The third great group preying on molluscs, the grazing fish, apparently provoke few responses, but this conclusion, justified from the literature, almost certainly represents a lack of observation rather than a real lack of response. The ubiquitous occurrence of escape responses to asteroid starfish, however, probably reflects the great antiquity of this particular predatory relationship (Carter, 1968).

Consideration of the ecological consequences of these behavioural adaptations is restricted by lack of information since few authors have commented on these aspects. One notable exception is Feder (1963) who in addition to his studies on escape responses also studied the feeding habit, of the ochre starfish, *Pisaster ochraceous* in the field and in the laboratory (Feder, 1959). The attached mussels (*Mytilus californianus*) and the barnacles (*Balanus glandula, B. nubilis, B. tintinnabulum, Tetraclita squamosa rubescens*, and *Mitella polymerus*) are fed upon more or less in direct proportion to their abundance and availability to *Pisaster*, but this is not true of the gastropods. Of these, only two species, *Tegula funebralis*, and *Acmaea scabra* are of real importance to *Pisaster* as food, and these are not fed upon in proportion to their abundance and availability. The case of *Acmaea scabra* is of particular interest since this species, which does not show an escape response, overlaps with *Pisaster* only at the bottom of its range of distribution on the shore. Yet it ranks relatively high on a percentage basis as food for the starfish. Feder concludes that the starfish may play a role in determining the lower limits of intertidal distribution for *A. scabra*, but that starfish predation does not appear to have provided sufficient selective pressure to encourage the evolution of defensive behaviour patterns in this species.

In Great Britain, of those starfish which may initiate escape responses, none occurs commonly on the shore. *Marthasterias glacialis* occurs sublittorally on rocks, and has been recorded feeding on *Haliotis tuberculata* (Forster, 1962) in the Channel Islands, indicating that the response of this species does not give it complete protection. This contrasts with the situation on the North American west coast where the abalone species are protected so well that authors have questioned whether a predator/prey relationship in fact exists between them and asteroid starfish (Feder, 1963; Montgomery, 1967). *Asterias rubens* is common at and below low water of spring tides, but migrates on to the shore during the winter (Lewis, 1964), locally in large numbers, feeding mainly on newly settled mussels and barnacles. The consequent mortality of the attached forms and the survival, aided by their specific defensive responses, of the limpets and top shells, must be a significant factor in the establishment of patterns of distribution on the lower shore, and an effective factor in maintaining the balance between grazing herbivores and attached suspension feeding forms at this level.

On soft substrata in Great Britain two starfish are important predators of molluscs, *Asterias rubens* and *Astropecten irregularis*. *Asterias rubens* opens the shell of its prey before consuming the meat while *Astropecten* feeds by engulfing small whole bivalves which are then digested as they succumb to the anaerobic conditions resulting from this treatment. Christenson (1962) has shown that this species shows a preference for metabolically more active forms, preferring for example *Spisula subtruncata* to *Venus striatula* (= *gallina*). Perhaps this preference is to some extent balanced by the leaping abilities of *Spisula* the attraction of the bivalve to the starfish and the specific response of the bivalve to the starfish serving to maintain a nice balance in the predator-prey relationshop between these two species of the *Venus*-community.

Asterias rubens on soft substrata is restricted to these types in which it can locate and open its prey. Divers at Millport have observed this species feeding on buried bivalves on coralline gravel, coarse sand and sand, the greater densities of starfish being found on the coarsest substrates. In the feeding position, the disk of the starfish may be several cm below the surface in a shallow pit (Dr. P. R. O. Barnett and Mr. B. L. Hardy, Personal Communication). Similar underwater observations have been made by Smith (1961) who watched the starfish *Pisaster brevispinus* seek out and unearth the burrowing clams *Saxidomus* and *Protothaca*. In

each of the bivalve groups which include members which respond to contact with *Asterias* by leaping, there is a tendency for those species which are found in coarser substrata to respond while those normally found on finer substrata, where *Asterias* does not occur, are not responsive. *Asterias* does not occur intertidally on soft substrata and most bivalves whose range is largely in the littoral zone show no response to starfish other than shell closure.

The responses to starfish are mediated through contact chemoreception, and in this respect differ from responses to predatory gastropods such as *Conus*, where the sensory mechanism involved is one of distance chemoreception. There is a clear adaptive advantage in the more rapid response which distance chemoreception involves, since initial contact with some carnivorous gastropods may, in fact, be fatal, for example, where this contact may involve the injection into the prey of a toxic secretion through the agency of radula teeth held in the eversible proboscis (Kohn, 1959, 1960; Smith, 1967). Feder and Lasker (1964) made preliminary attempts to extract the material from starfish which elicits escape responses. Their experiments showed that the active substance was located in the epidermis of the tube feet, and they succeeded in partially purifying an active substance from *Pisaster ochraceous* using the response of *Acmaea limatula* as a form of bioassay. Feder and Arvidsson (1967) obtained an extract of *Marthasterias glacialis* in preparative amounts, and have used this to examine the reactions of the large whelk (*Buccinum undatum*), as well as the reactions of an isolated *Buccinum* radula muscle (odontophore apparatus) which responds to the material by contracting (Fänge, 1963), this response being quantitative within a rather narrow range. Feder and Arvidsson found no noticeable discrepancy between results obtained when testing extracts on living animals and on radula muscle preparations, but note that they cannot exclude the possibility that the living animals and muscle preparations respond to different active substances from *Marthasterias glacialis* since the extract was only partially purified. More recently, Mackie, Lasker and Grant have shown that the muscle preparation does in fact respond to a different substance from the living animal, and they have gone some way towards determining the chemical identity of the two active substances involved.

This recent progress towards the identification of the chemical substance from starfish which is responsible for initiating defensive responses in molluscs is an important step towards making available significant new opportunities for the study of these examples of escape behaviour. It is hoped that this will act as a stimulus to the further study of this behaviour by biochemists, physiologists, ethologists and ecologists.

Discussion of the evolutionary significance of escape mechanisms may centre on the relationship of these specialised instances of behaviour with 'normal' behaviour patterns. Yonge (1936) in his discussion of the evolution of the swimming habit in the Pectinacea has shown that it represents no more than one line of evolution made possible by an initial change from the dimyarian to the monomyarian condition, and that the adaptations present in the swimming members are developments of those initially acquired for the efficient cleansing of the mantle cavity. Similar exploitation of pre-adapted structures or mechanisms is involved in the other escape responses. Indeed, the elaboration of existing behaviour patterns in the development of specific responses involving locomotion may well be a characteristic of all such responses.

Stasek (1965) concludes that the directed spurting of *Tridacna maxima* may represent specialisation of the shell adductions used by other bivalves in clearing pseudofaeces from the mantle cavity. In the Bivalvia, leaping movements show clear similarities with the movements employed in recovering the position at the surface of the substratum following accidental burial to a depth greater than the length to which the siphons can extend. The ability to recover the surface position is one showed by all bivalves which burrow in soft substrata. This must have been an essential attribute in the exploitation of this type of environment, and hence of great antiquity. The leaping movements are no more than the further exploitation of the same movements to develop a means of rapid locomotion over the surface. In the gastropods the rolling/leaping movements of *Calliostoma* and *Nassarius* appear to be, similarly, a development of those movements normally employed in recovering the erect position following a disturbance in which the shell has been overturned, the

so-called 'righting movement'. This involves rotation of the shell relative to the foot, twisting of the foot, and exaggeration and acceleration of the pedal locomotory wave, all components of the escape response. Failure of the foot to attain a grip during recovery often results in rolling movement over the sustratum. Comparisons of these types of movements in both bivalves and gastropods would provide a fruitful field of investigation using neurophysiological techniques, since the reactions involved can be easily elicited by clearly defined stimuli. The differences are postural, or lie in the different application of the forces involved, and possibly involve little change in the nervous pathways.

Finally, I would end with a request. The study of behaviour takes place at many levels of organisation. It may be largely anecdotal or it may be experimental and highly sophisticated. Provided the species involved are correctly identified, the former can act as a stimulus for the latter. First-hand information on the interactions of animals is important in ecological and morphological interpretation, and as the source of inspiration of further physiological and behavioural studies. I would hope that no one should consider such information too trivial to communicate in some way to his fellow-students of the infinite variety of animal behaviour.

Discussion

Dr. B. N. Desai: The nature of defensive mechanism in Archegastropods is by means of contact chemoreceptor and in the case of Mesogastropoda and Stenoglossa by means of olfactory sense. What observations you have made while studying these responses?

Dr. A. D. Ansell: I have not studied the nature of the chemoreceptor with reference to the systematics of the responding organisms. Perhaps the reaction in such cases might depend on the nature of the predator animals.

Mr. P. I. Chacko: Have you made any observation on the defensive adaptation with reference to attached bivalves. This would be of interest to Indian workers with reference to the well being of the pearl oysters.

Dr. A. D. Ansell: Attached bivalves show no special escape responses, but Dr. Hancock has made some interesting observation on the mussel, *Mytilus edulis* in Danish and British waters. Danish mussels have larger adductor muscles than British mussels of the same shell length, and in laboratory experiments the rate of feeding by starfish on British mussel is consistently higher than on Danish mussel. Perhaps the larger size of the Danish mussels enables them to ward off predation.

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APPENDIX

Family	Genera	Species	Defensive adaptation	Predator*	Principal references
		GASTROPODA—PROSOBRANCHIA			
ARCHAEO-GASTROPODA					
Zeugobranchia	*Haliotis*	*H. iris* *H. australis*	d, f.	*Coscinasterias calamaria* (Asteroidea)	Bennett (1927)
Haliotidae	*Haliotis*	*H. cracherodii*	a, c, d, e, f.	*Astrometis sertulifera, Pisaster ochraceus, P. giganteus, P. brevispinus, Pycnopodia helianthoides, Leptasterias aequalis* (Asteroidea)	Bullock (1953)
	Haliotis	*H. rufescens*	a, b, c, d, e, f, g, i.	*Pisaster ochraceus, Pycnopodia helianthoides* (Asteroidea)	Montgomery (1967)
		H. assimilis	a, c, d, e, f, g, i.		
	Haliotis	*H. iris*	a, c, d, e, f.	*Lepsia haustrum, Lepsiella scobina, Buccinulum strebeli, Corninella maculosa* (Gastropoda)	Clarke (1958)
	Haliotis	..	c, d, f.	Starfish	MacGinitie and MacGinitie (1949)
	Haliotis	*H. tuberculata*		*Marthasterias glacialis* (Asteroidea)	Forster (personal communication), Feder (1967)
	Haliotis	*H. cracherodii* *H. rufescens*	a, c, d, e, f, g.	*Pisaster ochraceus, P. giganteus* *Pycnopodia helianthoides* (Asteroidea)	Feder (1963)
FISSURELLIDAE	*Fissurella*	*F. volcano*	a, c, d, e, f.	see *Haliotis*	Bullock (1953)
	Megathura (= *Lucapina*)	*M. crenulata*	a, c, d, e, f.	see *Haliotis*	Bullock (1953)
	Diodora	*D. aspersa*	b.	*Pisaster ochraceus, P. brevispinus, P. giganteus, Leptasterias aequalis, Evasterias troschelii, Orthasterias koehleri, Pycnopodia helianthoides, Hippasteria spuriosa, Patira miniata* (Asteroidea) *Gorgonocephala eucnemis* (Ophiuroidea), *Strongylocentrotus purpuratus* (Echinoidea)	Margolin (1964 *a*)
	E. marginula	*E. reticulata*	f.	*Marthasterias glacialis, Asterias rubens* (Asteroidea)	Feder (1967)
Patellacea	*Patella*	*P. vulgata*	a, c, d, e, f.	*Asterias rubens* (Asteroidea)	Ansell (this paper)
Patellidae	*Patella*	*P. vulgata*		*Asterias rubens* (Asteroidea)	Connell (1959; in Margolin, 1964)

* Many of the predators noted are those which elicit the response in laboratory tanks and indicate the type of predator, but possibly not the actual natural predator. (For explanation of letters a-i, *see* pp. 491–492).

APPENDIX—(*Contd.*)

Family	Genera	Species	Defensive adaptation	Predator	Principal references
	Patella	*P. vulgata*	c, d, f, i.	*Marthasterias glacialis, Asterias rubens, Asterina gibbosa* (Asteroidea)	Feder (1967)
	Patina	*P. pellucida*	e, f, i.	*Marthasterias glacialis, Asterias rubens* (Asteroidea)	Feder (1967)
	Patina (= *Helcion*)	*P. pellucida*	a, c, e, f.	*Asterias rubens* (Asteroidea)	Ansell (this paper)
	Cellana	*C. radians* *C. ornata*	a, c, d, e, f.	*Lepsia haustrum, Lepsiella scobina, Buccinulum strebeli, Corninella maculosa* (Gastropoda)	Clarke (1958)
Acmaeidae	*Lottia*	*L. gigantea*	a, c, d, e, f.	see *Haliotis*	Bullock (1953)
	Acmaea	*A. scutum* *A. cassis* *A. limatula* *A. fenestrata*	a, c, d, e, f.	see *Haliotis*	Bullock (1953)
	Acmaea	*A. pelta*	a, c, e, f.	*Pisaster ochraceus, Leptasterias aequalis, Evasterias troschelli* (Asteroidea)	Margolin (1964 *b*)
		A. scutum	a, c, e, f.	*Pisaster ochraceus, P. brevispinus, Leptasterias aequalis, Evasterias troschelii, Orthasterias koehleri, Pycnopodia helianthoides* (Asteroidea)	Margolin (1964 *b*)
	Acmaea	*A. limatula*		*Pisaster ochraceus, P. giganteus,*	Feder (1963)
		A. scutum *A. pelta*	a, c, e, f.	*Pycnopodia helianthoides* (Asteroidea)	Feder and Lasker (1964)
	Acmaea	*A. tessulata*	a, c, d, e, f.	*Asterias rubens* (Asteroidea)	Ansell (this paper)
	Acmaea	*A. tessulata*	e, f.	*Marthasterias glacialis, Asterias rubens* (Asteroidea)	Feder (1967)
	Acmaea (= *Patelloidea*)	*A. virginea*	Toxic secretion from glands in mantle skirt		Fretter and Graham (1954)
Trochacea Trochidae	*Stomatella*	..	Autotomy: end of foot		Hedley (1902) Talmadge (in Stasek, 1967)
		S. auricula	Autotomy: end of foot		Quoy and Gaimard (1834)
	Calliostoma	..	d, f.	*Pisaster* (Asteroidea)	Heinsohn (in Passano, 1957)
	Calliostoma	*C. zizyphinum*	a, c, d, e, f, g, h.	*Asterias rubens, Marthasterias glacialis* (Asteroidea)	Ansell (this paper)
	Calliostoma	*C. zizyphinum*	d, e, f, h.	*Marthasterias glacialis, Asterias rubens* (Asteroidea)	Feder (1967)
	Gibbula	*G. magus*	a, c, d, e, f, g, i.	*Asterias rubens, Marthasterias glacialis* (Asteroidea)	Ansell (this paper)
		G. cinerea	a, c, d, e, f, g, i.		
	Gibbula	*G. cinerea* *G. umbilicalis*	c, d, e, f, h.	*Marthasterias glacialis, Asterias rubens* (Asteroidea)	Feder (1967)
	Cantharidus	*C. clelandii*	d, e, f.		
	Monodonta	*M. lineata*	c, d, e, f, h.		

	Monodonta	..	h.	*Coscinasterias tenuispinus* (Asteroidea)	Thorson (in Feder and Christensen, 1966)
	Monodonta (= *Melagraphia*)	*M. aethiops*	a, c, d, e, f.	*Lepsia haustrum* (Gastropoda) *Coscinasterias calamaria* *Astrostole scabra* (Asteroidea)	Clarke (1958)
	Monodonta (= *Zediloma*)	*M. digna*, *M. avida*, *M. atrovirens*	a, c, d, e, f.	*Lepsia haustrum, Lepsiella scobina, Buccinulum strebeli,* *Corninella maculosa* (Gastropoda)	Clarke (1958)
	Monodonta (= *Anisodeloma*)	*M. lugubris*			
	Tegula	*T. funebralis*, *T. brunnea*, *T. ligulata*	a, c, d, e, f, i.	see *Haliotis*	Bullock (1953)
	Tegula	*T. funebralis* *T. brunnea*	a, c, d, e, f, i.	*Pisaster ochraceus, P. giganteus,* *Pycnopodia helianthoides* (Asteroidea)	Feder (1963); Burke (1964); Yarnall (1964)
	Norrisia	*N. norrisii*	a, c, d, e, f.	see *Haliotis*	Bullock (1953)
	Trochus	*T. viridis*	a, c, d, e, f.	*Lepsia haustrum, Lepsiella scobina, Buccinulum strebeli,* *Corninella maculosa* (Gastropoda)	Clarke (1958)
	Trochus	*T. pyramis*	f.	*Conus textile* (Gastropoda)	Kohn and Waters (1966)
	Stomatia	..	Autotomy: end of foot		Allan (1941)
	Gena	..	Autotomy: end of foot		Adams and Adams (1858); Hedley (1902)
		G. varia	Autotomy: end of foot		Fishelson and Qidron-Lazar (1966)
MESOGASTROPODA					
Littorinacea Littorinidae	*Littorina*	*L. scutulata*	i. (but *see* Bullock, 1953)	*Pisaster ochraceus* (Asteroidea)	Feder (1963)
Calyptraeacea Calyptracidae	*Calyptraea*	..	Autotomy: end of foot		Pelseneer (1935)
	Calyptraea	*C. sinensis*	Toxic secretion from mantle skirt		Graham (1954)
Strombacea	*Crepidula*	*C. fornicata*	Toxic secretion from mantle skirt		Graham (1954)
Struthiolariidae	*Struthiolaria*	*S. papulosagigas*	..	*Sclerasterias mollis* (Asteroidea)	Crump (in Feder, 1967)
Strombidae	*Strombus*	*S. gigas*			
		S. costatus, *S. raninus*	Accelerated leaping locomotion	*Fasciolaria tulipa* (Gastropoda)	Robertson (1961)
	Strombus	*S. canarium*	Accelerated leaping locomotion	*Conus textile* (Gastropoda)	Kohn and Waters (1966)
	Lambis	*L. lambis*			
	Strombus	*S. gibberulus* *S. luhuanus*	Accelerated leaping locomotion	*Aulica vespentilio*	
	Lambis	*L. lambis*	Accelerated leaping locomotion	*Conus oramoreus* (Gastropoda)	Gonor (1966)
Naticacea Naticidae	*Natica*	*N. millepunctata*	Covering of shell by fold of foot	*Marthasterias glacialis* (Asteroidea)	Schiemenz (1896)

APPENDIX—(*Contd.*)

Family	Genera	Species	Defensive adaptation	Predator	Principal references
		N. catena		*Asterias rubens* (Asteroidea)	Thorson (1963, in Margolin, 1964)
	Natica	*N. intermedia* *N. montagui*	Covering of shell by fold of foot	*Marthasterias glacialis*, *Asterias rubens* (Asteroidea)	Feder (1967)
	Natica	*N. alderi*	Covering of shell by by fold of foot	*Marthasterias glacialis*	Ansell (this paper)
Lamellariacea Lamellaridae	*Velutina*	*V. velutina*	Secretion of acid from skin glands	..	Thompson (1960 *b*)
	Lamellaria	*L. perspicua*	Secretion of acid from skin glands	..	Thompson (1960 *b*)
Cypraeacea Cypraeiacea	*Trivia*	*T. arctica*	Covering shell	*Asterias rubens* (Asteroidea)	Ansell (this paper)
	Zonaria (= *Erronea*)	*Z. spadicea*	Accelerated pedal locomotion	see *Haliotis*	Bullock (1953)
STENOGLOSSA *Muricacea* Muricidae	*Acanthuria* (= *Nucella*)	*A. spirata*	Accelerated pedal locomotion	see *Haliotis*	Bullock (1953)
Buccinacea Buccinidae	*Buccinum*	*B. undatum*	Leaping	*Marthasterias glacialis* (Asteroidea)	Feder and Arvidsson (1967), Feder (1967)
Nassidae	*Nassarius* (= *Nassa*)	*N. reticulata*	Leaping	*Astropecten bispinosus* (Asteroidea)	Bauer (1914)
		N. mutabilis	Leaping	*Marthasterias glacialis* (Asteroidea), *Astropecten bispinosus*, *Coscinasterias tenuispinus*, *Asterina gibbosa* (Asteroidea) *Strongylocentrotus lividus* (Echinoidea)	Weber (1924)
		N. fossata	Leaping	*Pisaster brevispinus* (Asteroidea)	Bullock (1953)
		N. vibex	Leaping	..	Gore (1966; in Gonor 1966)
		N. reticulatus *N. incrassatus*	Leaping	*Marthasterias glacialis*, *Asterias rubens* (Asteroidea)	Feder (1967)
Volutacea Harpidae	*Harpa*	*H. ventricosa*	Autotomy: end of foot		Quoy and Gaimard (1832)
		GASTROPODA—OPISTHOBRANCHIA			
Cephalaspidae Acteonidae	*Acteon*	*A. tornatilis*	Purple secretion of hypobranchial gland		
			Toxic section from repugnatorial glands as *Acteon*	(small animals)	Fretter and Graham (1959)
Aceridae	*Acera*	*A. bullata*			
Philinidae	*Philine*	*P. quadripartita*	Discharge of acid fluid		Thompson (1960 *a*)
Anaspidea Aplysiidae	*Aplysia*	*A. depilans*	Discharge of purple secretion		Eales (1921); Engle (1957)
	Notarchus *Dolebella*		Jet swimming		Engel (1933)

Sacoglossa	*Lobiger*	*L. serradifalci*	Autotomy: end of foot		Gonor (1961)
Oxynoidae			Autotomy: natotory appendages of foot		Riggenbach (1903); Pelseneer (1935)
Caliphyllidae	*Caliphylla* (= *Phylbbranchus*)	*C. australis*	Autotomy: cerata		Risbec (1928)
		C. borgnini			Trinchese (in Risbec, 1928)
Stiligeridae	*Stiliger*	*S. fuscovittata*	Autotomy: cerata		Lance (1962)
		S. boodleae	Autotomy: cerata		Baba (1949)
		S. vanellus	Autotomy: cerata		Edmunds (1966)
NOTASPIDEA					
Pleurobranchidae	*Berthella*	*B. plumula*	Discharge of acid fluid		Thompson (1960 *a*)
	Pleurobranchus	*P. membranaceous*	Discharge of acid fluid swimming		Thompson and Slinn (1959), Thompson (1960 *a*); Thompson (1960 *b*)
NUDIBRANCHIA					
Doridacea					
Polyceridae	*Plocamophorus*	*P. ocellatus*	Swimming, luminous flashes; discharge of luminous cloud		Harvey (1952)
Doridae		..	Autotomy: mantle margins		Synopsis in Stasek (1967)
Aeolidacea					
Dironidae					
Tethyidae					
Zephyrinidae					
Dotonidae					
Flabellinidae			Autotomy: cerata		Synopsis in Stasek (1967)
Tergipedidae		..			
Aeolidiidae					
	Glaucus		Autotomy: end of foot		Forster: *see* Bergh (1884)
		GASTROPODA—PULMONATA			
BASOMMATO-PHORA					
Patelliformia					
Siphonariidae	*Siphonaria*	..	Toxic secretion from repugnatorial glands		Fretter and Graham (1954)
Hygrophila					
Physidae	*Physa*	*P. fontinalis*	Leaping	*Glossosiphonia*	Degner (1921); Wrede (1928)
Lymnaeidae	*Lymnaea*	..	Leaping	*Glossosiphonia*	Herter (1929)
Planorbidae	*Planorbis* (= *Helisoma*)	*P. nigricans*	Burrowing	*Pseudemys* †	Kempendorff (1942)
Ancylidae	*Latia*	*L. neritoides*	Discharge of luminous secretion		Bowden (1950)

† The stimulus eliciting burrowing in *P. nigricans* is apparently a chemical released when an individual is damaged, for example, by the turtle *Pseudemys*.

APPENDIX—(*Contd.*)

Family	Genera	Species	Defensive Adaptation	Predator	Principal references
STYLOMMATOPHORA					
Oncidiacea Oncidiidae	*Oncidella*	*O. celtica*	Toxic secretion from repugnatorial glands		Joyeux-Laffuie (1882) von Wissel (1898), Fretter (1943)
	Oncidium (= *Onchidium*)	*O. juanfernandiziana*	Discharge of acid fluid	(‡)	Semper (1881)
		O. floridanum	Discharge of acid fluid		Crozier and Arey (1919); Arey and Crozier (1921); Arey (1937)
Zonitacea Vitrinidae	*Vitrina*	*V. pellucida*	Leaping		Collinge (1890)
Arionidae	*Prophysaon*	All species	Autotomy: end of foot		Pilsbury (1948); Ingram (1948)
		P. coeruleum	Autotomy: end of foot		Cockerell (1890 *a*, *b*), Raymond (1890)
		P. foliolatum	Autotomy: end of foot		Binney (1892)
Limacidae	..	—	Discharge of fluid mucus; accelerated pedal locomotion	Land planarians	Froehlich (1955)
Ariophantacea Ariophantidae	*Xesta* (= *Nanina*)	..	Discharge of noxious fluid		Benson (1835; in Stasek, 1967)
	Helicarion	All species	Autotomy: end of foot Leaping		Semper (1881) Cooke (1895)
	Habroconus (= *Helix* [*Stenopus*])	—	Autotomy: end of foot		Guilding (in Semper, 1881)
Helicacea Pleurodontidae	*Pleurodonte* (*Polydontes*)	*P. imperator*	Autotomy: end of foot		Pfeiffer and Gundlach (1861)
	(= *Helix* [*Polydontes*])	*P. apollo*	Autotomy: end of foot		Pfeiffer and Gundlach (1861)
		P. crassilabris	Autotomy: end of foot		Pfeiffer and Gundlach (1861)
Helicidae	*Helix*	*H. depithouarisi*	Flight with loping gait		Carlson (1905), Parker (1937)
		H. asperso	Flight with loping gait		Parker (1937)
			BIVALVIA		
ANISOMYARIA *Pectinacea*		"Pectenacea" of Φresund	Swimming	*Astropecten irregularis*, *Luidia sarsii*, *Solaster endeca*, *Marthasterias glacialis*, *Asterias rubens* (Asteroidea)	(in Feder and Christensen, 1966)
Pectinidae	*Pecten*	*P. maximus*	Swimming	*Asterias rubens* (Asteroidea)	Dakin (1910)
	Pecten	*P. maximus*	Swimming	*Marthasterias glacialis* (Asteroidea)	Uexküll, 1912; Fänge, 1963

	Pecten (= *Chlamys*)	*P. jacobeus*	Swimming	*Asterias rubens* (Asteroidea)	Dakin (1910), Bauer (1912)
	Pecten	*P. jacobeus*	Swimming	*Astropecten auranciacus, Asterina gibbosa, Echinaster sepositus, Anseropoda placenta, Marthasterias glacialis*	Lecompte (1952)
		P. opercularis	Swimming	*Asterias rubens* (Asteroidea)	Dakin (1910)
		..	Swimming	Starfish, Octopus	MacGinitie and Mac Ginitie (1949)
		P. circularis	Swimming	*Pisaster ochraceus, P. gigantus* (Asteroidea)	Feder (1963)
		P. septemradiata	Swimming	*Asterias rubens* (Asteroidea)	Ansell (this paper)
	Pecten (= *Placopecten*)	*P. magellanicus*	Swimming	*Asterias vulgaris* (Asteroidea)	Dickie and Medcoff (1963)
Limidae	*Lima*	*L. hians*	Autotomy: mantle tentacles		Gilchrist (1895); Gilmour (1963, 1967)
			Swimming—(nest-building)		Gilmour (1967)
EULAMMELLIBRANCHIATA		*L. loscombi*	Swimming	*Marthasterias glacialis* (Asteroidea)	Feder (1967)
Trigoniacea Trigoniidae	*Neotrigonia*	*N. pectinata*	Leaping	..	Cooke (1895)
Cardiacea Cardiidae	*Laevicardium*	*L. crassum*	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1967 *a*), Feder (1967)
	Serripes	*S. groenlandicus*	Leaping	*Pycnopodia helianthoides* (Asteroidea)	Margolin (1964 *a*)
	Cardium	*C. echinatum*	Leaping	*Asterias rubens* (Asteroidea)	Thorson (1959), Ansell (1967 *a*), Feder 1967
	Cardium	*C. fasciatum*	Leaping	*Astropecten irregularis, Asterias rubens* (Asteroidea)	Christensen, Thorson (in Feder and Christensen, 1966)
	Clinocardium	*C. nuttalli*	Leaping	*Pisaster ochraceus* (Asteroidea)	Ingels (1958; in Margolin, 1964), Ray (1959)
Tridacnidae	*Tridacna*	*T. maxima*	Directed spurting	Grazing fish	Stasek (1965)
Veneracea Veneridae	*Venus*	*V. ovata*	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1968)
	Venus (= *Mercenaria*)	*V. mercenaria*	Burrowing	*Asterias forbesi* (Asteroidea)	Pratt and Campbell (1956)
	Venerupis	*V. rhomboides*	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1969)
Mactracea Mactridae	*Spisula*	*S. subtruncata*	Leaping	*Astropecten irregularis, Asterias rubens* (Asteroidea)	Thorson (in Feder and Christensen, 1967)
		S. elliptica	Leaping		Margolin (in Feder and Christensen, 1966)
		S. subtruncata	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1969)
		S. elliptica	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1969)
	Mactra	*M. corallina*	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1969)
	Spisula	*S. solida*	Leaping	*Lunatia nitida* (Gastropoda)	Ziegelmeier (1954)
	Mactra	..	Leaping	*Natica millepunctata, N. hebraea* (Gastropoda)	Hirsch (1915)

(‡) *Periophthalmus* was suggested by Semper as a possible predator, but this suggestion is not supported by Crozier and Arey's observations.

APPENDIX—(*Contd.*)

Family	Genera	Species	Defensive Adaptation	Predator	Principal references
Tellinacea Asaphidae	*Gari*	*G. tellinella*	Leaping	*Asterias rubens* (Asteroidea)	Ansell (1967 *b*)
	Solecurtus	*S. coarctatus*	Autotomy: siphons		Forbes and Hanley (1853)
		S. strigillatus	Autotomy: siphons		Rawitz (1892), Faussek (1897)
		S. scopula	Autotomy: siphons		Atkins (1937)
		S. chamasolen	Autotomy: siphons		Atkins (1937)
		S. gibbus	Autotomy: siphons		Morse (1919)
Tellinidae	*Tellina*	*T. tenuis*	Autotomy: siphons	*Pleuronectes platessa*	Ansell and Trevallion (1967)
Solenacea Solenidae	*Phaxus* (= *Cultellus*)	*P. pellucidus*	Leaping burrowing	*Asterias rubens* (Asteroidea)	Ansell (1962)
	Solen	*S. fonesi*	Autotomy: siphons		Bloomer (1967); Annandale (1916)
		S. kempi	Autotomy: siphons		Ghosh (1920)
		S. marginatus (= *S. vagina*)	Autotomy: siphons	*Pleuronectes platessa*	Pelseneer (1935); Atkins (1937); Rawitz (1892)
		S. rosaceus	Autotomy: siphons		Ricketts and Calvin (1952); Pohlo (1963)
		S. sicarius	Autotomy: siphons		Ricketts and Calvin (1952); Stasek (1967)
	Ensis	*E. directus*	Leaping	*Polynices duplicatus* (Gastropoda)	Turner (1955)
		E. arcuatus	Burrowing (leaping)	*Asterias rubens* (Asteroidea)	Ansell (1969)
		E. siliqua	Burrowing (leaping)	*Asterias rubens* (Asteroidea)	Ansell (1969)
DECAPODA			*CEPHALOPODA—DIBRANCHIA*		
Sepiacea Sepiidae	*Sepia*	*S. officinalis*	Colour changes, Ink discharge, flight		Holmes (1940)
Loliginacea Lologinidae	*Sepioteuthis*	*S. sepiola*	Colour change, Ink discharge, flight		Boycott (1965)
Architeuthacea Onychoteuthidae	*Doryteuthis*	*D. plei*	"		Boycott (1965)
	Onychoteuthis		"Jet flying"		Morton (1964)
OCTOPODA					
Octopodacea Octopodidae	*Octopus*	"*Octopus*" sp.	Autotomy: arms		Straughan (1957); Lane (1960)
		O. defillipi	Autotomy: arms		Jatta (1896)
	Octopus	*Octopus* sp.	Discharge of Ink, flight	*Gymnothorax mordax*	MacGinitie and MacGinitie (1949)
	Octopus	*O. vulgaris*	Colour change, discharge of ink, flight		Packard (1960); Wells (1962)

DISTRIBUTION, MOVEMENT AND BEHAVIOUR OF *NODILITTORINA GRANULARIS* (GRAY) ON A SHORE AT NEGOMBO, CEYLON

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ABSTRACT

A quantitative study was made of the distribution of *Nodilittorina granularis*, the common littorine of the west coast of Ceylon, on a selected rocky shore.

It was found that the main period of recruitment to the population occurred at the tail end of the South-West monsoon during the months of October and November. The main area of settlement was the middle of the mid-littoral region, which on the particular shore studied was marked by an oyster belt. Very little settlement took place outside this area.

During the period of the South-West monsoon, *i.e.*, from May to November littorines of shell length 3 mm. and below were found to occupy the shoreward half of the mid-littoral region though a few were found outside this region too. The larger littorines were found to be most numerous in the supra-littoral fringe region during this period.

During the intermonsoonal period November to April the larger sizes were found concentrated about the oyster belt. The supra-littoral fringe region became very dry and carried only a very sparse littorine population.

Some experiments were performed to test the behavioural response of various size groups to immersion and exposure.

The causes of the noted changes in distribution are discussed.

INTRODUCTION

THE population of *Nodilittorina granularis* (Gray) inhabiting selected sandstone rocks at Negombo, Ceylon, was studied over a period of one and a half years. The selected rocks formed part of a sandstone reef at Negombo on the west coast of the island. The selected site consisted of two large sandstone rocks extending from the shore to a distance of 85 ft. into the sea. The shoreward rock was 65 ft. broad and the seaward one 20 ft., these being separated by a deep gully. The upper surfaces of the rocks were quite flat. A clear zonation was evident and it was possible to distinguish a supra-littoral fringe approximately 20 ft. wide, followed by a mid-littoral or barnacle zone, and an infra-littoral fringe. The middle of the mid-littoral was occupied by a belt of the oyster *Ostraea cucullata* (Born). The infra-littoral fringe was covered by growths of *Sargassum*, *Gracilaria*, and *Padina*. The mid-littoral region itself carried low growths of algae like *Laurencia*, *Enteromorpha*, *Acanthophora*, and *Centroceros*

METHODS

Ten stations were established three of which were located on the seaward rock and seven on the shoreward rock. Of the three stations on the seaward rock only station 1 was located in the infra-littoral fringe. Stations 2 and 3 belong to the mid-littoral zone and were located 10 and 20 ft. respectively from station 1. Station 4 was located in the oyster belt. Stations 5, 6 and 7 were in the mid-littoral zone 10, 20 and 30 ft. shoreward of station 4. Stations 8 and 9 belong to the supra-littoral fringe. Station 10 was located on the vertical shoreward face of the rock. This tended to be more sheltered than stations 8 and 9 and retained moisture longer than the latter

Monthly samples were collected at each of these stations using the following method. A wire frame 1 ft. square was set down at each station and all individuals within this frame were carefully collected. These were preserved in 5% sea-water formalin and samples from each station counted and measured in the laboratory. Measurements were carried out using a vernier hand micrometer. The dimensions measured were the height (from apex to the posteriormost point of aperture) and width (from the periphery of body whorl to the margin of the outer lip). In the analysis that follows the height was used as a measure of size.

Recruitment and Growth of Population

In order to follow the growth changes taking place in the population the collections were considered in three size ranges (A) 0·10–0·20 cm., (B) 0·30–0·40 cm., and (C) 0·60–0·70 cm.

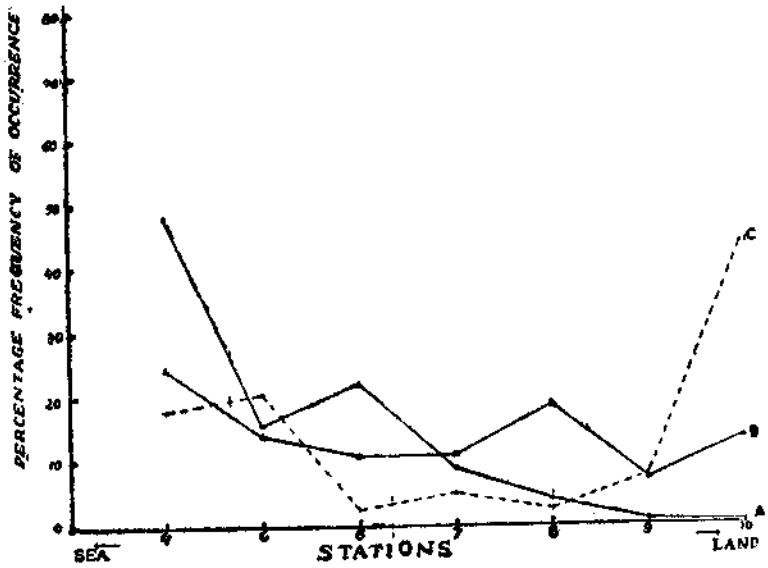

Fig. 1. Distribution of three size groups of *N. granularis* at stations 4 to 10 on the sandstone reef at Negombo, Ceylon, during the period August 1966 to July 1967.
A—0·10–0·20 cm.; B—0·30–0·40 cm.; C—0·40–0·50 cm.

Figure 1 shows the distribution of the different size ranges relative to the stations. This figure deals with stations 4 to 10 which are the only stations accessible throughout the year. Stations 1, 2 and 3 are accessible only between November and April. These will be dealt with separately. In preparing these results the frequency of occurrence has been worked out as follows. The total number of individuals in each size range collected at each station throughout the year was expressed as a percentage of the total number of individuals belonging to that size range collected at all stations. Figure 1 shows that the greatest concentration of shells belonging to group A occurred at station 4 and that the numbers of this size group showed a fairly regular decline with the lowest percentages appearing at stations 9 and 10 in the supra-littoral fringe. Considering the shells of group B a much more diffused pattern is seen. This size range is encountered at all stations the frequency of occurrence fluctuating between approximately 10 and 25%. The numbers belonging to group C were considerably smaller than those belonging to either of the other groups. The distribution of shells of this group appears rather more patchy. The highest frequency appeared at station 10. Stations 4 and 5 showed a frequency around 20%. At stations 6, 7 and 8 the frequency was less than 5%.

Figure 2 shows the analysis of frequency at the three stations on the seaward rock. The great majority of animals in group A occurred at station 3 which was the most shoreward station on this rock. The highest frequency in group B occurred at stations 2 and 3. Individuals of group C were found at all three stations with the highest frequency appearing at station 2. Considering this size range on both rocks it was found that the largest numbers occurred at stations 2, 1 and 3 in that order. The numbers at the other stations were rather small as has been pointed out earlier.

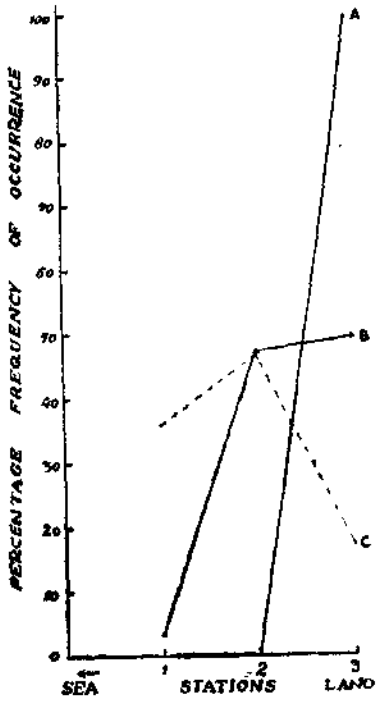

FIG. 2

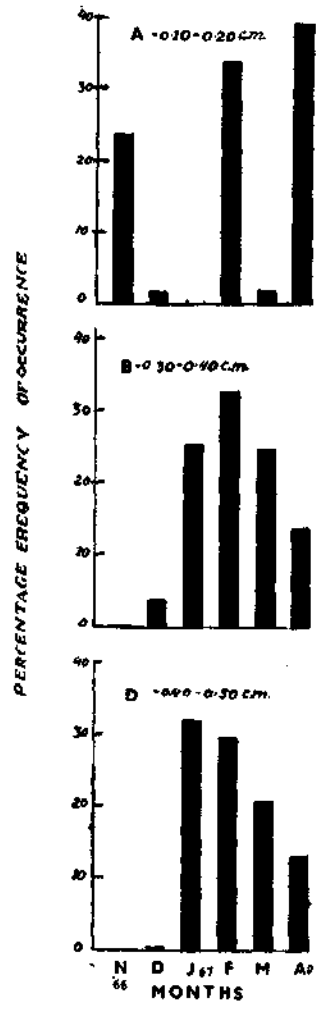

FIG. 3

FIG. 2. Distribution of three size groups of *N. granularis* at stations 1 to 3 on the sandstone reef at Negombo, Ceylon, during the period November 1966 to April 1967. A—0·10–0·20 cm.; B—0·30–0·40 cm.; C—0·40–0·50 cm.

FIG. 3. Frequency of occurrence of three size groups of *N. granularis* over the period November 1966 to April 1967, on a shore at Negombo, Ceylon. Histograms represent collections from stations 1 to 3.

It would appear from this analysis that the settlement of *Nodilittorina granularis* occurs predominantly around the middle of the mid-littoral zone (Stations 3 and 4). Since, however, the

next size range shows a more even frequency of distribution among the stations there seems to be some radiation of individuals both seawards and shorewards from the point of settlement. It does not seem, however, that the population that spreads in all directions meets with equal success at the different levels. Thus, at stations 6, 7, 8 and 9 very few individuals entering the third category are encountered. Animals of this category seem to predominate in areas which are either constantly moist or are fairly sheltered from the direct drying effects of the sun. The former situation seems to be the one that is preferred since the largest number belonging to group C occurs at stations 1, 2 and 3.

An attempt has been made to derive from this study some indication of the period of settlement of *N. granularis* on this shore and also to follow roughly the changes in size composition of the population over the period of survey. Figure 3 shows a series of histograms for the two size groups A, B, and a size group D of the range 0·40–0·50 cm. These histograms express the total number of a particular size range in a particular month expressed as a percentage of the total number of individuals of that size range collected during the period of survey.

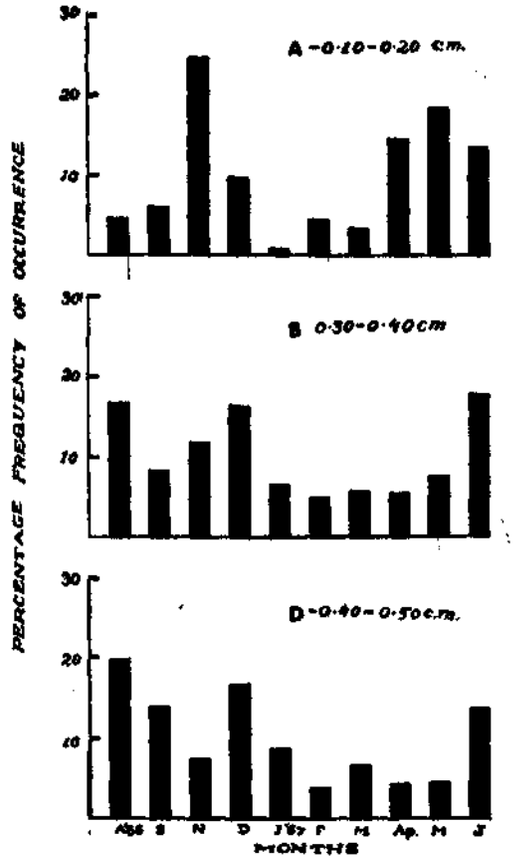

FIG. 4. Frequency of occurrence of three size groups of *N. granularis* over the period August 1966 to July 1967, on a shore at Negombo, Ceylon. Histograms represent collections from stations 4 to 10.

Considering the histogram for group A, one maximum occurs in November 1966 and another in May 1967. If one were to consider the occurrence of individuals below 0·10 cm. the occurrence

of these maxima is further supported. Thus, it is found that individuals of shell size below 0·10 cm. were encountered in October 1966 and April 1967. Of these two maxima the one encountered during April is larger than that occurring in November. The shore in question is subjected to the South-West Monsoon from May to early October. There is reason to believe that there is an extended period during which young littorines are settling but that there may be two peaks of settlement one around the beginning of the monsoon and the other near its end.

It is possible to see a continuation of these two waves of settlement in the histogram relating to the next size group. Thus there are two peaks, one in December 1966 and the other in July 1967, which may be related in turn to the settlement of October–November 1966 and the settlement of April–May 1967.

Since very few individuals seem to enter group C on this shore, histograms were constructed for the size range 0·40–0·50 cm. The pattern that emerges is very similar to that shown by group B Peaks are evident in December 1966 and also July 1967.

Discussion

Williams (1964) has studied the zonational pattern of various size groups of *Littorina littorea* (L.) on a shore in Wales. He found that shells below 13 mm. were concentrated around M.L.W.N. and shells ranging from 13·5 to 17 mm. were found at E.L.W.N. and the largest sizes above 17 mm. were found below M.L.W.N. In the case of the *Nodilittorina* population that was studied a rather similar pattern of distribution was evident. The smallest sizes below 0–30 cm. were concentrated in the oyster belt which was the middle of the mid-littoral zone. Sizes between 0·30 and 0·50 cm. were found to have a wider distribution over the whole mid-littoral region. The largest sizes, *i.e.*, above 0·60 cm. were most numerous in the infra-littoral fringe region. Williams confirms the view of Tattersall (1920), Hayes (1929), Rees (1934), and Moore (1940) and suggests that *L. littorea* can attain maximum size only on the lower regions of the shore. The fact that the largest sizes of *N. granularis* were found on the lowest parts of the shore ties in with this conclusion. Some of the larger shells were also found at the shoreward end on the vertical face of the reef. This region since it retains a fair amount of moisture perhaps affords more favourable conditions than the more exposed higher regions on the shore.

With regard to site of settlement, Smith and Newell (1955) have suggested that settlement takes place mainly in the sub-littoral region and that shells are subsequently washed into the intertidal region from where they spread shorewards. In the case of *N. granularis* the region of the oyster belt appears to be the main site of settlement. From this point it appears that the animals gradually move out to other parts of the shore. Since, the reef that was studied rises sharply up from the sea bottom any movement similar to that occurring in *L. littorea* cannot take place.

Results available at present do not permit a clear statement on growth pattern of this species. In a total of 17,122 shells collected the largest size encountered was 0·80 cm. and very few were found to reach this size. This seems to be the maximum size attained on this particular shore.

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DISCUSSION

Mr. P. J. Jose: What is the main factor controlling the settlement of *Nodilittorina granularis* in the middle of mid-littoral region?

Dr. D. H. Atapattu: I have not studied the factor.

Mr. K. V. Rao: This is a very interesting paper. I find a bimodal distribution of *N. granularis*. One may be due to South-West monsoon (August) and the other possibly due to North-East monsoon. You have stated that South-West monsoon extends from May–November. Just as in India, we have the South-West monsoon from May–August and the North-East monsoon period from October–March, Ceylon coast also should have the same monsoon cycle due to the vicinity of the Ceylon and Indian Coasts.

PESTICIDE CONCENTRATIONS IN CALIFORNIA BAYS AND ESTUARIES

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ABSTRACT

In a nation-wide programme to monitor organochlorine pesticide residues in estuaries, the U.S. Bureau of Commercial Fisheries in January 1966, contracted to the California Department of Fish and Game the responsibility to monitor selected estuaries for pesticides in California.

Native and Pacific oysters, *Ostrea lurida* and *Crassostrea gigas*, were selected as the bioassay organisms for estuaries since they are particularly efficient in concentrating these chemicals which are otherwise difficult to identify in large bodies of water. Oysters have been sampled monthly since January 1966 from commercial oyster beds in five California estuaries. Sampling stations to monitor pesticides in oysters have been established in five additional estuaries currently unsuitable for commercial shellfishing.

Since January 1967, the Asiatic clam, *Corbicula fluminea* has been used to monitor pesticides culminating in the freshwater of the Sacramento-San Joaquin River Delta draining over six million acres of extensively cultivated agricultural land.

Analysis of samples performed by the U.S. Bureau of Commercial Fisheries pesticide laboratory in Gulf Breeze, Florida, reveal DDT, DDD, DDE, dieldrin and endrin ranging from 10 to 1000 ppb in oysters and clams sampled in California. Levels of pesticide pollution have been found lower in northern California estuaries than in central California estuaries. Studies are being conducted to determine pesticide pollution in southern California estuaries influenced by extensive agricultural and urban wastes.

Analysis is performed by electron capture gas chromatography, capable of detecting as little as 1×10^{-12} grams of some organochlorine compounds. Extractions are accomplished by refluxing the desiccated sample with petroleum ether in a soxhlet extraction assembly. Partitioning with acetonitrile and eluting with six and fifteen per cent ethyl ether in petroleum ether on an activated florisil column prepares the extract for gas chromatography.

INTRODUCTION

ORGANOCHLORINE pesticides are used extensively in agricultural and urban areas. Eventually sub-lethal concentrations of these highly toxic, persistent organic compounds accumulate in bays and estuaries. Since pesticides are concentrated and persist in plant and animal tissues, they may become a hazard to aquatic resources. A continuous surveillance program enables investigators to observe trends in pesticide pollution and recommend corrective action for management.

As part of a nation-wide program, the U.S. Bureau of Commercial Fisheries contracted with the California Department of Fish and Game in January 1966 to determine the geographical and seasonal trends of pesticides in selected estuaries. Our approach is to use oysters and other shellfish as bioassay animals because they concentrate pesticide residues in their tissues. This method eliminates difficulties in identifying minute quantities of pesticides in large bodies of water and the problem of measuring pesticides in areas subject to tidal fluctuation and runoff from extensive agricultural areas.

PESTICIDE ANALYSIS TECHNIQUES

The pesticides tested for are aldrin, endrin, dieldrin, the ortho-para and para-para isomeres of DDT, DDD and DDE; heptachlor, heptachlor epoxide, methyoxychlor, benzene hexachloride

and lindane. Electron capture gas chromatography is capable of detecting some chlorinated compounds in quantities as small as 1×10^{-12} grams. Quantification of the small amounts of chlorinated pesticides found at low trophic levels in the marine environment and the extraction and cleanup of organochlorine residues from animal tissue require careful laboratory technique. To avoid discrepancies in laboratories we followed the procedure for extraction and cleanup of organochlorine residues from animal tissue set forth by the U.S. Bureau of Commercial Fisheries Laboratory, Gulf Breeze, Florida.

A four-hour soxhlet extraction of ground, desiccated tissue in petroleum ether prepares the extract for partitioning with acetonitrile. The acetonitrile fraction containing organochlorine pesticides is evaporated to dryness, and the remaining residues are quantitatively transferred to an activated Florisil cleanup column. A 200 milliliter elution of 6 per cent ethyl ether in petroleum ether then separates DDT, DDD, and DDE from endrin and dieldrin. Endrin and dieldrin are eluted with 200 milliliters of 15 per cent ethyl ether in petroleum ether. This eluate receives further cleanup on a magnesium oxide, celite column. Finally, a 100 milliliter elution with petroleum ether removes dieldrin and endrin residues from this column. The 15 per cent eluate is then ready for gas chromatography.

Shellfish were examined continuously for two years (January 1966 through December 1967). In 1966 analyses were performed by the U.S. Bureau of Commercial Fisheries. Expansion of the program in 1967 included facilities necessary for analysis by the California Department of Fish and Game.

Results

Giant Pacific oysters, *Crassostrea gigas*, were used as bioassay animals at 11 stations where oysters are harvested commercially. Native oysters, *Ostrea lurida*, bay mussels, *Mytilus edulis*, and Asiatic clams, *Corbicula fluminea*, were used in 6 estuaries not used for oyster culture. Asiatic clams are sampled in the freshwaters of the Sacramento-San Joaquin Delta.

Humboldt and Tomales Bays, Drakes Estero, and Bolinas Lagoon are not part of drainages for large urban or agricultural areas. Shellfish from these areas were relatively free from organochlorine pesticide residues. Although the oysters concentrated a high of 47 parts per billion (ppb) DDT in April 1966, they were seldom found with more than 20 ppb DDT, DDD, or DDE. At some stations, pesticides were seldom detected (Tables I–VI).

TABLE I

Concentrations of pesticides in parts per billion in oysters sampled from California bays, January-April 1966

	January				February				March				April			
	DDE	DDD	DDT	Di*	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di
Humboldt Bay																
Bird Island																
Gunther Island			Tr				10								47	
Tomales Bay																
Nicks Cove					12	Tr	Tr		11							
Tomales Bay Oyster Co.	Tr				Tr	Tr										
Drakes Estero																
Schooner Bay					Tr				Tr	Tr						
Berries Bar					13	15	Tr		Tr	Tr			10	17		
San Francisco Bay																
Marin Pier	13	19	13		30	36	13		47	60	19		52	83	23	14
Coyote Point	48	66	54		77	100	84	27	42	54	43	20	72	88	70	29
Elkhorn Slough																
Round Island	155	155	230		210	215	285	20	96	120	110	11	96	110	96	20
Morro Bay																
Baywood	73	32	22		73	31	24		82	35	26		75	25	20	24
Los Osos Creek	51	24	22		49	22	17		43	17	14		88	39	30	27

* Dieldrin. Tr. Trace only.

TABLE II

Concentrations of pesticides in parts per billion in shellfish sampled from California bays, May-August 1966

	May				June				July				August			
	DDE	DDD	DDT	Di*	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di
Humboldt bay																
Bird Island																
Gunther Island			11		Tr	Tr	14		11	17			Tr		11	
Tomales Bay																
Nicks Cove	Tr				Tr				11				11	Tr		
Tomales Bay																
Oyster Co.	Tr	11	Tr						14	Tr			11	Tr		
Drakes Estero																
Schooner Bay	Tr	Tr			Tr				Tr				Tr			
Berries Bar	13	16	Tr		10	10			17	13			11	11		
San Francisco Bay																
Marin Pier	69	120	45	20	59	92	38		37	47	24		52	82	43	
Coyote Point																
Native Oyster	71	91	74	23	47	66	46		30	38	39		35	46	42	
Bay Mussel													29	51	49	
Elkhorn Slough																
Round Island																
Pacific Oyster	89	95	85	18	88	82	65		86	79	64		72	77	55	
Bay Mussel													60	46	30	
Morro Bay																
Baywood	76	32	24	16	52	19	14		55	20			62	25	18	
Los Osos Creek	65	25	20	10	40	16			43	16			53	22	14	

* Dieldrin. Tr. Trace only.

TABLE III

Concentrations of pesticides in parts per billion in shellfish sampled from California bays, September–December 1966

	September				October				November				December			
	DDE	DDD	DDT	Di*	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di
Humboldt Bay																
Bird Island																
Gunther Island	Tr	21			Tr	Tr	18		Tr	Tr	14		Tr	Tr	20	
Tomales Bay																
Nicks Cove	Tr				Tr				Tr	Tr			Tr			
Tomales Bay Oyster Co.	14	11			14	12			Tr				Tr			
Drakes Estero																
Schooner Bay					Tr	Tr			Tr	Tr						
Berries Bar	12	Tr			Tr	Tr			Tr	Tr			11	10		
San Francisco Bay																
Marin Pier	57	90	49		51	88	33	11	55	84	40	15	55	110	98	20
Coyote Point	50	65	55		42	71	62	21	33	51	40	15	63	79	75	21
Elkhorn Slough																
Round Island	79	66	41		84	65	56	10	130	77	76		190	160	210	30
Morro Bay																
Baywood	59	35	26		69	34	24		69	33	25		100	37	46	
Los Osos Creek	73	34	23		10	27	23		71	33	25		72	31	37	
Bolinas Lagoon	10				Tr	Tr			Tr	10						

* Dieldrin. Tr. Trace only.

TABLE IV

Concentrations of pesticides in parts per billion in oysters sampled from California bays and rivers, January-April 1967

	January				February				March				April			
	DDE	DDD	DDT	Di*	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di
Humboldt Bay																
Bird Island																
Gunther Island	10	Tr	30		Tr		28		Tr		12		Tr	Tr	19	
Tomales Bay																
Nicks Cove	12	Tr	Tr		13	10	11		13	12	Tr		14	12	11	
Tomales Bay																
Oyster Co.	Tr		Tr		12	Tr	10		11	Tr	Tr		11	12	11	
Drakes Estero																
Schooner Bay	Tr				Tr				10	Tr			11	13	10	
Berries Bar	Tr	Tr			11	14			17	20	Tr		14	18	10	
Bolinas Lagoon		11				13			10	16			10	17	11	
San Francisco Bay																
Marin Pier	52	130	88	23	34	65	49	11	30	59	49	15	42	75	70	19
Coyote Point	41	60	58	26	49	68	72	26	51	74	79	28	61	82	89	23
Elkhorn Slough																
Round Island	200	160	260		220	230	440	25	200	200	390	29	230	260	690	30
Morro Bay																
Baywood	110	29	58		110	42	73		62	50	96		130	47	130	
Los Osos Creek	62	29	41		120	47	96		63	43	130		110	42	120	
San Joaquin River																
West Island	280	230	200		330	370	300	20	320	350	320	22	270	250	250	17
False River													470	410	970	24

* Dieldrin. Tr. Trace only.

TABLE V

Concentrations of pesticides in parts per billion in oysters sampled from California bays and rivers, May–August 1967

	May				June				July				August			
	DDE	DDD	DDT	Di*	DDE	DDD	DDT	Di	DDE	DDD	DT	Di	DDE	DDD	DDT	Di
Humboldt Bay																
Bird Island											24					
Gunther Island	Tr		19		Tr	Tr	19		Tr	Tr	24		Tr		12	
Tomales Bay																
Nicks Cove	10	Tr	Tr		Tr	Tr	Tr		11	Tr	Tr		Tr			
Tomales Bay Oyster Co.	Tr	Tr	Tr		11	14	13		Tr	Tr	Tr		Tr			
Drakes Estero																
Schooner Bay	Tr	13			11	13	Tr		Tr	Tr			15	18	10	
Berries Bar	16	17	11		12	14	10		13	15	Tr		Tr	18	10	
Bolinas Lagoon	11	16	14			14	12		11	21	13		13	20	11	
San Francisco Bay																
Marin Pier	23	55	34	13	39	85	64	21	45	120	89	19	53	130	63	17
Coyote Point	65	76	69	21	52	78	70	25	51	84	80	17	47	58	50	43
Elkhorn Slough																
Round Island	210	340	860	39	300	390	920	33	160	200	390	10	200	260	500	10
Morro Bay																
Baywood	80	34	67		120	49	70		51	29	37		82	40	49	
Los Osos Creek	93	57	92		130	56	80		64	46	52		81	44	49	
San Joaquin River																
West Island	320	250	260	12	230	210	270	18	170	130	150	Tr	140	93	130	15
False River	460	320	910	19	320	200	640	20	420	260	780	16	270	180	500	16

* Dieldrin. Tr. Trace only.

TABLE VI

Concentrations of pesticides in parts per billion in oysters sampled from California bays and rivers, September–December 1967

	September				October				November				December			
	DDE	DDD	DDT	Di*	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di	DDE	DDD	DDT	Di
Humboldt Bay																
Bird Island							Tr								Tr	
Gunther Island		Tr	16		Tr	Tr	15		Tr	Tr	21		Tr	Tr	22	
Tomales Bay																
Nicks Cove	Tr	Tr							14							
Tomales Bay Oyster Co.	Tr								11	Tr	Tr					
Drakes Estero																
Schooner Bay									Tr							
Berries Bar	13	16	10		15	18	Tr		14	17	Tr		Tr	Tr		
Bolinas Lagoon									Tr	11			Tr	Tr		
San Francisco Bay																
Marin Pier	30	74	38	11	31	68	36									
Coyote Point	39	58	50	13	33	37	43		46	86	11	16	46	84	51	16
Elkhorn Slough																
Round Island	190	210	390	15	62	55	110		19	15	34	14	25	23	37	17
Moro Bay																
Baywood	55	23	46		48	21	26		35	10	15		46	13	16	
Los Osos Creek					43	20	25		37	14	20		29	10	12	
San Joaquin River																
West Island	170	150	230	20	180	150	270	10	69	49	1·1	20	39	31	77	18
False River													40	35	21	17

* Dieldrin. Tr. Trace only.

Water used for irrigating over 6 million acres of agricultural land in the Sacramento and San Joaquin Valleys drains into San Francisco Bay where it becomes mixed with industrial and domestic wastes. Fluctuations in the quantity of DDT, DDD and DDE found in oysters of such large estuarine systems may be influenced by salinity, tidal action, estuarine productivity, pollution intensity, and oyster condition, rather than seasonal variation alone. In San Francisco Bay DDT in oysters varied from less than 20 to over 80 ppb during the sampling period (Fig. 1).

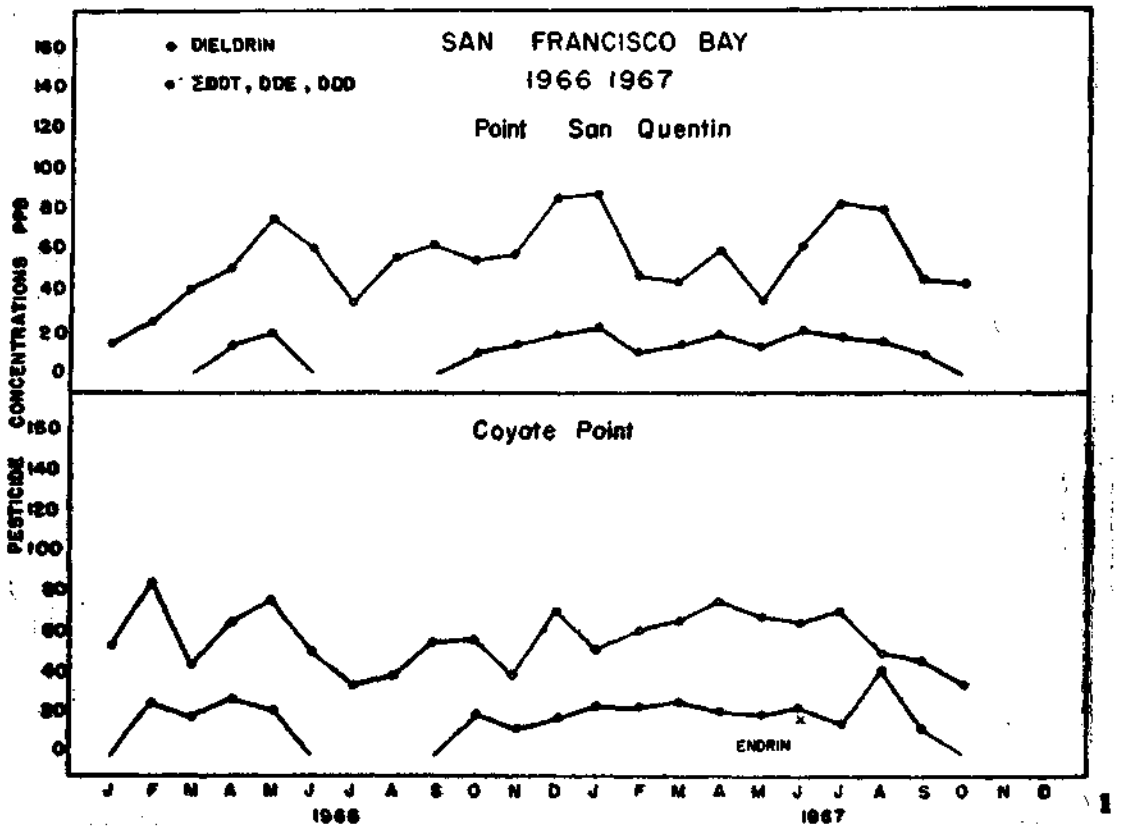

FIG. 1

In small estuaries the effects of changing environmental conditions may not be of sufficient magnitude to obscure locally heavy pesticide pollution. For instance, an increase in organochlorine pesticides in Elkhorn Slough, located in an intensively cultivated region, was observed beginning in November 1966. Unusually, heavy freshwater runoff from November 1966 through April 1967 may have caused the poisonous residues to accumulate in this small estuary (Fig. 2).

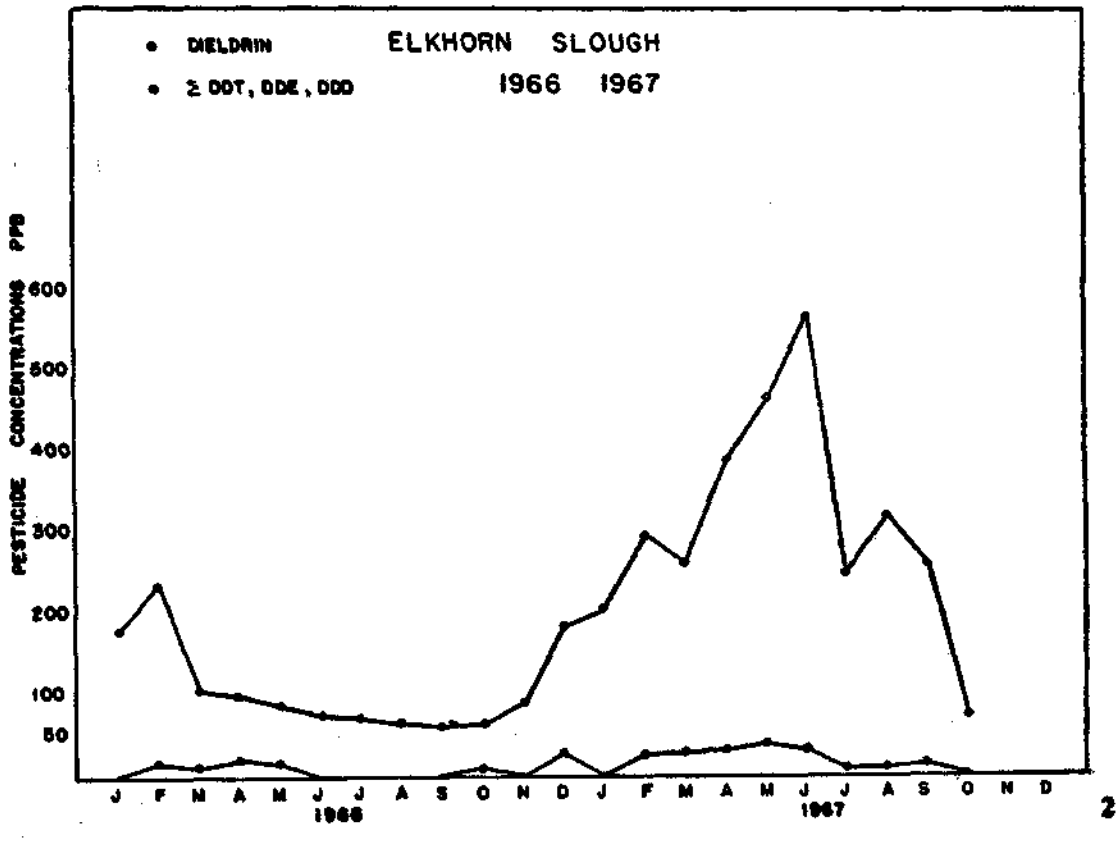

FIG. 2

Pesticide concentrations in Morro Bay approximated those in San Francisco Bay. An increase in pesticides in Morro Bay oysters was observed in 1967. Maximum DDT levels in 1967 were 100 ppb, an increase of 50 ppb over 1966 (Fig. 3).

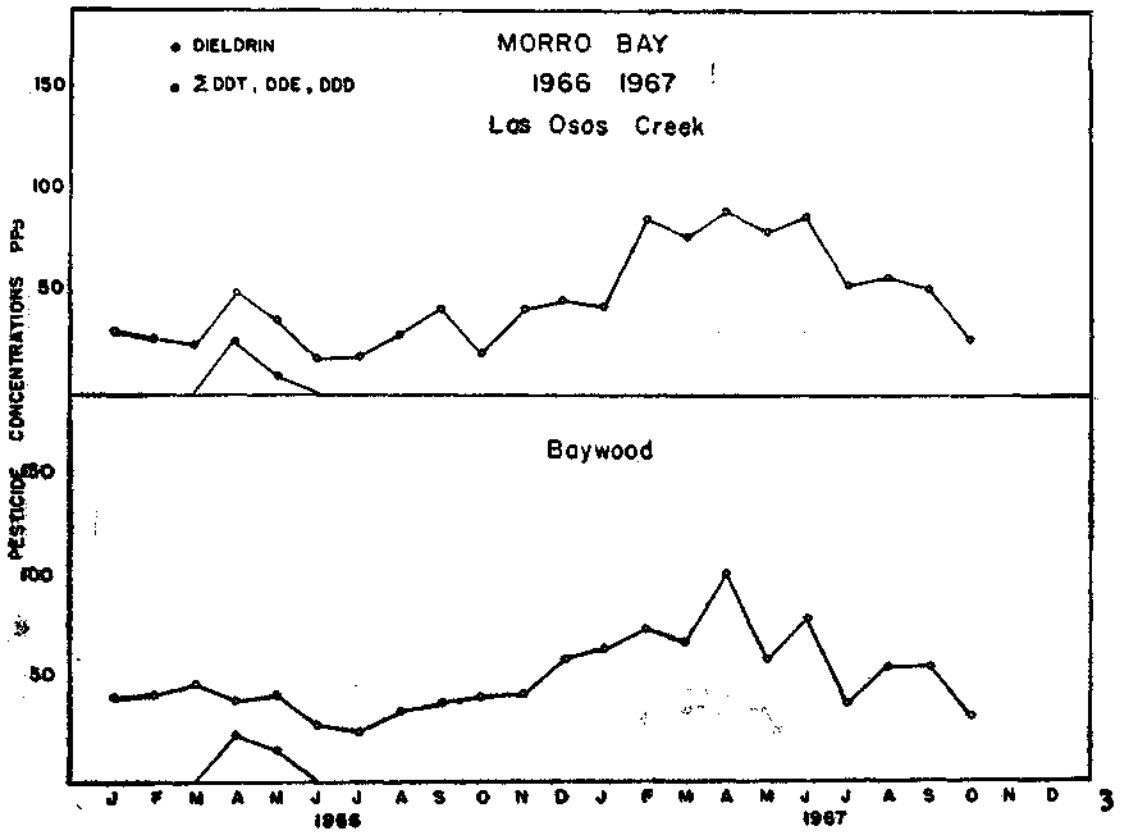

Fig. 3

An additional monitoring station was established near Los Angeles in September 1967, to determine the magnitude of pesticide pollution in a predominately industrial and urban area.

Pesticides in Crabs

An investigation was initiated in August 1967 to assess the effects of pesticides on Dungeness crabs (*Cancer magister*) near San Fransisco. Fishermen speculate that pesticides may be responsible for the declining catch in the San Francisco area which has dropped from 4,784,000 pounds in 1960 to only 389,000 pounds in 1966. Studying the effects of pesticides on crabs, including ova and larvae, may help determine if pesticides played a part in the decline. Initial studies of crabs taken within San Francisco Bay revealed the presence of 100 ppb DDE and 63 ppb DDD in crab tissues. DDT was not found.

SUMMARY

DDT, DDD and DDE are commonly found in shellfish from California bays and estuaries. Endrin and dieldrin are less frequently found. Pesticides in shellfish from estuaries which are not part of urban or agricultural drainages seldom exceed 20 ppb DDT, DDD, or DDE. Pesticides in shellfish from estuaries receiving runoff from agricultural regions frequently exceed 300 ppb.

LITERATURE CITED

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MEMORY TRACES IN *OCTOPUS VULGARIS*

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ABSTRACT

Octopus vulgaris Lamarck has proved to be an unusually suitable animal for work on learning and there is now a considerable literature on visual and tactile learning, sensory analysing mechanisms and the effect of brain lesions on learning in this animal. It is possible to localise the mechanisms for visual and tactile learning within specific lobes in the cephalopod brain and it is beginning to be possible to state where the changes caused by training must be taking place. Recent experimental work on the nature and probable whereabouts of these memory traces is discussed.

INTRODUCTION

ONE of the central problems in neurophysiology is the nature of memory traces. Animals change their behaviour as a result of individual experience. These behavioural changes are often more or less permanent and, in many instances, it can only be supposed that the changes we observe are due to alterations in the state of the central nervous system. Very little is known about the nature of these alterations. Nobody can yet specify precisely where to look or what to look for in the brain of any animal.

We are, however, slowly moving towards the point where it will be possible to do this, and some of the relevant experiments have been made with cephalopods and notably with *Octopus*. The account that follows is a review of the progress of these experiments.

THE BRAIN OF *Octopus vulgaris*

Cephalopod brains are all very much alike. Even that of *Nautilus*, which is clearly much more primitive than the others, is referable to the same overall plan (Young, 1965). The brain of the mediterranean *Octopus vulgaris* Lamarck (Fig. 1) has been studied in most detail. It consists of about thirty distinct lobes, each with a characteristic structure. Details of the neural arrangements in several of the lobes and of the pattern of connexions linking lobe to lobe have been worked out in a long series of careful anatomical studies (Young, 1964 and in preparation) so that the anatomy of this brain is probably as well known as that of any animal, vertebrates notwithstanding.

DISCRIMINATION EXPERIMENTS WITH OCTOPUSES

In parallel with these anatomical studies, a number of experiments has been made on the function of the brain of *Octopus*. Octopuses can readily be trained to discriminate between objects that they see or touch (various training methods will be discussed below), and there is now a considerable literature on the sensory capabilities of octopuses insofar as these can be assessed from the results of training experiments.

We know, for example, that the visual acuity of *Octopus vulgaris* approaches that of ourselves (Sutherland, 1963 *a*) and that this animal is capable of distinguishing between a wide variety of shapes

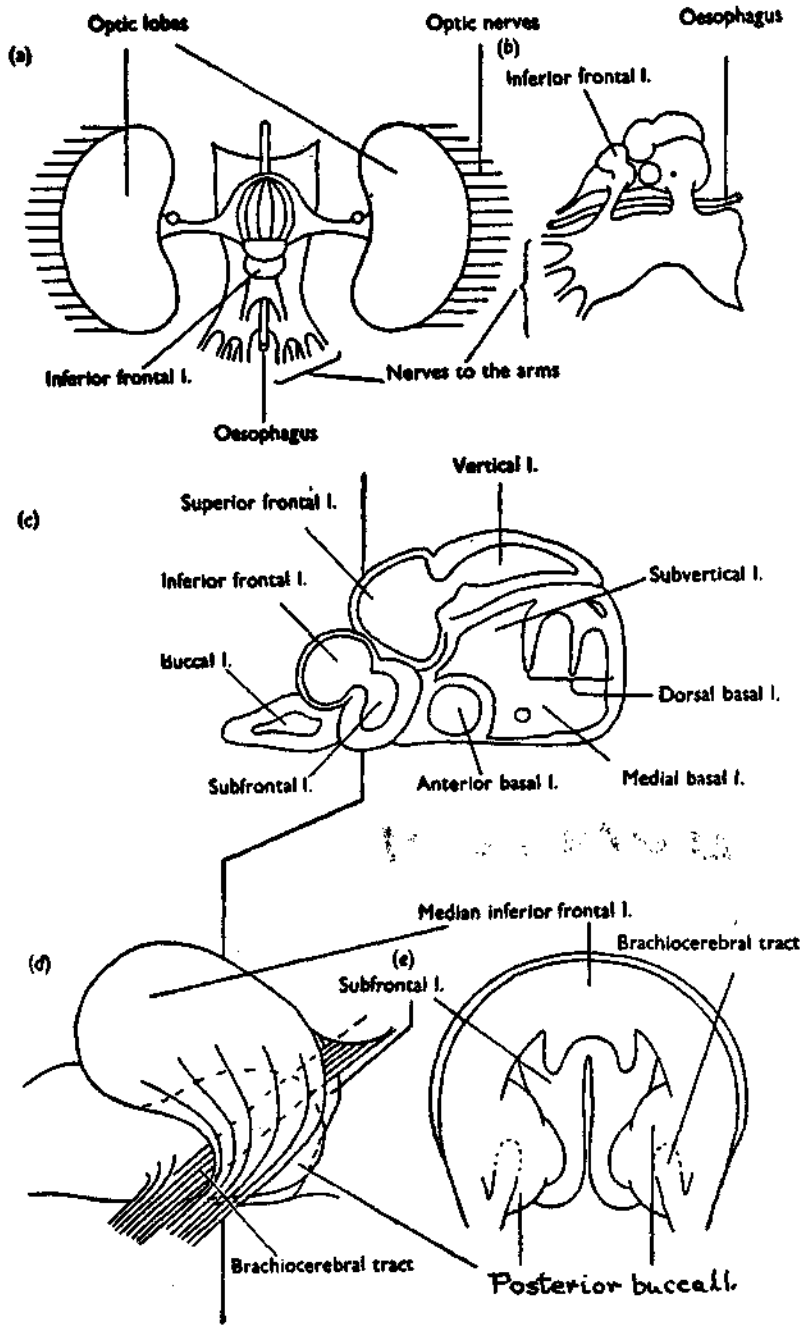

FIG. 1. The brain of *Octopus vulgaris*, showing the whereabouts of lobes mentioned in the text: (*a*) shows the brain from above, as it would appear after removal of the cartilagenous box surrounding the central regions; (*b*) is the same, from the left side; (*c*) is a longitudinal vertical section through the median part of the supraoesophageal brain; this diagram is used in Figs. 2 and 3 as a map on which to plot the extent of brain lesons. In an octopus of 250 g. this part would be about 3 mm. long; (*d*) and (*e*) show some further details of the inferior frontal touch-learning system (from Wells, 1959)

by sight, including mirror images and different orientations of the same figure (Sutherland, 1958; 1959, 1960 *a*, *b*, 1962 *a*).

It is known, moreover, that octopuses distinguish between light sources emitting polarised light when the plane of vibration is different (Moody and Parriss, 1961; Rowell and Wells, 1961) and that the animals very possibly also distinguish between colours (Goldsmith, 1917). From the orientations pattern of errors that they make in visual training experiments it is possible to make deductions about the organisation of the octopuses visual analysing mechanisms (*see* reviews by Sutherland, 1957; 1962 *b*, 1963 *b*).

A similar range of information is available about the animal's tactile capabilities. Octopuses, blinded by cutting the optic nerves, can distinguish between objects of different texture and between objects of different size (Wells, 1964 *a*). They apparently classify the shapes of objects that they touch on a basis of the degree of bend that contact imposes on the suckers grasping the objects, so that there are many shapes alike to *Octopus* that appear different to ourselves. Texture, which is also assessed from the degree of distortion of the suckers in contact, is confused with shape (Wells, 1964 *a*). The animals are extremely sensitive to the taste as well as to the texture of objects that they touch, and their capacity to distinguish between chemical differences, using only the suckers on the arms, easily surpasses that of the human tongue (Wells, Freeman and Ashburner, 1965).

The results of the numerous experiments on sensory capabilities and sensory analysing mechanisms have been reviewed by Sutherland (1962 *b*), Wells (1962, 1966 *a*, *b*) and Young (1961, 1964).

It will be noted that all these findings have arisen from training experiments made under laboratory conditions. Usually, the objects to be distinguished are shown or presented one at a time and the animals trained to respond by feeding them if they attack or accept the test object (in tactile experiments the objects are presented by touching them against one of the arms of blinded animals). This treatment rapidly results in the animals attacking or taking all objects presented to them and in order to establish discriminant responses they have to be punished as well, for 'incorrect' positive responses. Octopuses are sensitive to electric shocks and a convenient form of punishment is 6–9 volt A.C. shocks, given by touching the animals on the arms or body with a live probe which is plunged into the water when the octopuses makes a mistake. This training technique, originally devised by Boycott (1954), has formed the basis of most of the training experiments made so far, including nearly all of those on the effects of brain lesions to be described below.

We thus have an extensive, but lopsided, picture of the function of the brains of cephalopods. We know a great deal about the behaviour of one species (*O. vulgaris*) in training experiments in the laboratory, and comparatively little about the use that this or any other cephalopod makes of its abilities under natural conditions.

Brain Lesions and Learning

In the laboratory, trained as described above, octopuses learn rapidly. An octopus, confronted with a simple visual or tactile discrimination problem begins to respond differently in the two test situations within a few trials and within 20 or 30 trials may be making 75 or 80% of correct responses. Individuals vary, but all learn. It is therefore possible to discover which parts of the brain are concerned in learning by taking bits out until learning fails.

Over the past twenty years many experiments have been made to discover the minimal lesions that prevent octopuses from learning simple discriminations. These experiments have revealed the existence of critical areas, all of which are located within the supraoesophageal part of the brain (Fig. 1). They have also shown, perhaps rather surprisingly, that the octopus has not one, but two learning systems, one apparently concerned only with touch learning, the other mainly with vision. Some typical experiments are shown in Figs. 2 and 3.

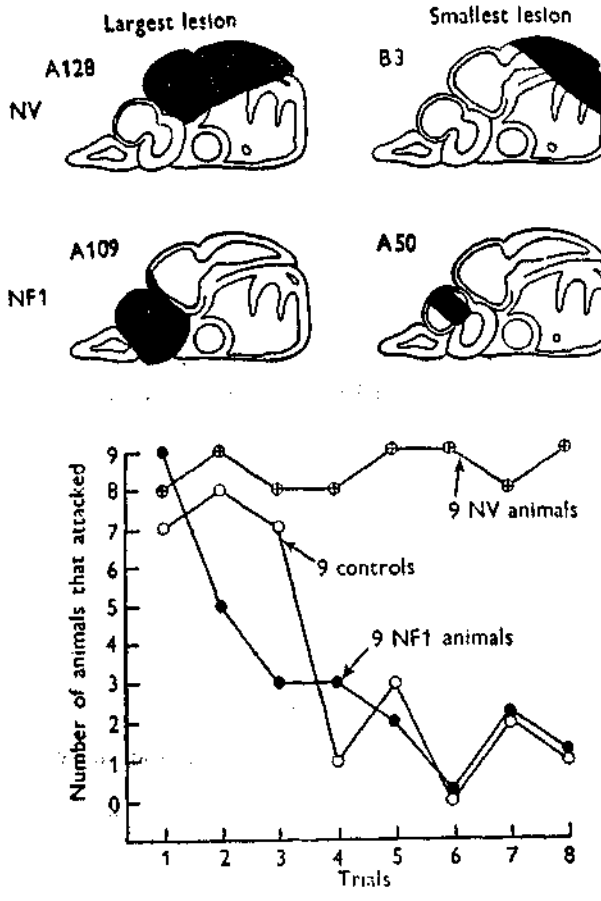

FIG. 2. A summary of the results of a series of experiments in which octopuses were punished for attacking crabs. Most of the control animals learned to leave crabs alone within four trials, at each of which they were given a 6–9 volt A.C. shock for attacking. Animals with the inferior frontal system destroyed or damaged (lesions of the types shown in A50 and A109 above—'NFI animals') learned not to attack crabs as readily as controls. This type of lesion eliminates touch learning (*see* Fig. 3) but does not affect visual learning. Octopuses with the vertical lobe removed or damaged (lesions such as those in A128 and B3 above—'NV animals') did not learn to leave crabs alone in this set of experiments (from Wells, 1961).

SMALL CELL REGIONS IN THE BRAIN

In both the tactile and the visual learning systems there are regions of many small cells without which learning fails altogether. Lesions to the optic, superior frontal and vertical lobes impair performance in visual learning experiments and the critical small-cell regions are found within the optic and vertical lobes. The vertical lobe has a densely staining rind of some 25 million small neurones, most of which have processes that end within the vertical lobe itself; visual learning is very considerably slowed if the vertical lobe is removed. In the tactile system, the equivalent area is the subfrontal lobe, a region containing 5½ million small cells, and here the situation is clearer,

since tactile learning fails altogether as the last few thousands of the small cells are removed from the region where the subfrontal merges with the posterior buccal lobe (Figs. 1 and 3).

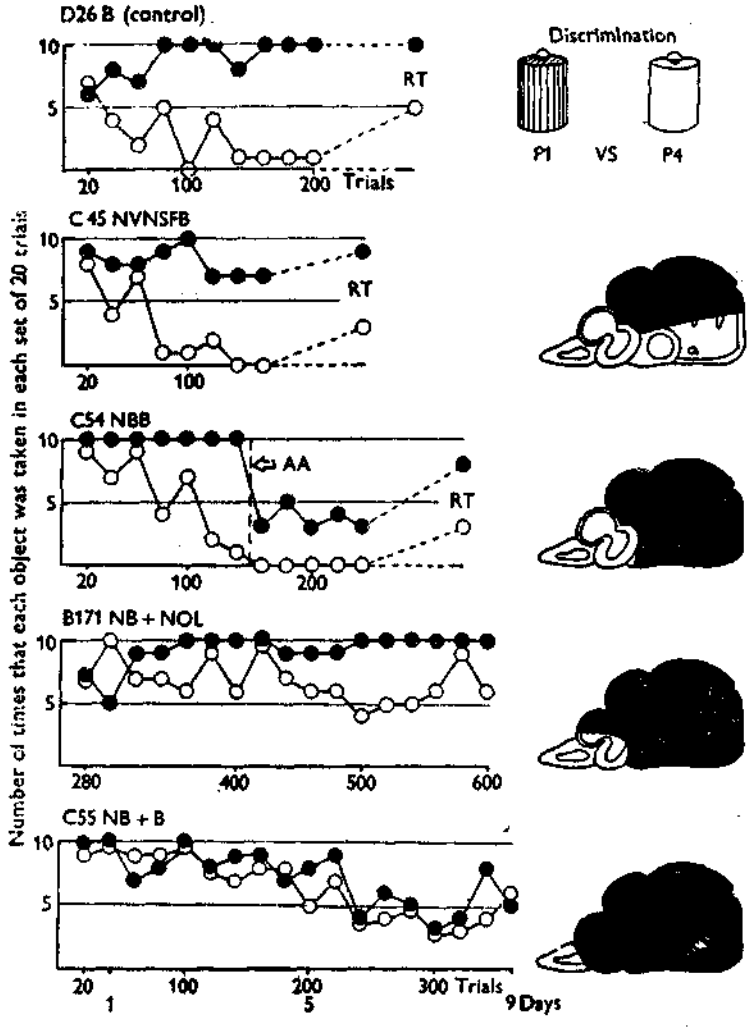

FIG. 3. Some typical experiments showing the effect of brain lesions on touch learning. Animals blinded by cutting the optic nerves were used. In each example the number of times the 'positive' object was taken in each 10 trials is plotted • and the number of takes of the 'negative', O. Black areas show parts of the brain that were removed in operations prior to training. Note that the capacity to learn to make this rough-smooth discrimination is eliminated by removal of the inferior frontal system (*see* Fig. 1). 'R.T.' in the first three records shows the scores in retention tests carried out five days after the end of training. In the record of C54 'AA' means 'Arm amputated', an operation that reduced the proportion of takes without altering the capacity to discriminate. As in Fig. 2 all lesion plots are made on a standard diagram derived from Fig. 1 (*c*) (from Wells, 1959).

Minimal Cell Assemblies and Learning

Partial removal of the small-cell lobes from octopus brains produces defects in performance proportional to the number of cells removed. The situation with regard to the vertical lobe is summarised in Fig. 4. In the case of the comparatively easy visual discrimination (horizontal and vertical rectangles) used for this series of experiments, little deficit was produced by surgical removal of as much as 50% of the vertical lobe. With more difficult discriminations a greater difference between operated and control animals was found (Young, 1958).

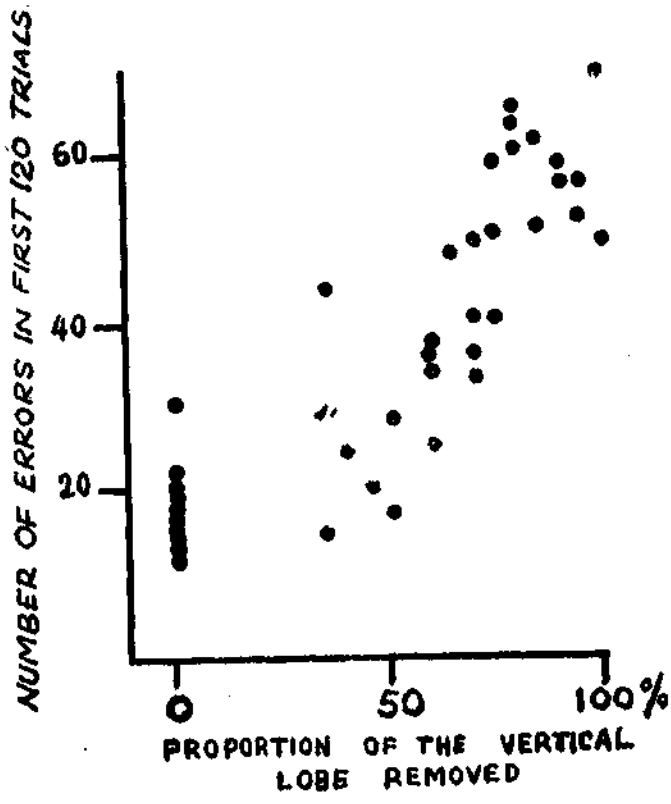

Fig. 4. Showing the relation between cell numbers in the vertical lobe and the capacity to learn a visual discrimination. Each point shows the score made by one *Octopus* in training to discriminate between a rectangle shown with its long axis horizontal, and the same rectangle shown with its long axis vertical. Unoperated controls made between 70 and 90% correct responses. The vertical lobe contains 25 million small cells nearly all of them having processes that terminate within the lobe. Performance is clearly related to the number of these cells that remain after operation (from Young, 1958).

A similar state of affairs is found in relation to touch-learning, which is prevented by removal of the subfrontal lobe. Halving the subfrontal, by splitting the supraoesophageal brain, considerably slows touch learning and produces an immediate reduction in the quality of performance in discriminations that the octopus has already learned to make. The deficit produced is only slowly made good by further training (Fig. 5).

The split-brain technique has proved fruitful as a means of investigating the effects of very large lesions, since longitudinal vertical division of the supraoesophageal brain prevents the spread

of information from one side of the brain to the other. There is, apparently, no communication between parts of the touch learning system at a suboesophageal level, so that it is possible, after this operation, and provided that experience of the test objects is limited to the arms on one side at a time, to train the two halves of the octopus independently.

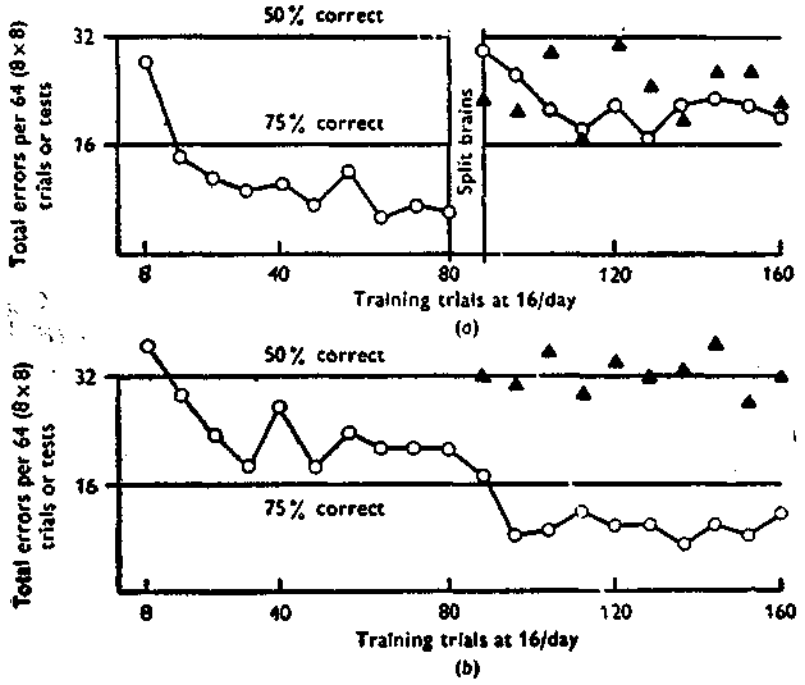

FIG. 5. Results from two series of experiments with split-brain octopuses. The animals were blinded and trained to discriminate between rough and smooth spheres by touch, the spheres always being presented to arms on the same side of the body. All had their supraoesophageal brains divided by a longitudinal cut. In the upper series (*a*) the brain was split in mid-training, in (*b*) the operation was carried out before training was begun. Plots O show total errors made per 8 trials by the 8 animals in each group. Plots ▲ record the results of unrewarded transfer tests made by presenting the test objects to arms on the untrained side of the body; animals split before training [series (*b*)] did not discriminate with the untrained side. Octopuses trained and then split discriminated with the untrained side; the memory trace must normally be bilateral. Note that in (*a*) the operation produces an immediate increase in errors and that the animals operated before training [series (*b*)] learn more slowly than intact octopuses; both sides of the brain must normally contribute to the determination of responses made by the arms on either side of the body (from Wells, 1966 *a*).

The performance of one-half of the split-brain octopus can be used as a control for the effect of a lesion made in the other side of the brain. Experimentally this is very convenient; it eliminates variations in performance due to side effects of the operation, since the two sides of the same animal are, presumably, equally sick or equally hungry.

Using the split brain technique it has been possible to show (1) a relation between the extent of a one-sided lesion and the subsequent performance of the animal similar to that produced by partial removal of the vertical lobe in visual experiments, and (2) that learning is still just possible with very large lesions indeed. Removal of the whole of the inferior frontal and all but a few

thousands of the small cells from the region where the subfrontal merges with the posterior buccal lobe leaves octopuses that can still just manage a simple rough-smooth tactile discrimination. Animals with lesions very slightly bigger, including removal of all the small cells from this region, do not learn at all (Wells and Young, 1965, 1966).

The Whereabouts of the Memory Trace

The small-cell regions in the subfrontal-posterior buccal region in the vertical and in the optic lobes are therefore, quite possibly, the actual sites of the changes that must take place when an octopus learns. Strictly speaking it is, of course, impossible to prove this; short of pinpointing structural alterations at the microscopic or chemical level, one can never be absolutely certain that a lesion has done more than simply cut tracts leading to or from information stores located elsewhere. But this sort of explanation seems rather unlikely in view of the anatomical situation of the regions concerned. The critical areas for visual and tactile learning are all in the uppermost parts of the brain, superimposed upon regions that can be shown to be concerned with movement control. The latter continue to operate in a satisfactory manner after total destruction of all the parts known to be concerned in learning. An octopus with its visual or tactile learning system removed is not recognisably abnormal until an attempt is made to train it in discrimination experiments or in other tests for memory retention. If the memory stores are located outside the superior frontal-vertical-optic or inferior frontal-subfrontal-posterior buccal lobe areas, it is hard to imagine a function for the millions of small cells in these regions other than sensory sorting (since they are certainly not involved in motor control). If sensory analysis were their sole function the storage elements of the system must be buried in the motor control machinery, and it is then not at all clear why the effect of removing the small-cell areas from the tactile and visual learning systems *after* training should so effectively eliminate learned responses while leaving other activities of the animals (which must depend on some measure of sensory discrimination) unimpaired. On balance, and in the absence of direct proof for or against, it would seem reasonable to accept that the changes brought about by training an octopus are in fact located in the areas demonstrably concerned with learning. And that in all probability, they are taking place within the subfrontal/posterior buccal region and in the vertical and optic lobes.

The Nature of the Memory Trace

We can thus identify at least two regions in the octopus brain that are vital to the establishment of whatever long-term changes take place when an octopus learns. Quite possibly, the small-cell areas are the actual site of the changes themselves. We ought, in principle, to be able to examine the subfrontal and vertical lobes of trained and untrained octopuses and to identify some difference between the two. But so far we cannot do this. We do not know what to look for, and it is, therefore, necessary to begin by considering evidence that might provide us with clues as to the likely nature of memory traces in animals.

Discriminations are learned by octopuses under conditions that would lead to associative learning in a vertebrate. That is, the signals to which the animal is reacting must occur *before* the rewards or punishments given for the animals correct or incorrect responses. In the octopus case the reward or punishment given for accepting an object touched or attacking a figure seen must arrive within about thirty seconds of the action taken if a significant discriminatory performance is to be achieved within a matter of 100–200 trials (Wells and Young, 1968). Rewards or punishments given before the response is made, or at a longer interval after the response, do not lead to longterm cumulative changes in behaviour.

Thus in *Octopus*, as in vertebrates, one finds that permanent changes to the central nervous system occur only if the animals are subjected to a specific sequence of events; the changes may

then endure for a considerable fraction of the animal's lifetime (Saunders, Wells and Young, 1968). Other sequences—if, for example, feeding or punishment occurs mid-way between or immediately before trials—may well result in an animal becoming more, or less, likely to respond to objects that it sees or touches. But these effects are comparatively short-lived, lasting for a matter of a few hours at most, and characteristically unspecific; responses to all objects seen or touched are altered together and in the same direction. There is no question of a specific cumulative response to a particular stimulus. An example is given in Fig. 6.

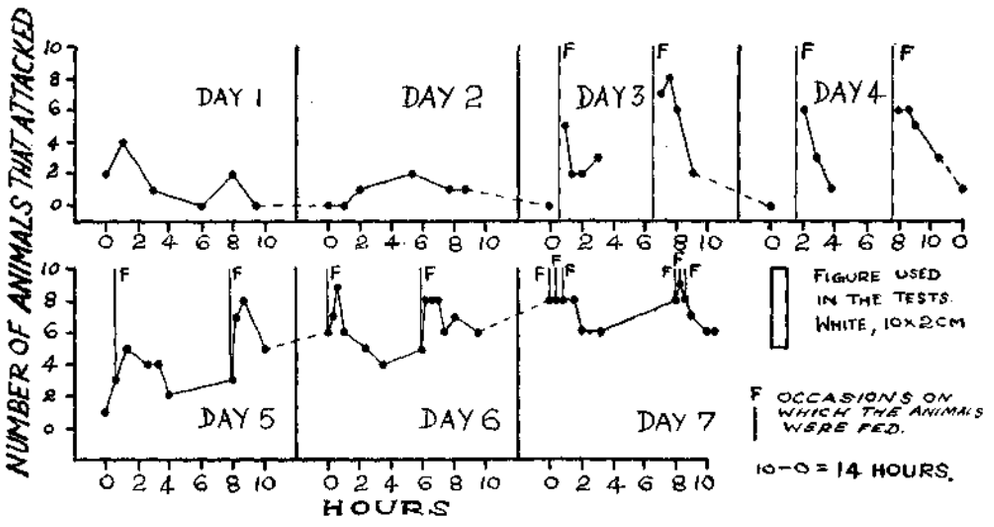

FIG. 6. Learning and sensitisation in *Octopus*. This record summarises responses made by 10 octopuses over 7 days during which they were tested at intervals by showing them a white vertical rectangle at the far ends of their tanks. At first (days 1 and 2) the animals rarely attacked the rectangle. Their level of response was greatly raised by feedings (days 3 and 4), even though the food was given in the octopus's home and between trials when the rectangle was absent. Response levels raised in this way, however, soon declined to the baseline shown on days 1 and 2. On day 5 and subsequently, the animals were fed immediately after showing them the rectangle on the occasions indicated. This treatment leads to a progressive and enduring rise in the level of response to the rectangle (after Young, 1960).

Such ' sensitised ' responses, enhanced by food or punishment, may well be adaptive in that they increase the chances of the animal making correct responses provided that biologically favourable and unfavourable events do not occur at random or in strict alteration, both improbable conditions in nature (Wells, 1968 *a*, *b*). The possible significance of sensitisation in the evolution of associative learning mechanisms is discussed by Wells (1968 *a*).

Memory traces formed as a result of paired action-reward sequences are very difficult (and may be impossible) to eradicate once established. A trained *Octopus* can be anaesthetised until breathing and heart-beat have ceased (a condition in which the central nervous system is, presumably, electrically silent) or it can be subjected to faradic stimulation of the exposed brain, a treatment that quite certainly disrupts any organised electrical activity that is going on. Neither treatment eliminates the records that have been established within the brain as a result of training (Boycott and Young, 1955). Long-lasting memory traces cannot therefore depend upon the maintenance of electrical activity within the central nervous system; it cannot, for example, be attributed to the activities of self-reexciting chains of neurones. Something has been altered as a result of training that can survive temporary disruption of the functioning of the nervous system.

Long and Short-Term Memories

It has already been pointed out that learned responses to specific sensory inputs may persist for months after the end of training (Saunders, Wells and Young, 1968). In addition to these long-term memories, there are clearly also occasions on which the central representations of specific events in the outside world disappear comparatively quickly. A cuttlefish (*Sepia*) that has been pursuing a prawn will behave as though hunting for the prawn for several seconds after the prey has vanished from its sight; it will swim around a corner apparently in pursuit of food which is no longer in its field of view (Sanders and Young, 1940). Clearly it would be uneconomic for the animal to continue hunting for the prey for very long; what in fact happens is that the cuttlefish abandons the search after a matter of a few seconds, and settles down to wait for the next prawn. It is impossible to be certain that the animal has now forgotten about the prawn that eluded it, but it certainly behaves as though this were the case.

Experiments have also been done on *Octopus* where, again, there is evidence of short-term memories with an effective duration of a minute or so only. The situation is comparable with that to be observed in vertebrates like ourselves where too there seems to be a distinction between long-term learning and the sort of short-term memories that are established between, say, reading and dialling a telephone number. Such short-term memories are readily disturbed by further sensory inputs during the retention period and they seem to vanish without trace; their relation to processes resulting in the establishment of long enduring memories is uncertain.

One means of demonstrating the existence of short-term processes in octopuses was explored by Dilly (1963). He tested them in a discrimination experiment. The animals, restrained behind a transparent partition, were shown two identical figures at the far end of their tanks. At each trial a crab was shown briefly next to one or other of the figures, and then removed. An opaque screen was placed between the octopuses and the test figures. Some seconds later the partition and screen was raised so that the octopus could approach the figures. If it approached and grasped the 'correct' figure, indicated by the crab, it was fed. This was not, apparently, a difficult task for octopuses and näive animals made 80–90% correct responses from the start of the experiment following delays of 10 seconds; with further experience of the experimental situation the delay period could be extended to 20–30 seconds. Disturbing the posture of the octopus during the delay period did not prevent correct responses. Since the correct figure at each trial was determined by chance, the animal had no means of predicting the 'correct' side from its past experience of the experimental situation. One must suppose that the animals' choice of figure was determined by some representation of the crab persisting in the octopus brain between removal of the crab and release of the octopus.

Detour experiments constitute a somewhat more elaborate means of demonstrating short-term memories and estimating their possible duration. Using the apparatus shown in Fig. 7 Wells (1964 *b*) showed that octopuses would detour through an opaque corridor in order to reach a crab seen through one or other of two transparent windows to the side of the corridor. In order to make a correct response the octopus had to make an appropriate left-right choice at the far end of the corridor. The response seems to be guided visually, since blinding the animals in one eye leads to systematic errors that are most readily explained by supposing that the animal normally fixates on the appropriate wall as it goes into the corridor. Removal of the rotation receptors, in contrast, does not upset the response, so that it is unlikely that the animal remembers the turn it made on going into the corridor as a means of determining which direction of turn it should make at the far end (Wells, 1964 *b*).

If the animals are delayed by shutting them into the corridor during the course of a run, the chances of an incorrect response rise with the length of delay. Up to about two minutes total running time (including enforced delays, timed from the moment of entering the corridor, until the moment of entry into one or other of the feeding compartments) octopuses make mainly correct

responses. Since the side that the crab is shown is determined by chance (tossing a coin) the animal is unable to predict which side to run to on the basis of its pact experience of the maze. It could, in principle, learn to recognise left- and right-sided runs as separate experiences requiring different sequences of movement in response, but it seems very unlikely that octopuses in fact do this. For one thing the probability of a correct response does not rise with practice. Many octopuses complete detours swiftly and successfully at the first trial in the apparatus. Others require to be led through on one or more occasions. But in any event, once an octopus has run successfully it will generally continue to do so. From the start, the great majority (80% *plus*) of all runs by unoperated animals are correct. It has also been found that a long succession of runs in one direction in no wise interferes with performance when the octopus is abruptly required to detour in the other (Wells, 1964 *b*). This evidence tends to confirm the view that the responses made by an octopus in the detour apparatus are guided be memories of the last crab that it has seen.

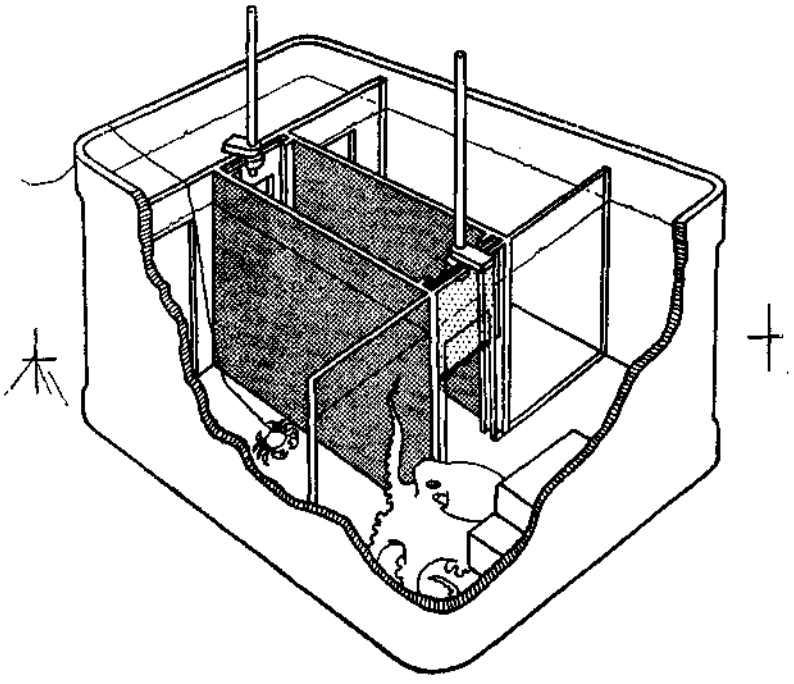

FIG. 7. Detour apparatus used in experiments on short-term learning by octopuses. The animals were shown a crab in one or other of two 'feeding' compartments visible through the transparent wall of the 'home' compartment. To get the crab the octopus had to move down the corridor, out of sight of the crab, and make a correct left-right choice at the far end. The animal could be delayed in the corridor by lowering shutters at either end (from Wells, 1967).

The representation of the crab seen that guides the octopus as it detours fades away in time. The chances of a correct response fall progressively until, at about two minutes, runs are equally likely to end up in the correct and incorrect feeding compartments (Fig. 8). The fact that many of the animals will complete detours after two minutes in spite of this can be interpreted as a result of accumulated experience in the maze; the octopus does not now remember the whereabouts of the last crab, but past experience has taught it that running through the maze generally results in its getting fed.

The length of time that it is possible to delay the octopus in the maze and still obtain correct responses can be drastically reduced by surgical removal of the vertical lobe. After this operation-

octopuses still perform as well as normal animals in rapid detours. But their capacity to make correct responses following enforced delays is severely impaired. Detours taking longer than one minute are equally likely to be correct and incorrect (Fig. 8). The operation has reduced the duration of short-term memory traces to about half of that in intact animals (Wells, 1967).

This is interesting because the vertical lobe is known to play an important part in the establishment of long-term memory traces in octopods, as has already been described. Without their vertical lobes, octopuses must be subjected to several times as many training trials as normal octopuses in order to reach the same standards of accuracy in simple discrimination experiments (Young, 1961). The implication is clearly that since long- and short-term memories depend upon the same parts of the brain, they may well represent aspects of the same process. This is important since there is no *prima facie* reason for supposing that the two processes are other than independent.

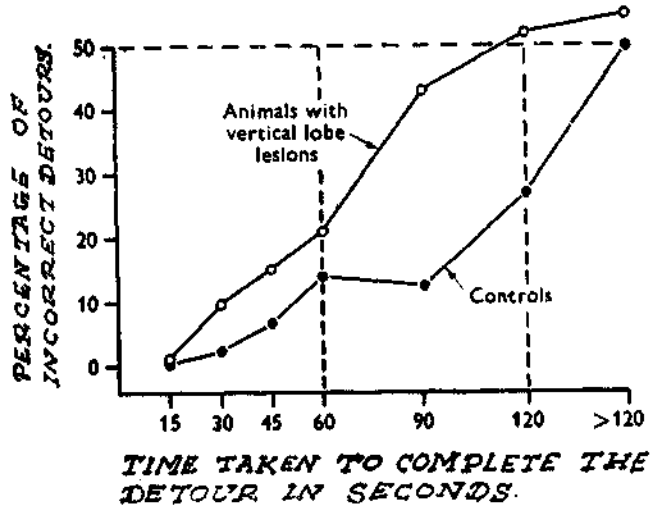

FIG. 8. Summarising the performance of octopuses in detour experiments (*see* Fig. 7). In all 1071 runs were made by 27 octopuses. Unoperated animals normally run through the maze within 30 seconds, but will still perform mainly correctly if delayed for up to 2 minutes. Octopuses deprived of their vertical lobes run equally rapidly and are almost as accurate as controls in these short runs. But they perform comparatively badly if delayed, apparently forgetting the whereabouts of the crab in a matter of ninety seconds or so (from Wells, 1967).

DISCUSSION

Evidence has been presented to show that octopuses learn under the same conditions and in a similar number of trials as the higher vertebrates and that, once taught, they remember for similar lengths of time. It has also been noted that their behaviour is changed by sequences of action and reward other than conditioned stimulus-unconditioned stimulus and that these other sequences produce changes that are transient and unspecific. Effects analogous to short- and long-term learning in vertebrates can be demonstrated. The learning systems found in *Octopus* have, in short, properties very like those of the learning machinery in a vertebrate.

In part, of course, this must be a result of convergence, brought about by selection in the past. For animals living in the same world there are presumably optimum properties for learning machines that depend on the way of life of the species concerned. An octopus lives a remarkably 'vertebrate' existence. It is active, predatory and unarmoured. It must assess when to attack

and when to retreat, when to move on to new feeding grounds and when to stay put. As with vertebrates in a similar situation it is advantageous for it to be able to learn, and important that it does not learn too quickly; the number of experiences required to produce a persistent change in behaviour must, in general, be sufficient for anomalies in the action—result sequence to be weeded out statistically; one-trial learning can be very dangerous and is comparatively rare in animals. As with vertebrates, lasting changes in behaviour are brought about only by sequences of action and reward that make it reasonably certain that the reward is the result of the action the animal has taken.

But the resemblance between vertebrate and cephalopod nervous systems does not end here. One finds, for example, that in each case there are two distinct time-scales apparent from learning experiments. Memories of past events either last a matter of minutes, or for a matter of months. One finds that the long-term sort of memory cannot readily be eliminated. Anaesthesia and electric shock treatment do not erase memory traces in vertebrates or in cephalopods. It is hard to imagine selective processes that could give rise to this sort of parallelism and to the essentially similar anatomies associated with the development of learning in the two groups. Massive numbers of small cells appear to be required for both vetebrate and cephalopod learning machines.

There is no phylogenetic reason for these similarities; molluscs and chordates could hardly be more remotely related, and the more primitive members of the two groups show little evidence of learning. One must suppose that cephalopods and vertebrates have, quite independently, evolved machinery for associative learning that happens to have similar properties and, so far as we can tell, similar structure. Their remarkable parallelism could have arisen by chance, but it seems more likely that it is a consequence of constructing learning machinery from neurones. It is not at all improbable that the parallels we find are inevitable, in the sense that there is only one way of constructing a learning machine from neurones if it is to have the necessary properties already discussed. If this is so, there are interesting possibilities in comparing the detailed structure of the two learning systems. Much of the construction of either could be a reflexion of the past history of the group from which it comes; in animals no design develops afresh, it is always an adaptation of some previous design and the structure of the brain of any animal must include elements that are a result of past history rather than immediate functional necessity. This is a major problem in searching for memory traces and learning systems. We know that the relevant structures are somewhere concealed in the nervous networks of the brain. But we do not know how much of what we see or record is relevant.

Given two unrelated learning systems to compare, the situation alters. Similar detailed structures could occur by chance, but are not very likely to do so. If, therefore, we have the good fortune to discover similar structures associated with similar functions in the two, there is good reason to suppose that these structures, at least, are of immediate functional significance.

So far the comparison of the structure of vertebrate and cephalopod brains has been limited to the gross anatomical and light microscope level. Electron microscope and neurochemical studies are beginning but we are not yet in a position to compare the patterns of connexion in the two sorts of brain, as we shall eventually need to do. We are still in the process of whittling down the areas of search and defining the properties of the mechanisms concerned, attempting to reduce the anatomical problem to a scale where it is feasible to attack it.

It is, however, already apparent that the two machines are very much alike. So that facts gleaned from a study of one are very probably applicable in explaining the function of the other. Work on vertebrates is in some areas well ahead of that on cephalopods. In vertebrates, for example, we know that the establishment of enduring memories can be prevented by anaesthesia or electric shock or oxygen deficiency, provided that these treatments occur within seconds or minutes of the sequence of stimuli concerned; later the same treatments fail to eradicate the effect of past experiences (references, *see* Weiskrantz, 1966). We know that in at least some instances,

increased brain activity can be correlated with synaptic changes and with increases in protein synthesis, and there are experiments which suggest that the establishment of permanent records in the CNS depends upon these, (*see*, for examples, Agranoff, 1966, Cragg, 1967, Gaito, 1966, Rose, 1967). The implication is that there are short-term processes in learning that normally lead to growth processes if allowed to continue for more than a few minutes. The orthodox view is that the former are electrical in nature, since the establishment of long-lasting traces is prevented by treatments that stop electrical activity. It is tempting to identify short-term learning with electrical events and long-term learning with growth processes, although there is, strictly speaking, no experimental evidence to prove that this is the case.

In summary one can say that the central problem of the nature of memory traces remains unresolved. We move nearer to a solution, to the point where we can specify the sort of needles that we are seeking in the neuronal haystack. We have discovered a mollusc with a mechanism for learning that has properties closely resembling the equivalent mechanism in vertebrates, and it has become apparent that in the mollusc this mechanism is separated from the rest of the brain in a manner that promises to be easier to analyze, functionally and structurally, than the more complex brains of vertebrates where learning is more closely tied up with movement control. The cephalopods have already proved invaluable to neurophysiologists; practically, all that we know about the mechanism of nerve impulse propagation has come from studies of the giant nerve fibres of squids. It is possible that in *Octopus* we have a preparation that will allow us to break through to the solution of a second great problem that is ultimately far more important to all of us; how is it that nervous systems can be permanently changed by use?

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PHAGOCYTOSIS AS AN INTERNAL DEFENSE MECHANISM IN THE MOLLUSCA : WITH AN EXPERIMENTAL STUDY OF THE ROLE OF LEUCOCYTES IN THE REMOVAL OF INK PARTICLES IN *LITTORINA SCABRA* LINN.

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ABSTRACT

It is well known that molluscan leucocytes are involved in digestive and transportive processes in addition to shell repair and as internal defense mechanisms against biotic and abiotic invading materials. Since opsonins are known to enhance the cellular uptake of foreign particles in the mammalian reticuloendothelial system, and since phagocytosis is one of the major types of internal defense mechanisms in molluscs despite the fact that a reticuloendothelial system is absent, it is believed that the opsonic effect of a hemagglutinin, which has been recently discovered associated with the plasma of the American oyster, *Crassostrea virginica* may play an important contributory role in molluscan phagocytosis.

As the result of the study by Stauber (1950, *Biol. Bull.*, **98**: 227–241), it is known that *C. virginica* leucocytes are highly efficient in phagocytizing experimentally introduced ink particles. Furthermore, Stauber has demonstrated that ink-laden leucocytes are eliminated from oysters by migration to the exterior through various epithelial borders. Comparable studies by us have been carried out to determine the role of leucocytes in phagocytizing and eliminating India ink particles in the marine prosobranch *Littorina scabra*.

Ten series of *L. scabra* were exposed to India ink particles *via* intrapedal sinusal injections. Members of the series were fixed at 30 min., 1, 3, 6, 12, 24, 36, 96, 144 and 192 hours post-injection. Microscopical examinations of serial sections revealed that after an initial clotting of ink particles in the pedal sinus and associated vessels, most of the particles become phagocytized by leucocytes and are gradually eliminated from the body *via* migration through the epithelial surfaces of the foot, alimentary tract, gills, and *via* the normal excretory passages of the kidney, primarily the latter. Moreover, the mantle surface also serves as a border through which ink-laden leucocytes migrate but to a much lesser extent. Not all ink particles are phagocytized simultaneously. Non-phagocytized particles are circulated in the hemolymph and are distributed throughout the entire circulatory system, including the intertubular and interlobular spaces of the hepatopancreas and gonads. These are gradually phagocytized, with free particles being almost completely eliminated by the 4th day. While within the hepatopancreas, ink particles stimulate the hypersecretion of yellowish-brown spherules by certain cells and the appearance of numerous conspicuous vacuoles in secretory cells. Snails are completely devoid of ink particles by the 6th day post-injection.

INTRODUCTION AND HISTORICAL REVIEW

ALL molluscs possess leucocytes. Some pelecypods possess hemoglobin-containing erythrocytes (Griesbach, 1891; Dawson, 1932; Sato, 1931; Hill and Welsh, 1966). In this paper we are concerned only with leucocytes, specifically their function as associated with the arrest and elimination of foreign materials, biotic or abiotic, although their roles in digestion, locomotion, excretion, growth, regeneration, and repair, including shell repair, are known (*see* review by Wagge, 1955).

Cell types

Leucocytes within molluscs are distributed throughout their vascular systems and since the majority of molluscs possess open circulatory systems, leucocytes are also found in the body tissues. There still does not appear to be any agreement relative to the types of leucocytes present, although

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this topic has been the subject of several studies. Moreover, there is yet no agreement on the terminology employed to designate the types of leucocytes. Among the earlier investigators, Cuénot (1891), who has examined the blood of a number of species of cephalopods, gastropods, and pelecypods, has considered all the blood cells to be of one type, granular amoebocytes. Kollmann (1908) has stated that, with the exception of *Paludina vivipara*, none of the gastropods examined by him possess granular leucocytes. Furthermore, he has distinguished gastropod leucocytes as belonging to two classes: stage I cells which are characterized by a small amount of hyaline cytoplasm surrounding a spherical nucleus, and stage II cells which include more cytoplasm and a polymorphic nucleus. Kollmann has expressed the opinion that stage II cells arise from stage I cells. Haughton (1934) has reported the occurrence of one type of amoeboid cell in *Helix aspersa*.

Among more recent investigators, George and Ferguson (1950), after examining the marine gastropods *Busycon carica*, *B. canaliculatum*, and *Fasciolaria tulipa*, primarily the first species, have categorized their leucocytes into three types; lymphoid cells which may have either a thin layer of hyaline cytoplasm or a thick layer of granular cytoplasm surrounding the nucleus; granular macrophages which are the most numerous and which have more granular cytoplasm around a bean-shaped or bilobed nucleus; and eosinophilic granular amoebocytes which include large numbers of eosinophilic granules in their cytoplasm. Another detailed account of the blood cells of gastropods is that by Müller (1956). She has examined *Lymnaea stagnalis* and has stated that four types of cells occur, namely, normal cells, which are round or oval, 10–15 μ in diameter, and which possess one or more flat pseudopodia; leucocytes, which represent an actively amoeboid phase of normal cells; small cells, which she has postulated to have resulted from the breaking up of normal cells; and wandering cells, which she believes to be phagocytic normal cells.

Among pelecypods, de Bruyne (1895), working with *Mytilus edulis*, *Ostrea edulis*, *Unio pictorum*, and *Anodonta cygnea*, has recognized seven types of cells. Drew (1910) has found that the blood cells of *Cardium norvegicum* include finely granular eosinophils (48%), coarsely granular eosinophils (44%), and small basophils (8%). The latter is characterized by a slight amount of cytoplasm surrounding a spherical nucleus. Takatsuki (1934), who has examined *Ostrea edulis*, has only found two types of leucocytes: granular leucocytes and lymphocytes. Dundee (1953), who has studied eleven species of freshwater mussels, *Amblema costata*, *Quadrula quadrula*, *Uniomerus tetralasmus*, *Tritogonia verrucosa*, *Carunculina parva*, *Ligumia substrata*, *Proptera alata*, *Leptodea fragilis*, *Lasmiggona complanata*, *Lampsilis fallaciosa*, and *Anodonta grandis*, has reported that their blood cells can be designated as belonging to three types: granular eosinophilic amoebocytes, granular basophilic amoebocytes, and macronucleocytes. It is significant, however, that she has stated that: " The cells of mussels studied here do not directly fit any one of the above descriptions, *i.e.*, the cells described by Cuénot (1991), de Bruyne (1896), Drew (1910), George and Ferguson (1950), and Takatsuki (1934) but rather have some characteristics in common with several of them. Therefore, it seemed desirable to employ a different terminology and apply the existing terms wherever possible." This, unfortunately, although perhaps unavoidably, has been the practice of all others who have studied molluscan blood cells. In the American oyster, *Crassostrea virginica*, Galtsoff (1964) has definitely stated that two types of leucocytes occur, granular and hyaline amoebocytes, with the former frequently referred to as granulocytes because of the large number of granules in their cytoplasms. He has noted, however, that hyaline amoebocytes are not completely devoid of cytoplasmic granules.

In addition to gastropods and pelecypods, leucocytes of one or more types have been identified in monoplacophorans (Lemche and Wingstrand, 1959), amphineurans (Arvy and Gabe, 1949), scaphopods (Arvy and Gabe, 1951), and cephalopods (Cuénot, 1891). The leucocytes of these, however, have not been examined from the functional viewpoint and will not be considered in detail in this paper.

From the abbreviated review given above, it is apparent that the hematology of molluscs is in need of a great deal of further study. From what is known, it may be concluded that there is

no agreement as to the exact number of types of leucocytes occurring in molluscs except that some possess granular cytoplasm while others possess agranular or only slightly granular hyaline cytoplasm. Furthermore, the practice of identifying the types of cells by different designations has created an extremely difficult problem for those interested in homologies and comparative studies. This is true to some degree even for the usage of the general terms "amoebocyte", "leucocyte", and "phagocyte". There is yet no clear-cut evidence that one type of cells is more phagocytic than another, although some are of the opinion that granular cells are more actively so (Galtsoff, 1964). Thus, based on currently available information, it appears more appropriate to follow Stauber (1950) in considering amoebocytes, leucocytes, and phagocytes of molluscs as synonymous terms. In addition, since intravascular cells are known to migrate into extravascular tissues, we cannot agree with those who consider cells found migrating in tissues to be distinct from those found within blood vessels and sinuses.

Although some authors have suggested that granular and agranular or less granular leucocytes may not represent distinct types of terminal cells but stages of a developmental series (Kollmann, 1908; Müller, 1956), until more direct information becomes available on molluscan hemopoesis, preferably by employing specially designed experiments involving culture methods, one cannot state with certainty that one morphological type arises from another. It bears mentioning that some progress is being made in this area. Aside from the earlier studies by Gatenby (1931, 1932), Gatenby and Duthie (1932), Gatenby *et al.* (1934), Bohuslav (1933 *a, b*), and Haughton (1934), Perkins and Menzel (1964), Tripp *et al.* (1966), and Kramer *et al.* (unpublished), the last mentioned in this laboratory, have been able to maintain leucocytes from heart explants of *Crassostrea virginica* for extended periods, and Rosenfield (1965) and Li *et al.* (1966) have been able to maintain heart tissues, with proliferation by amitosis (?) in the latter case.

Hemopoesis and Ontogenesis

Relative to the hemopoetic sites and ontogenesis of molluscan leucocytes, again, there are no definitive answers. Wandering phagocytic cells have been described as originating in connective tissues and from the epithelial layers of various tissues (*see* review by Wagge, 1955). Briefly, Wagge (1951) has reported that in *Helix aspersa*, certain amoebocytes originate from the mantle epithelium and others from the connective tissue of the mantle. Millott (1937) has described the formation of phagocytes by budding from the hepatopancreas of *Jorunna tomentosa*, and Potts (1923) has described a similar phenomenon in *Teredo*. More recently, Müller (1956) has reported that blood cells of *Lymnaea stagnalis* are formed continuously in connective tissue, particularly in the lung, and Pan (1958) has suggested that the amoebocytes of *Biomphalaria glabrata* may be formed from fibroblasts situated in the trabeculae of the mantle blood sinuses and from a cellular reticulum found in the wall of the kidney near the pericardium.

In addition to the hemopoetic sites reported, several other investigators have commented on this subject. These investigators have studied amoebocyte production *in vitro* rather than *in vivo*. Specifically, Gatenby and Hill (1934), Haughton (1934), Bourne (1935), and Crawford and Barer (1951), all of whom have examined tissues of *Helix aspersa* maintained in culture, have reported that amoebocytes differentiate from connective tissue, particularly that found in the mantle.

Although the reports cited above, especially by those who have studied amoebocytes migrating from tissue explants, would indicate that various connective tissue cells and epithelia are capable of differentiating into amoebocytes, there is some hesitation to accept this as an irrefutable fact. The reason for this is that certain types of molluscan cells maintained in culture, particularly epithelia and connective tissue elements such as fibroblasts, are capable of becoming dissociated from surrounding cells, rounding up, and even becoming phagocytic. The question being raised is: do these processes represent true ontogenetic development of blood cells or do they represent dedifferentiation so common among certain types of cells maintained in culture? If the former is true, then we do have some direct evidence for hemopoesis in molluscs. However, if the latter is true,

what earlier workers have observed in tissue cultures do not represent true hemopoesis. This problem is in need of critical re-examination.

In addition to the formation of amoebocytes at presumably specific hemopoetic sites, various investigators have reported that these cells are capable of multiplying by division. This belief has again arisen primarily from observations on molluscan tissues maintained in culture. Bohuslav (1933 *a*, *b*) has expressed the opinion that amoebocytes migrating from tissues maintained in culture can divide both by mitosis and amitosis while Gatenby (1932), Gatenby and Hill (1934), Gatenby *et al.* (1934), and Bourne (1935) have described only amitotic division of amoebocytes associated with cultures of the mantle of *Helix aspersa*. In addition, Gresson (1937), while studying *Modiolus*, has reported finding nuclei that appeared to be at early prophase, and Fretter (1939) has described amoebocytes of *Philine aperta* undergoing division. In addition to nuclear division, Bourne (1935) has described cytoplasmic fragmentation as another mechanism for the formation of new amoebocytes. It would thus appear that mature molluscan amoebocytes are capable of dividing, either by mitosis or amitosis, if the latter truly occurs. Furthermore, cytoplasmic fragmentation may be another method. It appears appropriate to call attention to the fact that among the original observations reported herein, we have observed an ink-laden leucocyte of *Littorina scabra* stretched out in such a manner so as to suggest division (Fig. 19).

Intracellular enzymes

Since it is known that phagocytic leucocytes of molluscs are capable of degrading digestible particles intracellularly, it is of considerable interest to know what enzymes occur within leucocytes and the factors which govern the actions of these.

Relative to enzymes which have been detected, Wagge (1951) has found alkaline phosphatase activity in the nuclei of small amoebocytes of *Helix aspersa* and Cheng (unpublished) has demonstrated alkaline phosphatase in the cytoplasm of amoebocytes of *Crassostrea virginica* by employing histochemistry. Yonge (1926) and George (1952) have reported that phagocytes can take up and hydrolyze fats in *Ostrea edulis*, *Crassostrea virginica*, and *Modiolus demissus*. In addition, Zacks and Welsh (1953) and Zacks (1955) have reported lipase activity in the leucocytes of *Mercenaria mercenaria*.

One of the more extensive studies of this nature is that by Takatsuki (1934). He has found that the amoebocytes of *Ostrea edulis* include carbohydrases which can reduce starch, glycogen, maltose, lactose, sucrose, and the glucosides amygdaline and salicine. The hydrolysis of starch and glycogen is particularly evident and the amylase has an optimum pH of about 7·0. In addition, Takatsuki has also demonstrated the occurrence of both lipoclastic and proteoclastic enzymes. The latter can degrade peptone and casein and has an optimum pH of about 8·0.

In addition to hydrolytic digestive enzymes, a few other categories of enzymes have been demonstrated in molluscan leucocytes. Jatsenko (1928) has demonstrated an indigo carmine-reducing oxidase system in *Anodonta* and Takatsuki (1934) has reported that a complete oxidase system occurs in the cells of *Ostrea edulis* as revealed by the reduction of indigo carmine and by the guaiacum reaction method. More recently, Zacks and Welsh (1953) have demonstrated the occurrence of cholinesterase and Zacks (1955) has demonstrated the presence of a dehydrogenase and cholinesterase in the amoebocytes of *Mercenaria mercenaria*.

Phagocytosis

The primary concern of the physiologic role of molluscan leucocytes in this report is of their role as internal defense mechanisms, specifically phagocytosis and elimination of foreign materials. In addition to this role, leucocytes are also known to be involved in encapsulation of larger foreign bodies (*see* review by Cheng, 1967).

In order to determine the mechanisms responsible for phagocytosis, the problem of leucocytic accumulation at certain tissue sites must first be considered. According to one theory, when the organism is subjected to a foreign stimulus, its affected tissues release a chemotactic substance (phlogistine, leucotaxine) that attracts leucocytes into the area (Humphrey and White, 1963). It has been shown that in mammals, the reticuloendothelial system is influenced by such a substance as well as by the nature, size, and surface charges of the foreign particles to be phagocytized (Benacerraf *et al.*, 1957; Dobson, 1957; Zilversmit *et al.*, 1952; Wilkins and Myers, 1964). Furthermore, components of the plasma known as opsonins can also enhance the cellular uptake of foreign particles (Wright and Douglas, 1903; Jenkin and Rowley, 1961; Filkins and Smith, 1965; Normann and Benditt, 1965; Saba and Lucio, 1965; Saba *et al.*, 1967). It appears that the main common denominator in opsonic action is the ability to coat the surface of the foreign particle so as to decrease the surface electrical potential and to promote the adhesion of coated particles to the surface of phagocytes. Among the most active opsonins known are the specific antibodies that occur in the plasma of "immunized" organisms. However, naturally occurring opsonins may occur in "non-immunized" animals. Hypothetically, it is possible that although molluscs do not include a reticuloendothelial system, invading foreign particles may stimulate the affected tissue(s) to secrete some substance which attracts leucocytes directly thus accounting for the accumulation of phagocytic leucocytes at specific tissue sites. Furthermore, it is possible that yet unidentified opsonic agents may occur in molluscan plasma which enhance phagocytosis. Indeed, Tripp (1966) has recently found that a natural hemagglutinin occurring in the plasma of the oyster *Crassostrea virginica* exhibits opsonic properties. He has been able to demonstrate a decrease in the time required for the phagocytosis of vertebrate erythrocytes experimentally injected into the oyster if the erythrocytes had been coated with the hemagglutinin. It is is not known whether a similar opsonic property is associated with natural agglutinins in other species of molluscs, but if this is true, then one would expect the opsonic effect to be present in other species in which agglutinins and precipitins have been reported (*see* Huff, 1940; Cheng and Sanders, 1962; Cheng, 1967). It should be noted, however, that Bang (1961) has demonstrated that certain flagellated marine bacteria are bound to oyster amoebocytes mechanically by their flagella.

The ability of molluscan leucocytes to phagocytize foreign materials has been known since Haeckel (1862) demonstrated that the blood cells of *Thetis fimbria* can ingest particles of indigo. Since then, several reports of this phenomenon in a number of species have become available. De Bruyne (1893, 1895) has verified phagocytic activity by the leucocytes of various molluscs. Cuénot (1914), in connection with his report on phagocytosis in molluscs, particularly the amphineuran *Acanthochites discrepens*, has reported that ink particles introduced into the blood stream are removed *via* phagocytic activity of blood cells. His primary concern, however, was with the immediate removal of the ink particles rather than with their ultimate disposal. Takatsuki (1934) has reported the phagocytic uptake of ink and carmine particles injected into the body of *Ostrea edulis* by amoeboid leucocytes.

Modern experiments on the role of molluscan leucocytes in the uptake and disposal of foreign materials commenced with the studies by Stauber (1950). He injected India ink intracardially into *Crassostrea virginica* and traced the fate of the ink particles in histological sections. He has found that, starting as soon as 15 minutes after injection, the ink particles agglomerate and form emboli in the arterial vessels of the viscera, mantle, and adductor muscle. Subsequently, the particles become phagocytized by mobile leucocytes and are distributed to all parts of the oyster with concomitant resolution of the emboli. Eventually, around the 8th day post-injection, the ink particles are eliminated from the oyster by the migration of ink-laden leucocytes through the epithelial layers of the alimentary tract, digestive diverticula, palps, mantle, and pericardium from whence they are voided to the exterior. It is of interest to note that Stauber has reported that the epithelia of the gonoducts, nephridia, and shell-forming mantle do not serve as routes of elimination. Stauber's studies were followed by those of Tripp (1958 *a*, *b*; 1960) who has employed heterologus biotic materials. He has injected erythrocytes of the rabbit, weakfish, duck, duck erythrocytes infected with the avian malaria parasite *Plasmodium lophurae*, bacterial spores, vegetative bacteria, and yeast cells into *C. virginica* and has found that digestible particles, *i.e.*, erythrocytes, vegetative bacteria,

some yeast cells, are eliminated *via* the migration of particle-laden leucocytes across the same epithelial layers as well as are digested intracellularly. Non-digestible particles, *i.e.*, malarial pigments, most yeast cells, bacterial spores, are eliminated almost completely by migration of leucocytes across epithelial borders. Feng (1959, 1965, 1966) also has carried out parallel studies. He has injected soluble starch, bovine hemaglobin, human serum albumin, diphtheria antitoxin, and rhodamine-labelled proteins, all non-particulate materials, into *C. virginica*. He has found that all of these materials become pinocytized and are either digested intracellularly in leucocytes or are removed through epithelial layers. In the 1966 paper, he has reported that bacteria injected intracardially into *C. virginica* are quickly phagocytized and are eventually rendered non-viable by intracellular digestion. These studies have clearly demonstrated that oyster leucocytes are capable of the uptake, either by phagocytosis or pinocytosis, of foreign materials and eliminate them either intracellularly by digestion or by migration across certain epithelial borders.

In *Biomphalaria glabrata*, a pulmonate gastropod, Tripp (1961) has demonstrated that leucocytes can eliminate foreign particles introduced either by injection or implantation. Specifically, he has reported that yeast cells, polystyrene spheres, bacteria, chicken erythrocytes, carmine particles, and willow pollen become phagocytized and digested intracellularly if digestible, or are voided across epithelial layers. Some, however, are retained within tissue phagocytes.

Since *Crassostrea virginica*, a pelecypod, and *Biomphalaria glabrata*, a pulmonate gastropod, have been shown to possess highly efficient systems, in the form of phagocytic leucocytes, for removing foreign particles and molecules small enough to be phagocytized, it appeared to be of considerable interest to determine whether a prosobranch gastropod also possesses a comparable system. In order to test this, we have carried out parallel studies on the marine prosobranch *Littorina scabra*.

Materials and Methods

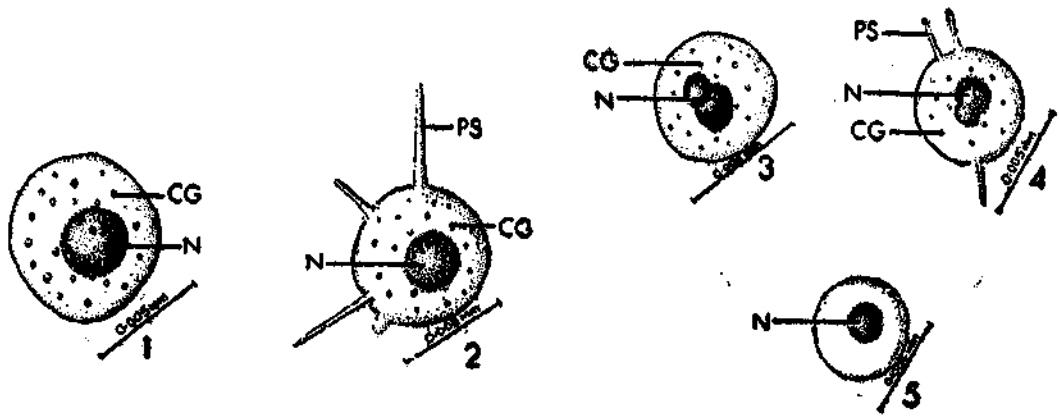
The specimens of *Littorina scabra* used during this study were all collected from concrete embankments at the Hawaii Institute of Marine Biology on Coconut Island (Mokuoloe Island) in Kaneohe Bay, Oahu, Hawaii, U.S.A. They were selected by size so that all of the specimens used measured between 17 and 20 mm. in length. After being brought into the laboratory, the snails were maintained at 21–22° C. in an aerated sea-water aquarium with a salinity of 32 ‰ until employed.

The India ink injected into each snail consisted of 0·1 c.c. of a 1 : 10 sea-water dilution of Higgins American India ink. The injection process was carried out in the following manner. Specimens actively crawling along the sides of the aquarium were removed, and while one person held its foot and operculum back so as to prevent withdrawal into the shell, another injected the ink suspension directly into the pedal sinus. Ten series, five in each series, were thus injected. After injection, the members of each series were maintained in a finger-bowl the bottom of which was covered with 5 mm. of filtered sea-water. After an initial period of severe contraction, all of the snails began to move around normally. All of the members of each series were fixed in 10% sea-water formalin at the same time interval post-injection. The 10 time intervals were 30 min., 1, 3, 6, 12, 24, 36, 96, 144 and 192 hrs. The shell and operculum of each snail were removed prior to fixation after which the specimens were embedded in high temperature (melting point 56° C.) paraffin, serially sectioned at 8 μ, and stained with Delafield's hematoxylin and counterstained with eosin.

In addition to the 10 experimental series, five uninjected snails of comparable size were identically fixed, sectioned, and stained as controls.

The morphology of the normal blood cells of *L. scabra* was also studied. This was accomplished by removing the shells from several uninjected snails and after cutting their soft parts in

several places, their hemolymph was allowed to drain into a container. Drops of the hemolymph were then examined under a coverglass with both light and phase-contrast microscopy.



FIGS. 1–5. Fig. 1. Large granular leucocyte of *Littorina scabra* with spherical nucleus. Nuclear details not shown. Fig. 2. Large granular leucocyte of *L. scabra* with fine pseudopodia. Nuclear details not shown. Fig. 3. Small granular leucocyte of *L. scabra* with characteristic bilobed nucleus. Nuclear details not shown. Fig. 4. Small granular leucocyte of *L. scabra* with fine pseudopodia and characteristic bilobed nucleus. Nuclear details not shown. Fig. 5. Small agranular leucocyte of *L. scabra* with spherical nucleus. Nuclear details not shown.

CG, cytoplasmic granule; N, nucleus; PS, pseudopodium.

RESULTS

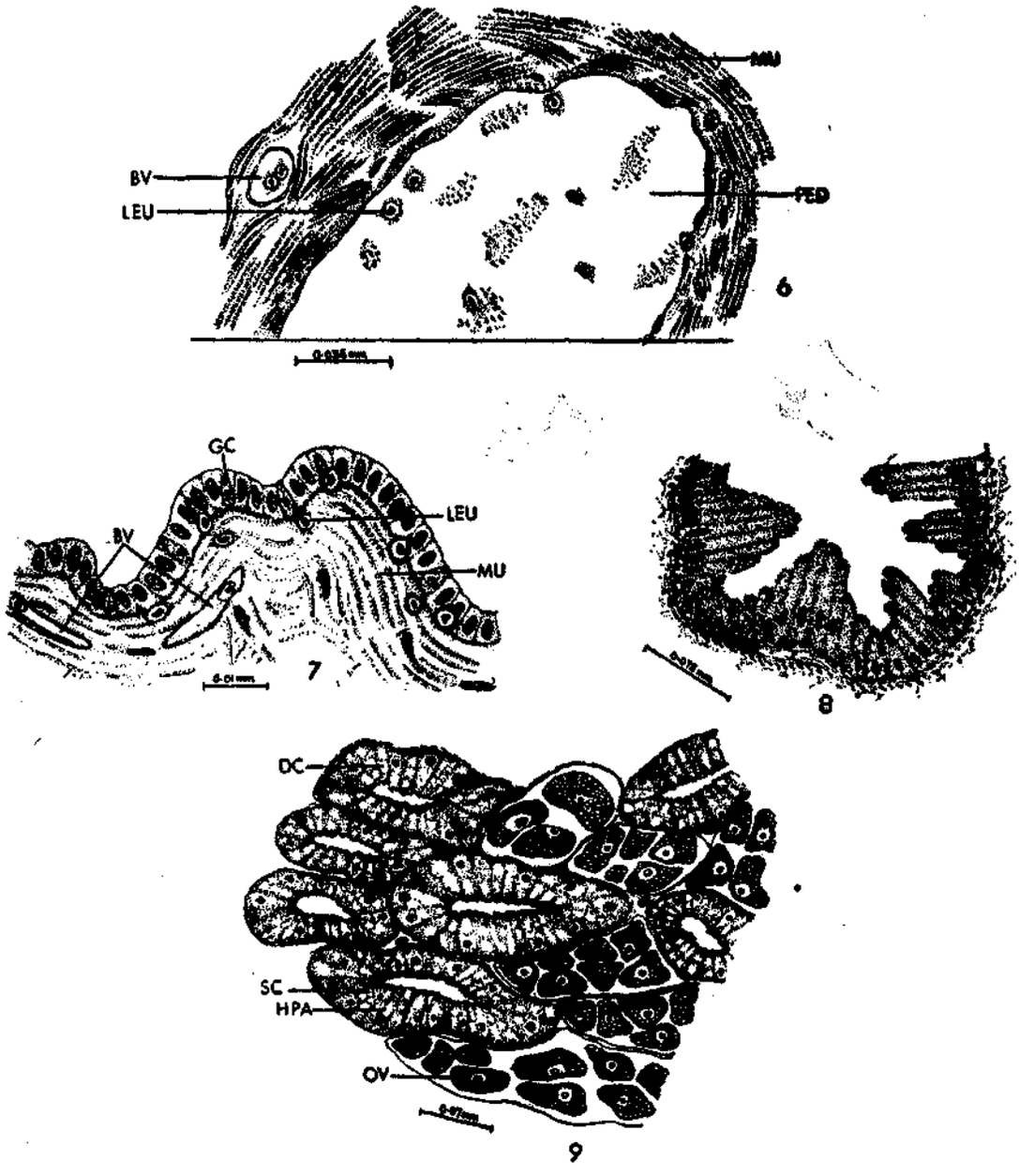
Normal Histology

It is not our intention to give a detailed account of the internal anatomy and histology of *Littorina scabra* although the major organs of this species were studied. The internal anatomy was very similar to that of *L. littorea* as given by Fretter and Graham (1962). However, brief histological descriptions of those organs and tissues now known to be involved in the temporary retention and ultimate elimination of ink particles are being given to facilitate presentation.

Leucocytes.—When examined under both the light and phase-contrast microscopes, three morphologically distinct types of leucocytes were recognized (Figs. 1–5). The first was a comparatively large spherical cell, measuring from 0·006 to 0·007 mm. in diameter. It included a nonlobate subspherical nucleus and coarse cytoplasmic granules (Fig. 1). These produced fine filamentous pseudopods when permitted to rest on glass slides for several minutes (Fig. 2). The second type of leucocyte was smaller, measuring from 0·004 to 0·005 mm. in diameter. It too was spherical but included a bilobed nucleus (Fig. 3). Its cytoplasm included fine granules. When permitted to remain on a glass slide for several minutes, this type of cell also produced fine filamentous pseudopods (Fig. 4). The third type of cell was the smallest. Each cell averaged 0·004 mm. in diameter and included a subspherical nucleus which was not lobed. This type of cell was readily distinguished from the second type in that its cytoplasm was devoid of granules (Fig. 5).

Pedal sinus.—The pedal sinus in *L. scabra* was a rather large cavity within the foot which was not lined with special cells but was boundered by myofibers of the foot (Fig. 6). Surrounding this sinus, cross-sections of several true blood vessels, lined with mesothelium, could be seen. Within the sinus, leucocytes were randomly scattered throughout. Furthermore, in some sections an amorphous eosinophilic film, which represented fixed plasma, could be seen within the sinus. In

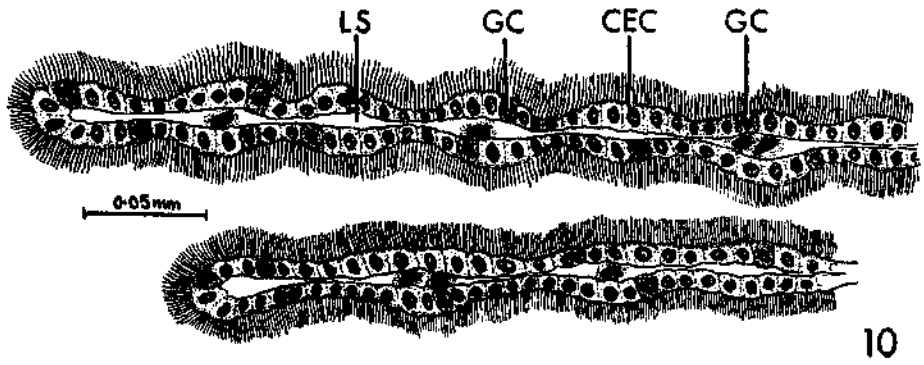
addition to the lumen of the pedal sinus, leucocytes were also found intermingled with the surrounding myofibers.



FIGS. 6–9. Fig. 6. Cross-section of portion of pedal sinus of *Littorina scabra* showing pedal muscles forming wall. Fig. 7. Section of dorsal foot surface of *L. scabra*. Fig. 8. Cross-section of intestine of *L. scabra*. Fig. 9. Section showing cross-sections of hepatopancreatic acini of *L. scabra* intermingled with ovarian lobes. BV, blood vessel; DC, digestive cell of hepatopancreas; GC, gland cell of foot surface; HPA, hepatopancreatic acinus; LEU, leucocyte; MU, muscle; OV, ovum; PED, pedal sinus; SC, secretory cell of hepatopancreas.

Foot surface.—Since the flat sole of the foot was not involved in the elimination of ink particles, it will not be described. The upper of dorsal surfaces of the foot, however, were involved.

The dorsal surfaces of the contractile foot were commonly folded. The acuteness of these folds was dependent upon the degree of contraction. In histological sections, the surface was covered by a layer of non-ciliated columnar epithelium (Fig. 7). Each of the cells measured from 0·005 to 0·006 mm. in height. Sparsely intermingled among the epithelial cells were a few goblet-shaped gland cells. The epithelial layer rested on a thin basement layer of connective tissue fibers. Beneath this layer were found myofibers and loosely arranged Leydig cells. Sections of blood vessels and sinuses were also present. Occasionally, free leucocytes were seen migrating in between the myofibers to the exterior through the surface epithelium.



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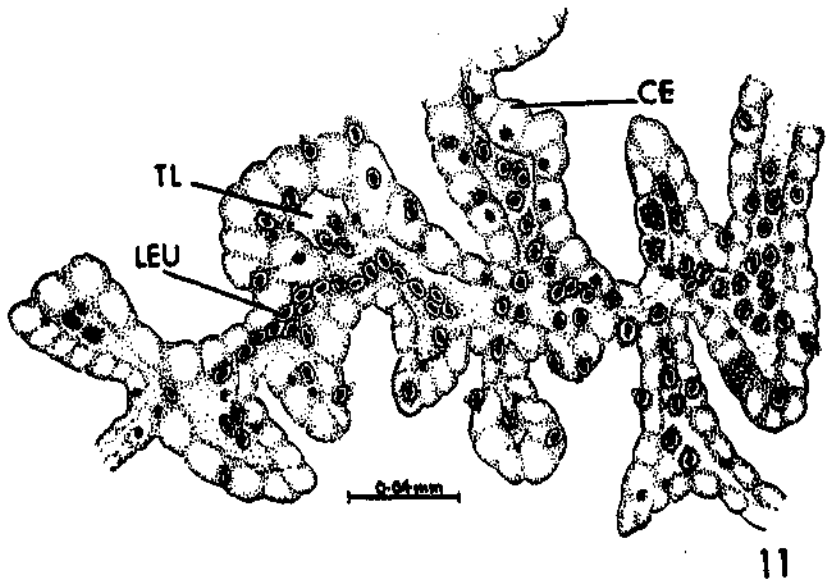

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FIGS. 10–11. Fig. 10. Drawing of two adjacent gill filaments of *Littorina scabra*. Fig. 11. Drawing of longitudinal section of nephridial tubule of *L. scabra*.

CE, covering epithelial cell of nephridial tubule; CEC, covering epithelial cell of gill filament; GC, gland cell of gill filament; LEU, leucocyte; LS, lumen or matrix of gill filament; TL, lumen of nephridial tubule.

Intestine.—The intestine of *L. scabra* was lined with a layer of thin, tall, and ciliated columnar epithelium (Fig. 8). The lumen was characteristically stellate resulting from the contraction of certain portions of the circumintestinal myofibers and also as the result of the irregular heights of the lining cells. The tallest cells were as long as 0·075 mm. while the shortest as low as 0·022 mm. The widths of the cells were rather consistent, measuring 0·004–0·006 mm. Surrounding the lining epithelium was a layer of basement membrane which separated the epithelium from the circumintestinal muscles.

Hepatopancreas.—The hepatopancreas of *L. scabra* was typical of gastropods. It was comprised of numerous tubular acini (Fig. 9). When observed in cross-section, at least two types of cells were recognized as comprising each acinus. The more abundant were the digestive cells. These columnar or wedge-shaped cells, each averaging 0·034 × 0·020 mm., were slightly lighter stained and included conspicuous vacuoles, some enclosing amorphous materials. The second type of cell, each measuring 0·040 × 0·026 mm., were the excretory (= secretory) cells. These were darker stained and included large, dark brown excretory granules in their cytoplasm.

In sexually mature snails, the gonadal lobes were intermingled among the hapatopancreatic acini (Fig. 9). The interacinar spaces of the hepatopancreas and the interlobular spaces of the gonads were occupied by blood vessels, sinuses, connective tissue elements, and free leucocytes.

Ctenidium.—Each ctenidal filament of *L. scabra* was comprised of a double layer of ciliated squamous to low cuboidal epithelium (Fig. 10). Each cuboidal cell averaged 0·007–0·010 × 0·003–0·006 mm. while the cilia measured 0·013 mm. long. The space between the two epithelial layers appeared empty except for occasional leucocytes.

Nephridium.—The kidney of *L. scabra* was readily appreciated as tubular folds projecting into the mantle cavity. Each fold was comprised of an irregular saccular extension lined with a single layer of cuboidal cells, each measuring 0·014 × 0·011 mm. (Fig. 11). Some of these cells enclosed minute light spherical bodies identified as excretory granules. The lumen of each tubular fold enclosed numerous free leucocytes, some of which were observed migrating into the mantle cavity from between lining epithelia.

Results of Experiments

30 *minutes.*—Examination of snails fixed at 30 min. post-injection revealed ink clots forming emboli in the pedal sinus and in the lumina of blood vessels in the vicinity. Furthermore, smaller clots were present between the myofibers (Fig. 12) and in between the Leydig cells present in the foot (Fig. 13). Associated with these clots were a number of leucocytes. This was particularly apparent in the pedal sinus and blood vessels where the blood cells were either intermingled with or surrounded the emboli. In addition, leucocytes were also associated with clots present in the foot (Fig. 14). Many of these cells enclosed phagocytized ink particles.

In addition to the tissues mentioned, ink particles not within leucocytes were observed between the hepatopancreatic acini and gonadal lobes. Some were also found on the periphery of the hepatopancreas, beneath of tunica propria (Fig. 15). The occurrence of free ink particles on the periphery of hepatopancreatic acinar cells was reflected in the secretory cells by the appearance of minute yellowish-brown spherules, each measuring 0·002 mm. in diameter. These spherules, although synthesized within hepatopancreatic secretory cells, were not limited to therein. Some were found in the interacinar spaces, in the vicinity of ink particles.

The sinuses at the bases of the gills (Fig. 16), as well as the spaces between the two epithelial layers of the gill proper, were filled with ink-laden leucocytes (Fig. 17). The surface epithelium of the gills appeared normal.

Relatively few free ink particles were found associated with the alimentary tract. On the other hand, ink particles within leucocytes were consistently present around the periphery of the stomach and intestine.

The nephridial tubes included a few ink-laden leucocytes. The lining cells appeared normal.

1 *hour.*—In specimens fixed one hour post-injection, emboli within the pedal sinus and associated blood vessels had dissolved. Numerous ink-laden leucocytes occurred in their place.

The ink particles found in the musculature and Leydig tissue in the foot were almost exclusively within leucocytes. Comparatively speaking, there was less ink at these sites than in the 30-minute series. For the first time, however, large numbers of ink-laden leucocytes were observed migrating through the epithelial layer lining the dorsal surfaces of the foot (Fig. 18).

Non-phagocytized ink particles could still be found in the interacinar and interlobular spaces in the region of the hepatopancreas and gonads. It is of interest to note that there was an increase in the number of yellowish-brown spherules in hepatopancreatic secretory cells and in the interacinar spaces, and an increase in the number of vacuoles in digestive cells. Furthermore, occasionally, a few ink particles were observed within the vacuoles of digestive cells. This, however, was not common. As the result of one fortuitous section, a single ink-laden leucocyte in the interacinar space was observed to be long and drawn out (Fig. 19). Its appearance was suggestive that it was in the process of dividing.

In the gills, ink-laden leucocytes were no longer confined to the spaces between the surface epithelium. Many were in the process of migrating to the exterior between the epithelial cells although these cells appeared normal (Fig. 20).

Ink-laden leucocytes, as well as free ink particles, were still present in relatively small numbers around the stomach amd intestine. The most dramatic appearance of ink-laden leucocytes occurred within the nephridial tubes. These spaces were completely packed although the lining cells appeared normal (Fig. 21).

3 *hours.*—By the third hour post-injection, all of the ink particles found within the pedal sinus were within leucocytes. Furthermore, most of the ink-laden leucocytes in the pedal sinus were found adhering to the muscle fibers lining the sinus (Fig. 22). Many were in the process of migrating into the muscular portion of the foot. Numerous ink-laden leucocytes also occurred in the pedal Leydig tissue. The appearance of the dorsal surfaces of the foot was identical to that in 1-hour specimens, *i.e.*, numerous ink-laden leucocytes were found in the process of migrating through the epithelial borders (Fig. 23).

Free as well as phagocytized ink particles still occurred in the interlobular and interacinar spaces of the gonads and hepatopancreas. Furthermore, yellowish-brown spherules of host origin were also present, both in secretory cells and intermingled among the extracellular ink particles (Fig. 24). The number of vacuoles in digestive cells remained increased.

The appearance of the gills was comparable to that in 1-hour specimens. Furthermore, the lining cells appeared normal. For the first time, ink-laden leucocytes were observed migrating into the intestinal lumen through in between the lining epithelium (Fig. 25). The appearance of the kidney tubules was equally as dramatic as in 1-hour specimens but the lining cells remained normal in appearance.

6 *hours.*—No free ink particles were found within the pedal sinus. What ink remained was within leucocytes. Most of the ink particles encountered in blood vessels were also within leucocytes, but some free particles were observed also.

Very few free ink particles were intermingled among the pedal myofibers and Leydig cells. Most were within leucocytes. As in 1-hour and 3-hour specimens, ink-laden leucocytes were observed migrating to the exterior through the epithelial lining of the dorsal surfaces of the foot (Fig. 26); however, their number was considerably smaller and as the result, the epithelial edge did not appear as a homogeneous black layer.

Free and leucocyte-enclosed particles, along with yellowish-brown spherules, were still present in the spaces surrounding hepatopancreatic acini and gonadal lobes. Ink-laden leucocytes occurred in the sinuses situated at the gill bases, in the gills, and were observed migrating to the exterior through the surface epithelium (Fig. 27). Some of these had become entrapped in the mucus secreted by the gland cells and adhered to the outer gill surfaces. It is of interest to note that the ctenidial epithelial cells were hypertrophied. Each cell averaged $0{\cdot}014 \times 0{\cdot}009$ mm. Ink-laden leucocytes continued to migrate into the intestinal lumen. The nephridial tubes were still packed with ink-laden leucocytes.

In addition to the structures mentioned, ink-laden leucocytes were observed intermingled with the connective tissue cells within the tentacles and in the mantle.

12 *hours.*— The distribution and appearance of both free and phagocytized ink particles in specimens fixed at 12 hours post-injection were essentially the same as those found in 6-hour specimens, including the occurrence of ink-laden leucocytes in the mantle and tentacles. Furthermore, the ctenidial epithelium continued to be hypertrophied with each cell averaging $0{\cdot}017 \times 0{\cdot}010$ mm. The only appreciable differences were (1) that very few ink particles remained in the pedal sinus and all of these were within phagocytes (Fig. 28); (2) yellowish-brown spherules of hepatopancreatic secretory cell origin were consistently found within blood vessels and sinuses throughout the body; and (3) the cells lining the nephridial tubules were hypertrophied, with each cell now averaging $0{\cdot}017 \times 0{\cdot}012$ mm. (Fig. 29).

24 *hours.*—By the 24th hour post-injection, although the distribution of ink-laden leucocytes remained essentially the same as in 6- and 12-hour specimens, the number of cells was recognizably less in the foot musculature, in the pedal Leydig tissue, migrating through the dorsal surfaces of the foot and alimentary wall, in blood vessel lumina, in the gills (Fig. 30), in the mantle and tentacles, and in the kidney tubules. The cells lining the ctenidial surfaces and nephridial tubules, however, still remained hypertrophied. Free ink particles still persisted in the spaces surrounding the hepatopancreatic acini and gonadal lobes.

As in 12-hour specimens, yellowish-brown spherules, originating in hepatopancreatic secretory cells, were found widely distributed in the circulatory system (Fig. 31).

36 *hours.*—The distribution pattern of ink-laden leucocytes in specimens fixed at 36 hours post-injection was again essentially the same as that in 6–, 12–, and 24–hour specimens although the number of ink-laden leucocytes was again reduced. No ink-laden cells, however, were observed in the tentacles, mantle, and pedal sinus. A few free ink particles were randomly distributed in various sinuses, especially in the spaces surrounding the hepatopancreas and gonads. Yellowish-brown globules, although less abundant, were still found distributed through the circulatory system and occasionally in tissues, and the ctenidial and nephridial cells still remained hypertrophied.

96 *hours.*—By the 96th hour post-injection, the amount of ink particles had become dramatically reduced. No particles existed in the pedal sinus and blood vessels. Moreover, none existed in the Leydig tissues and myofibers in the foot. A few ink-laden leucocytes, however, were observed in the process of migrating to the exterior through the dorsal surfaces of the foot. The gills and associated sinuses, as well as the alimentary wall, were devoid of phagocytes enclosing ink particles while the number of ink particles, enclosed in leucocytes, was greatly reduced in the nephridial tubules (Fig. 32). Furthermore, the nephridial cells had returned to their normal sizes and appearance.

The most conspicuous difference between 96-hour specimens and those fixed at earlier intervals was that no free ink particles existed in the interacinar and interlobular spaces of the hepatopancreas and gonads. The few particles present were within phagocytes. The hepatopancreatic secretory cells enclosed large numbers of brownish-yellow spherules, some measuring as large as 0·009–0·010 mm. in diameter.

144 *hours.*—No ink particles were present in specimens fixed at 144 hours. The hepatopancreatic secretory cells, however, still enclosed relatively large number of brownish spherules.

192 *hours.*—No ink particles occurred in specimens fixed at 192 hours. The various tissues of the body had returned to normal.

Discussion and Conclusions

From our observations, it is apparent that the prosobranch *Littorina scabra,* like the pelecypod *Crassostrea virginica* and the pulmonate gastropod *Biomphalaria glabrata,* is capable of removing particulate foreign materials, in this case India ink particles, experimentally introduced into its body. It has been demonstrated that specimens of L. *scabra* measuring between 17 and 20 mm. in length can rid themselves of 0·1 c.c. of 1 : 10 dilution of India ink injected into the pedal sinus by the 144th hour post-injection. The mechanism involves the phagocytic uptake of the ink particles and eventual migration of ink-laden leucocytes across certain epithelial borders to the exterior. As Stauber (1950) has shown, this is the same mechanism that occurs in *C. virginica,* and as Tripp (1961) has shown, it also occurs in *B. glabrata.* Although the principle is the same, certain differences in the epithelial borders involved are being noted. Stauber has reported that in *C. virginica* intracardially introduced ink particles are voided through the epithelial layers of the alimentary tract, digestive diverticula, palps, mantle, heart, and pericardium. Tripp has reported that in *B. glabrata* indigestible yeast cells are voided through the outer epithelium of the mantle and mantle collar, the inner surface of the mantle above the mantle cavity, the epithelium of the rectal ridge bordering the mantle cavity, the digestive gland (hepatopancreas), the rectum, and the epithelium subjacent to the columellar muscle. In *L. scabra,* the upper surfaces of the foot, the alimentary tract, particularly the intestinal wall, the gill surfaces, and especially the nephridia are the main exodus routes. The mantle and tentacles may be involved to a limited extent but this is not based on direct observation but the finding of ink-laden leucocytes in the connective tissues in the matrices of these structures in snails of the 6-, 12-, and 24-hour series. Then again, such leucocytes may have been carried to these sites in the circulation but are not voided to the exterior through the surfaces of these structures. The fact that only in one series of snails, the 1-hour series, were ink particles observed in hepatopancreatic cells renders it highly doubtful if this organ is an important one in *L. scabra* for the elimination of ink particles.

The dissemination of ink particles originally introduced into the pedal sinus to all parts of the body is undoubtedly accomplished primarily *via* the circulatory system and associated sinuses. For example, the finding of free ink particles in the interacinar spaces within the hepatopancreas can only be accounted for by their being transported to that site *via* the minute blood vessels that infiltrate the area. On the other hand, ink particles within leucocytes need not be transported in blood vessels exclusively. The finding of ink-laden leucocytes in Leydig tissues at various sites strongly suggests that such leucocytes are capable of extravascular migration. This, however, raises some doubt relative to Liebman's (1946) classification of leucocytes into two types: lymphoidocytes, which incorporate mainly excretory material, and trephocytes, which deal with the transport and release of nutritive material. The uptake of indigestible ink particles are without doubt by the same amoebocytes which phagocytize digestible particles such as vegetative bacteria, etc. Thus, to distinguish cells on the basis of the nature of the foreign materials handled, whether they be "excretory material" or "nutritive material" appears to be artificial.

It is of interest to note that the lumina of the nephridial tubules are major sites at which ink-laden leucocytes become concentrated. These spaces become packed with phagocytes by the first hour and are the last areas from which intracellular ink becomes cleared. Although some of the phagocytes have been seen migrating to the exterior between the cells lining the tubules, the relative rare occurrence of this phenomenon suggests that this is not the principal route for the exodus of such cells. Rather, most of the cells undoubtedly are emptied into the mantle cavity *via* the slit-like aperture which connects the terminal pouch into which the tubules empty with the mantle cavity. This is the normal excretory passage of the kidney (*see* Fretter and Graham, 1962). Thus, unlike in *C. virginica* and *B. glabrata*, the nephridia of *L. scabra* play a major role during the elimination of phagocytes.

Although what appears to be three morphologically distinct types of leucocytes occur in *L. scabra*, it has not been possible to distinguish in histological sections which one, or more, of these cell types are involved or are more active in the uptake of ink particles. Such information will have to await appropriate *in vitro* studies. Similarly, as to whether the three types of cells recognized really represent three terminal types or merely represent developmental or physiological stages cannot be ascertained by our experiments. A definitive answer must await other kinds of studies.

The appearance of yellowish-brown spherules in hepatopancreatic secretory cells in snails one hour post-injection strongly suggests that these spherules have been formed in response to the foreign material. Earlier, Cheng and Snyder (1962) have reported an increase of what have been termed "ferment cell granules" in the freshwater gastropod *Helisoma trivolvis* parasitized by the larvae of the trematode *Glypthelmins pennsylvaniensis*. In the same paper, these investigators have mentioned that Faust (1917), Agersborg (1924), and Hurst (1927) had also noticed similar granules in other parasitized gastropods. Morphologically, similar spherules have been also reported in pelecypods (*see* Cheng and Burton, 1965). The fact that such spherules have been found in the general circulation is somewhat puzzling. These may be globules of excretory material accumulated in secretory cells resulting from the digestion of some component of the ink, but since no secretory cells have been observed to be ruptured or lysed, thus permitting the escape of the spherules, it cannot be explained at this time how they have become incorporated in the hemolymph. At any rate, it appears to be quite definite that their formation within secretory cells has resulted from the introduction of ink as is the appearance of increased number of vacuoles in digestive cells.

It is also of interest to note that not all of the introduced ink particles are simultaneously phagocytized. In fact, as stated, non-phagocytized ink particles occur in the region of the gonads and hepatopancreas even in 36-hour specimens.

Another question which may be raised is: does the elimination of indigestible phagocytized particles *via* migration across epithelial borders constitute a specific characteristic of phagocytes enclosing such particles? It is difficult to conceive how this could be true. Rather, it is believed that molluscan leucocytes are being continuously lost to the exterior through epithelial borders among other methods, but the rate of exodus is dependent upon internal factors. That molluscan leucocytes without foreign materials do pass to the exterior by diapedesis is known (Orton, 1924; Yonge, 1928; Breder and Nigrelli, 1933). In the case of ink-laden leucocytes, the initial occurrence of leucocytosis in response to the introduction of ink, coupled with the fact that intracellular ink particles serve as readily visible markers, render the exodus much more evident. Furthermore, as Feng (1966) has shown, if sought for critically, phagocytes enclosing digestible bacteria can be observed migrating through epithelia. Thus, the process should not be considered characteristic of cells enclosing indigestible particles although the rate and number of cells being eliminated may be greater.

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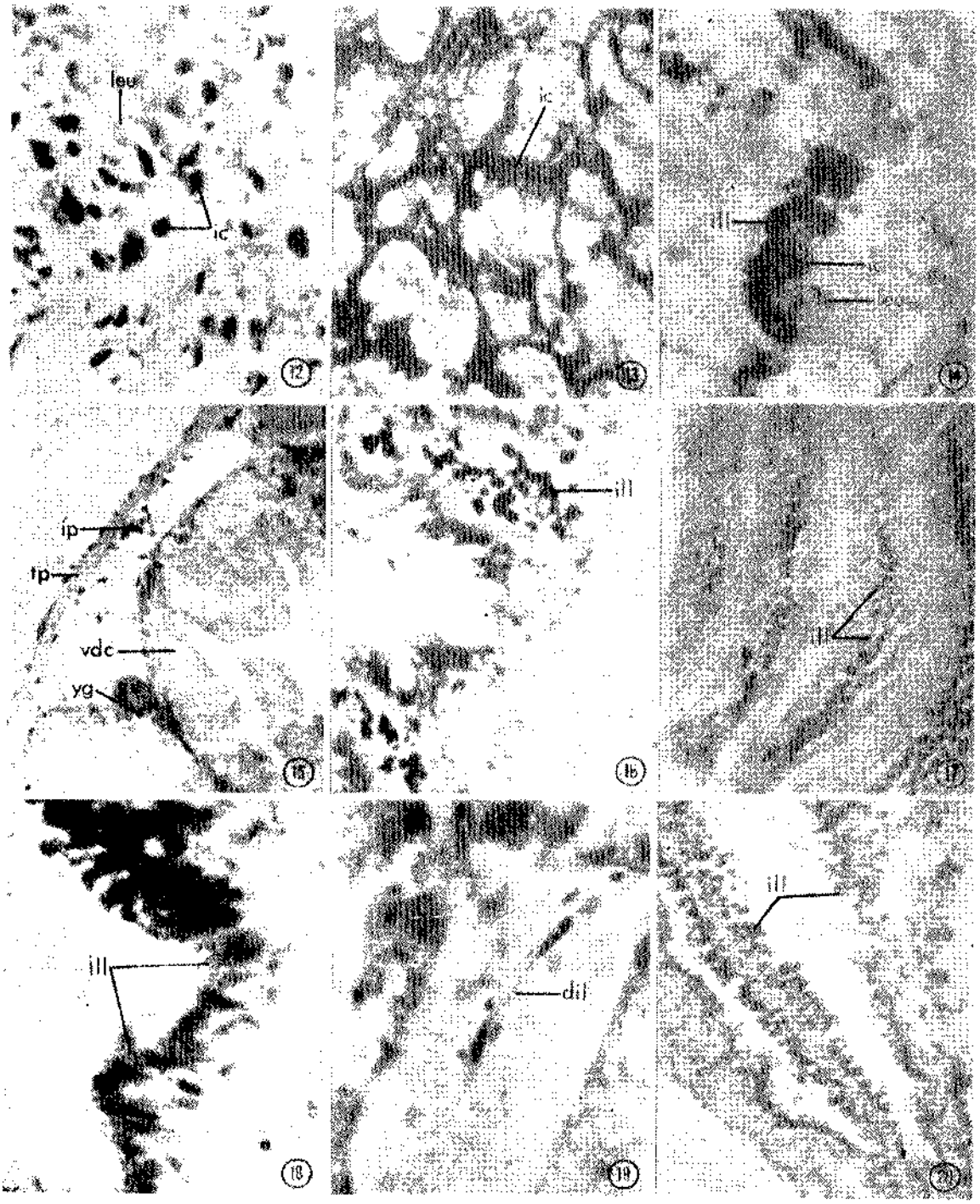
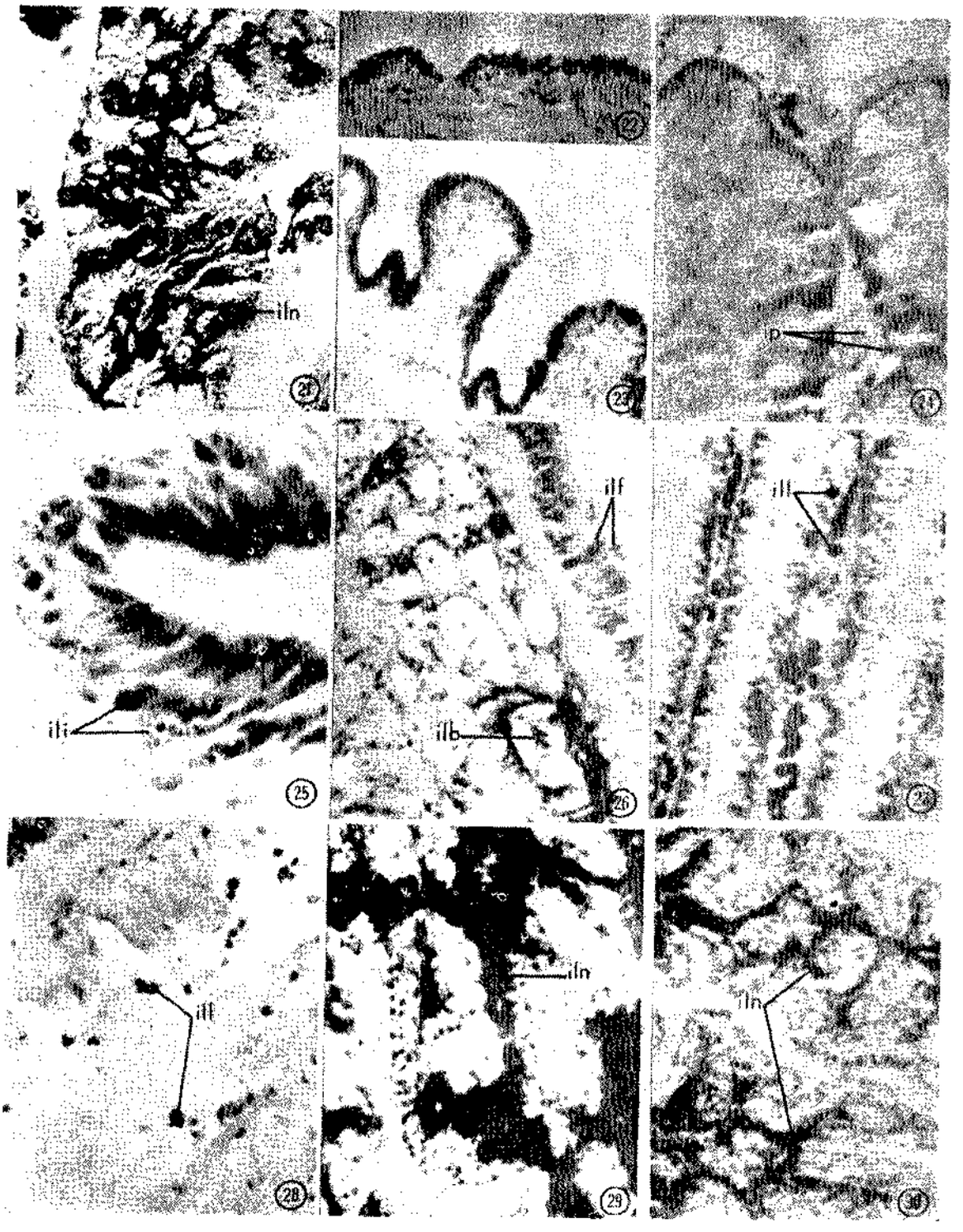
LEGEND TO PLATES

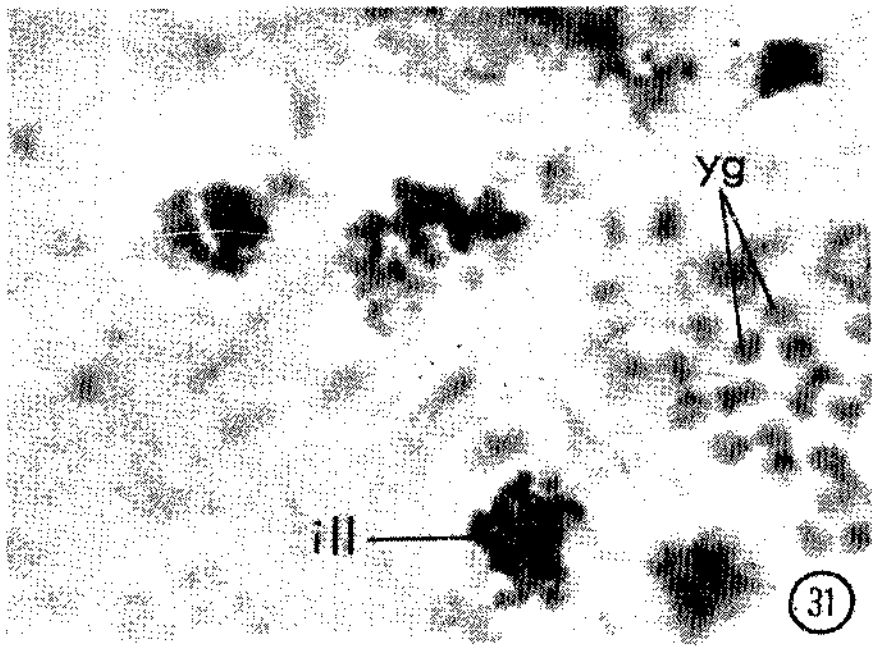
FIGS. 12–20. Fig. 12. Ink clots situated between myofibers in the foot (30 minutes after injection, 90×obj.). Fig. 13. Ink clots situated between Leydig cells in the foot. (30 minutes after injection, 90×obj.). Fig. 14. Leucocytes associated with ink clot situated between Leydig cells in the foot (30 minutes after injection, 90×obj.). Fig. 15. Free ink particles situated in interacinar space beneath tunica propria of hepatopancreas. Notice

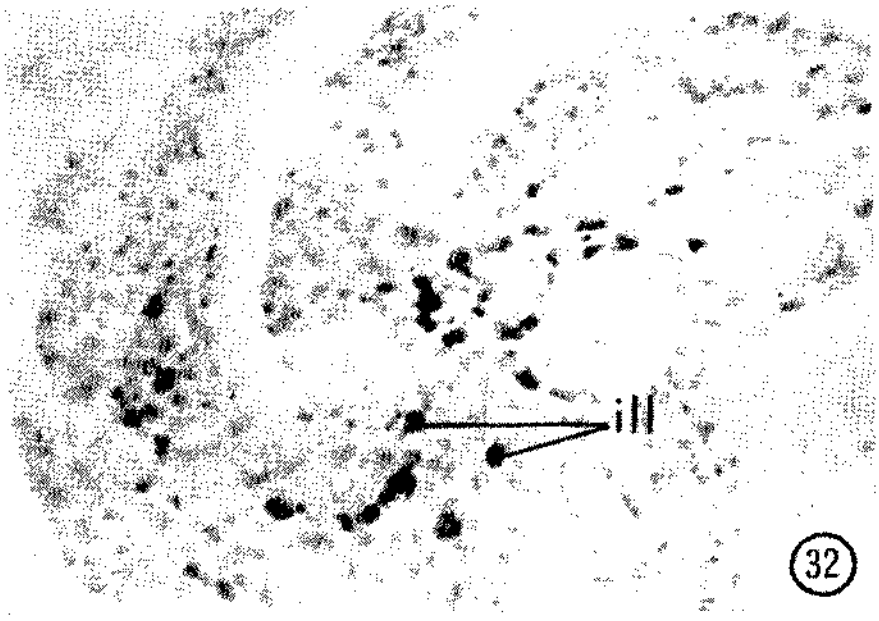


FIGS. 12–20



FIGS. 21–30





FIGS. 31-32

occurrence of yellowish-brown spherules in certain hepatopancreatic cells (30 minutes after injection, 40×obj.). Fig. 16. Ink-laden leucocytes in sinuses situated at the bases of gill filaments and in gill filaments (30 minutes after injection, 90×obj.). Fig. 17. Ink-laden leucocytes in gill filaments (30 minutes after injection, 40×obj.). Fig. 18. Ink-laden leucocytes migrating through epithelial lining of dorsal surface of foot (1 hour after injection, 90×obj.). Fig. 19. Ink-laden leucocyte situated in interacinar space within hepatopancreas in process of dividing (1 hour after injection, 90×obj.). Fig. 20. Ink-laden leucocytes in process of migrating to exterior from between surface epithelial cells of gill filaments. (1 hour after injection, 40×obj.) *dil*, dividing ink-laden leucocyte; *ic*, ink clots; *ill*, ink-laden leucocyte; *ip*, non-phagocytized ink particle; *leu*, leucocyte; *tp*, tunica propria of hepatopancreas; *vdc*, vacuole of digestive cell of hepatopancreas; *yg*, yellowish-brown spherule.

All photomicrographs are of sections of *Littorina scabra* injected with India ink.

FIGS. 21–30. Fig. 21. Ink-laden leucocytes filling lumen of nephridial tubule (1 hour after injection, 10×obj.). Fig. 22. Ink-laden leucocytes adhering to and migrating through myofibers lining pedal sinus (3 hours after injection, 40×obj.). Fig. 23. Ink-laden leucocytes along border of dorsal surface of foot (3 hours after injection, 40×obj.). Fig. 24. Non-phagocytized ink particles situated between hepatopancreatic acini. (3 hours after injection, 40×obj.). Fig. 25. Ink-laden leucocytes in the process of migrating through epithelial lining of intestine, (3 hours after injection, 90×obj.). Fig. 26. Ink-laden leucocytes migrating to exterior through surface epithelium of foot. Notice occurrence of ink-laden leucocytes in blood vessel (6 hours after injection 90×obj.). Fig. 27. Ink-laden leucocytes in lumen (matrix) and migrating through lining epithelim of gill filament (6 hours after injection, 40×obj.). Fig. 28. Ink-laden leucocytes in pedal sinus (12 hours after injection 40×obj.). Fig. 29. Ink-laden leucocytes filling lumen of nephridial tubule. Notice hypertrophied lining epithelium (12 hours after injection, 40×obj.). Fig. 30. Ink-laden leucocytes in lumen of nephridial tubule. Notice hyypertrophied epithelium (24 hours after injection, 40×obj.). *ilb*, ink-laden leucocytes in blood vessel; *ilf*, ink-laden leucocytes migrating through surface epithelium of foot; *ili*, ink-laden leucocytes migrating through intestinal epithelium; *ill*, ink-laden leucocytes; *iln*, ink-laden leucocytes in lumen of nephridial tubule; *ip*, non-phagocytized ink particles. All photomicrographs are of *Littorina scabra* injected with India ink.

FIGS. 31–32. Fig. 31. Ink-laden leucocytes and yellowish-brown spherules in lumen of blood vessel (24 hours after injection, 90× obj.). Fig. 32. Few ink-laden leucocytes remaining in lumen of nephridial tubule (96 hours after injection, 40× obj.). *ill*, ink-laden leucocytes; *yg*, yellowish-brown spherules.

HISTOCHEMICAL STUDIES OF MUCUS IN THE MANTLE OF TWO SPECIES OF VENERID CLAMS

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ABSTRACT

The results of a series of histochemical reactions designed to localize and analyze the mucosubstances in the mantle of the venerid clams, *Mercenaria mercenaria* and *M. campechiensis* revealed copious amounts of several different types of mucus in the mantle margin. Although the physiological roles of these substances are not as yet fully defined, the unusually large amounts of varied mucosubstances, and their locations within the mantle margin, indicate that they may be fulfilling several important functions.

There appears to be little difference in the types of mucus secreted between *M. mercenaria* and *M. campechiensis* but the amounts of material may vary both intra- and interspecifically from one environment to another.

INTRODUCTION

THE morphological complexity of the mantle margin of *Mercenaria mercenaria* has been described earlier (Hillman and Shuster, 1962, 1966; Hillman, 1964). The presence in the mantle margin of a prominent fourth fold filled with mucus appears thus far to be unique to *M. mercenaria* and *M. campechiensis*, although the fourth fold as a structure has been described for other species of venerids (*e.g.*, Oldfield, 1955; Ansell, 1961; Ockelmann, 1964: Selmer, 1967). It was this relatively large amount of mucus in the fourth fold which led us to initiate studies of mucosubstances in general in the mantle of the two species of *Mercenaria*.

The need for invertebrate mucus research was recently pointed out by Jakowska (1965) and Jeanloz (1966). The mucus-producing ability of mollusks is well known and this group has contributed greatly to what little is known about the secretion and function of invertebrate mucosubstances (*see, e.g.*, the review by Kent, 1964).

This report is intended as a general introduction to more specific studies of mucosubstances in venerid clams.

MATERIALS AND METHODS

Representatives of *M. mercenaria* from Duxbury Bay, Massachusetts and Daytona Beach, Florida and *M. campechiensis* from Daytona Beach were fixed for 48 hours in 10% formalin with 2% calcium acetate and 1% celytpyridinium chloride, following which the mantle were removed for routine paraffin embedding and transverse sectioning at 5 μ.

The following histochemical techniques for the localization and analysis of mucosubstances were utilized:

1. Alcian blue (AB) at pH 1·0 and 2·5 (Spicer, 1960). At pH 2·5 sialomucins and weakly acid sulfomucins stain blue; the most strongly acidic sulfomucins stain weakly or not at all. At pH 1·0 weakly and strongly acid sulfomucins stain blue selectively.

2. Periodic acid-Schiff (PAS) with diastase controls (Hotchkiss, 1948).
3. Alcian blue/PAS (AB/PAS) (Spicer, 1960). Neutral mucosubstances are colored magenta. Alcian blue, periodate unreactive acid mucosubstances stain blue. Alcian blue and periodate reactive materials color blue-purple.
4. High iron diamine/alcian blue (HID/ B) (Spicer, 1965). Sulfated mucosubstances stain black, sialomucins and hyaluronic acid stain blue.

Results

The mantle margin of the two species of quahogs is an extremely complex area, both histologically and histochemically. The results of the histochemical reactions for mucus in the mantle of *M. mercenaria* and *M. campechiensis* are summarized in Table I. The mucosecretory cells of the two species are virtually indistinguishable, however, the secretory areas seemed to vary somewhat in the amount of material secreted in that the second fold on *M. campechiensis* appeared to contain more PAS-positive material than *M. mercenaria*, and the fourth fold of *M. campechiensis* appears to be more prominent. This may be due to individual variation, to interspecific differences, or to some extent, environmental control.

TABLE I

Summary of histochemical reactions for mucus in the mantle edge of M. mercenaria *and* M. campechiensis

	First Fold	Second Fold		Third Fold		Fourth Fold				Secretory Ridge		
		Cell I	Cell II	Cell I	Cell II	Cell I	Cell II	Cell III	Cell IV	Cell I	Cell II	Cell III
AB pH 1·0	+++	++	−−	+++	−−	+++	++	−−	+	++	+	+
AB pH 2·5	+++	+++	−−	+++	−−	++	++	++	++	++	+	+
PAS	+	−−	+++	−−	+	−−	+	++	++	−−	tr	tr
D-PAS	+	−−	++	−−	tr	−−	+	tr	+	−−	tr	tr
AB-PAS	Purple	AB ++	PAS +++	AB ++	PAS +	+++	Purple	PAS +	Reddish-purple	AB ++	Purple	AB+to purple

Plus signs indicate degree of intensity of reaction. +++ = most intense. Minus signs indicate no reaction.

First Fold.—Mucus secretion in the first fold appears limited to flask-shaped connective tissue cells, the bases of which are relatively deep within the first fold. Mucus is transported to the outer surface of the mantle through neck-like extensions of these cells. The mucous material, while being strongly alcianophilic, is also PAS-positive and diastase resistant.

Second Fold.—From the various staining combinations, it is apparent that there are at least two different reactions for mucus taking place, indicative of two different types of secretory cells. One cell secretes a strongly alcianophilic, PAS-negative material. The degree of alcianophilia is greater at a pH 1 than at pH 2·5. A second type of cell, irregular in shape, is strongly PAS-positive, and does not stain at any pH with alcian blue. Following diastase digestion, the PAS-positive aspect of these cells is only slightly reduced.

Third Fold.—This fold is primarily a muscular fold, however, there are some mucus-containing cells which appear for the most part to be continuous with those cells in the second fold. Both cell types I and II as described for the second fold can be observed in the third fold.

Fourth Fold.—The unusually large amounts of mucosubstances in this fold have been described previously (*e.g.*, Hillman, 1964). Contrary to this earlier report, there is a considerable amount of alcianophilic material in the fourth fold. These results could be attributed to the differences between the Steedman (1950) technique and the modification of this technique used by Spicer (1960), or it may be due to the differences in reliability between the alcian blue 8 GS, used previously, and the alcian blue 8 GX employed in this study. At any rate, this fold is also histologically and histochemically very complex. Here again, there appears to be at least three different types of mucus being secreted. The first type of mucus is secreted by a relatively large goblet-like cell and is alcianophilic, more strongly at pH 1·0 than at pH 2·5. Frequently, morphologically similar cells can be seen, the contents of which are more alcianophilic at pH 2·5 than at a pH of 1·0. The material in these cells is also slightly PAS-positive and diastase-resistant. When subjected to a combination of both alcian blue and PAS, the resultant color is purple.

Less abundant are large irregular or flask-shaped cells which are PAS-positive and do not react with alcian blue. Following diastase digestion, they lose most of their reactivity with Schiff's solution. These cells, like the two types just described for the fourth fold, have a bulbous basal portion deep within the fold.

A fourth cell type, smaller and closer to the outer (leading) epithelium, secretes a material which is strongly alcianophilic at pH 2·5 and less so at pH 1·0. It is also PAS-positive and stains reddish-purple following the alcian blue-PAS combination.

Secretory Ridge.—The general histology of the secretory ridge, like the fourth fold, was described previously (Hillman, 1964). Mucosubstances are secreted in this area by three different cell types. A relatively large goblet cell secretes a PAS-negative, alcianophilic material at pH 1·0 and 2·5. A smaller cell secretes a substance which is also alcianophilic, although it does not stain as strongly as the material in the goblet cell.

The third cell type is a narrower cell secreting relatively large granules. The granules stain with alcian blue and occasionally weakly after the PAS reaction. Following the alcian blue-PAS sequence, the granules vary from solely alcian blue positive to purple.

There seems to be very little histological difference between *M. mercenaria* and *M. campechiensis*. In both species, the various cell types are in general juxtaposition within the general areas just described. The proportions of different mucosubstances within an area, *e.g.*, the fourth fold, may vary between species under a given environmental condition. These differences are difficult to quantify however. In addition, there is a considerable amount of individual variation between representatives of the same species under the same set of environmental parameters.

Discussion

The results of the variety of histochemical techniques applied to the mantle edges of the clams, *M. mercenaria* and *M. campechiensis* indicate that there are areas within the mantle in which relatively large amounts of mucus are secreted. These areas are for the most part quite complex in that different types of mucosubstances are secreted by different types of cells within a given area. The first fold area is an exception in that a single type of mucopolysaccharide is formed. This material appears to be of connective tissue origin and is rather strongly acidic as evidenced by the alcian blue staining. The fact that it is strongly alcianophilic at pH 1·0 indicates that it is a sulfated rather than a non-sulfated mucopolysaccharide. It is possible that, because of its location

in the shell-secreting portion of the mantle, it is associated in some way with calcification since acid mucopolysaccharides are generally found in calcifying tissues. However, such acid mucosaccharides are seldom PAS-positive in higher organisms, whereas the material found in the clam is. It may therefore represent an evolutionary step towards an acid mucosaccharide molecule aiding in calcification of bones and teeth.

Little can be said at this time about the function of the material in the second and third folds. The acidic mucosubstance responds to the histochemical reactions in a manner similar to heparin, and may be a heparin-like substance known to be secreted by some other species of clams (Love and Frommhagen, 1953). Its role in the clam is unknown, but it may function, as Love and Frommhagen (1953) suggest, in handling calcium in the mantle.

The function of the mucus in the fourth fold and secretory ridge area may be to aid in the cleansing of debris from the clam (Hillman, 1964). The variety of mucosubstances present, however, could suggest a more sophisticated role.

It is quite evident from the foregoing results that much more work needs to be done on the role of mucus in the quahogs and in invertebrates in general.

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PHYSIOLOGY OF CHROMATOPHORES IN CEPHALOPODA

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ABSTRACT

Rapid strides have been made in the last 20 years in several aspects of the physiology of chromatophores. The number of investigators interested in colour changes appears to be greater now than at any time in the past. Chromatophores of cephalopods are regulated by nerves, with endocrines as a secondary, slower mode of control. Although cephalopods show waves of colour passing from one end of the body to the other, they tend to maintain a degree of adaptation to the colour of the background on which they have been placed. The eyes are the major sense-organs sending stimuli to the colour change centres. Tactile stimulation, particularly of the suckers on the tentacles, can also initiate colour displays. Sereni (1930) found evidence of three chromatic centres in the nervous system, a general colour center, a motor center dominated by the former and an inhibitory center. Bozler (1928) and Sereni (1930) both reported that the muscle fibres of cephalopod chromatophores are dually innervated. The blood-borne substances operate primarily on the nervous centers and secondarily on the chromatophores themselves. Tyramine from the salivary glands causes darkening while betaine causes blanching in intact forms, primarily by stimulating the inhibitory center. Kahr (1959) observed that serotonin concentrates the chromatophoric pigments but acetylcholine is a potent chromatophore expanding agent.

THE ability to change colour through movements of pigments within certain integumentary cells or organs is widely distributed among animals. It has been observed for numerous cyclostomes, fishes, amphibians, and reptiles among the vertebrates; among the invertebrates it is exhibited by many higher crustaceans, cephalopods, and leeches and a few insects, echinoderms, and polychaetes.

The chromatophores of cephalopods comprise a central uninucleate cell filled with pigment and possessing a highly elastic cell membrane (Bozler, 1928). Radiating out from the central cell in the plane of the skin are from 6 to 20 or more uninucleate smooth-muscle fibres. All the fibres of a chromatophore usually contract simultaneously, stretching out the small, spherical, central pigmented cell into a disc having a diameter fifteen to twenty times that of the original sphere. The spherical form is restored by the elasticity of the membrane of the central pigmented cell after relaxation of the radiating fibres. A single nerve fibre is said to supply each muscle fibre (Hofmann, 1907); its terminal arborizations disperse broadly over the surface of the muscle.

Chromatophores in cephalopods are to be found as a rule much more abundantly on the dorsal than on the ventral aspects of these animals. Such colour organs occur in the derma of the skin, never in the epidermis. In the main, they are limited to this situation, but they are also associated in small numbers with some of the deeper organs as, for instance, the ink sac. In *Eledone* and *Octopus* they are small, but in *Sepiola, Argonauta,* and *Ommastrephes* they are so large that they may be seen even by the unaided eye.

Cephalopod chromatophores show considerable diversity in colour. In *Loligo* they may be brown, red, or yellow (Bozler, 1928). In *Sepia,* Kuhn and Heberdey (1929) have noted black, orange, and yellow colour cells. In both these instances, the dark chromatophores, either brown or black, are largest and the yellow ones smallest. Some of the older investigators have reported violet or even blue colour organs, but such have not been mentioned by recent workers. From the prevailing tints one would expect carotenoid substances to be present in these colour cells, but Lonnberg (1936) was unable to identify such materials in the chromatophores of *Eledone.*

In addition to chromatophores, many cephalopods possess iridophores. These are cells which contain glistening, often iridescent material which form sheets not only in the skin but on many internal organs. In the skin of *Sepia*, for instance, the iridophores occupy a position under the three types of chromatophores by which they are entirely hidden from view. Each iridophore consists of a flat, oval, nucleated cell containing twisted interlocking chains of platelets; these platelets are formed of reflecting material, guanine-like in character, by which an iridescence is conferred on its possessor. The iridophores are quite motionless during the normal colour changes of cephalopods. They are either exposed to view or cut-off from it by the action of the superjacent chromatophores.

Colour changes

The colour changes in cephalopods are commonly rapid and often sweep wave-like over the body of the animal so that at one moment a given part may be fully pale and another fully dark. These changes, therefore, are not easily followed in detail. When cephalopods are resting on a coloured background, they may be more constant in tint. Kuhn and Heberdey (1929) have recorded the states of the different chromatophores in *Sepia* on backgrounds of various colours. In green surroundings, the orange chromatophores are fully contracted. The yellow and black elements work together with the deep reflecting iridophores rather greenish in tone and produce a green coloration. In a yellow or red environment, the yellow and orange chromatophores are so completely expanded as to fill the spaces between the black ones. In consequence, the greenish tone of the reflecting iridophores is quite excluded. On white, grey, or black backgrounds, the red and yellow chromatophores are little in evidence and the darker or lighter grey of the animal is determined by the degree of expansion of the black chromatophores. In *Sepia*, tints of pure red or blue are not possible. Nevertheless, this cephalopod can adapt its tint well to the colour of its surroundings.

Control of chromatophores

Chromatophores of cephalopods are controlled primarily by nerves. Blood-borne substances have a secondary role. The eyes are the main sense-organs sending stimuli to the colour change centres. When one eye is removed, the entire animal continues to change colour but on the blinded side the changes are not as striking (Sereni, 1930 *a*). Complete blinding further reduces but does not abolish colour changes. Tactile stimulation, such as stimulation of the suckers on the tentacles, may result in a colour display. Postural changes, probably involving tactile and equilibrium centres, also contribute significantly to colour changes. For example, the chromatophores on the functional ventral surface are normally more concentrated than on the dorsal surface. Ventral illumination does not reverse this condition but turning an animal over will.

Sereni (1930 *a*) obtained evidence for the existence of three chromatic centres in the nervous system. One, situated in the supraoesophageal ganglia, is a motor centre and consists of two portions, one for each side of the body. These motor centres cannot substitute for each other. The second, a general colour centre in the central ganglia, controls the first one. The third is an inhibitory centre located in the cerebral ganglia. The general colour centre and inhibitory centre are also symmetrically placed, but each portion may act for the opposite side of the body as well as for its own. Evidence for excitatory and inhibitory fibres innervating each chromatophore has been presented by Sereni (1928, 1930 *a*). Bozler (1928) has also presented evidence for dual innervation of cephalopod chromatophores. However, he believed the nerve fibres were tonic and tetanic. The muscle fibres of the chromatophores appeared to contain two types of myofibrils, peripheral coarse ones and central fine ones. In 1931, Bozler reported these muscle cells can exhibit both tetanic and tonic contractions. Furthermore, the tetanic contractions appeared to be carried out by the peripheral coarse myofibrils and the tonic contractions by the thin central fibrils.

Substances that appear to be hormones operate primarily by influencing the nervous centres and secondarily by acting directly on the chromatophores. Tyramine has been found in the blood,

This material, produced by the posterior salivary glands, appears to cause darkening primarily by increasing the tonus of the motor centres (Sereni, 1929 *a*, *b*, *c*; 1930 *b*, *c*). Removal of the posterior salivary glands results in paler specimens and loss of chromatophore tone. Tyramine is more concentrated in the blood of dark species of *Octopus* (*e.g.*, *macropus*) than of light species (*O. vulgaris*). Furthermore, blood from *O. macropus* will darken *O. vulgaris*. However, blood from *O. vulgaris* will blanch *O. macropus*, suggesting the presence of a lightening substance. Betaine is the lightening substance found in cephalopods that appears to be involved in endocrine control of the chromatophores. This principle causes paling (decreases the chromatophore tone), presumably by stimulating the inhibitory centre. Evidence that tyramine and betaine may operate secondarily directly on the chromatophores comes from studies on portions of denervated skin. Both substances under such conditions cause chromatophore dispersion. The opposite actions of betaine, *i.e.*, blanching as a result of stimulation of the inhibitory centre in intact specimens and darkening by direct stimulation of isolated chromatophores, certainly deserves further consideration.

More recently, Erspamer and Asero (1953) found large quantities of serotonin in the posterior salivary glands of *O. vulgaris*. Smaller quantities were noted in the blood. Kahr (1959) attempted to determine the role of serotonin in the chromatics of the same cephalopod. He found that serotonin concentrated all of the chromatophoral pigments. He also reported that histamine had no effect on the chromatophores, but acetyl choline was a potent chromatophore-expanding agent whose action was antagonized by serotonin. The actions of these drugs were the same whether intact specimens or pieces of skin were used for the assays. The roles of histamine and acetyl choline described by Kahl were different from those reported by Sereni (1930 *b*, *c*) for the same animal. Sereni had stated that histamine caused darkening of intact specimens whereas acetyl choline caused them to blanch. Obviously, much remains to be learned about the control of colour changes in cephalopods, especially the role of blood-borne substances. Also, modern electrophysiological techniques should yield very interesting information concerning the dual innervation of these chromatophores.

Summary

The review is aimed to present the up to-date knowledge about the physiology of chromatophores in cephalopods. Chromatophores of cephalopods are regulated by nerves with endocrines as a secondary, slower mode of control. Although cephalopods show waves of colour passing from one end of the body to the other, they tend to maintain a degree of adaptation to the colour of the background on which they have been placed. The eyes are the major sense-organs sending stimuli to the colour change centres. Tactile stimuli, particularly of the suckers on the tentacles, can also initiate colour displays. Sereni found evidence of three chromatic centres in the nervous system, a general colour centre, a motor centre dominated by the former, and an inhibitory centre. Bozler and Sereni reported that muscle fibres of the chromatophores are dually innervated. The blood-borne substances operate primarily on the nervous system and secondarily on the chromatophores themselves. Tyramine from the salivary glands causes darkening while betaine causes blanching in intact animals, primarily by stimulating the inhibitory centre. Kahr observed that serotonin concentrates the chromatophores, but acetyl choline is a potent chromatophore expanding agent.

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STUDIES ON NEUROSECRETION IN MOLLUSCA

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ABSTRACT

In molluscs that have been studied so far, there are some indications of the presence of neurons with specialized staining properties that are possibly neurosecretory. However, there appears to be no stains specific for neurosecretion and certain inclusions in the cells can look like secretory products. Evidence of cyclical activity of neurosecretory cells is most frequently related to reproductive phenomena, but there are also instances of control of water balance and hibernation. The presence of neurohaemal organs within nerves and commissures has been described in several species of gastropods. Possible non-neural endocrine glands adjacent to the central ganglia have been described. In pulmonates granule-laden fibres are associated with the mediodorsal bodies.

THE discovery by Dahlgren (1914) of ' gland-like ' cells in the spinal cord of sharks was the earliest observation of secretory activity in nerve cells. These observations were greater confirmed by Speidel (1919) on skates. In addition to neurohumoral substances which function in the transmission of nervous impulses, certain neurons secrete long-range, long-acting agents. The latter, termed as neurosecretory substances, may act at some distance from the point of release and for relatively long periods of time. During the last 25 years Ernst and Berta Scharrer have accumulated an impressive body of evidence for the existence of neurosecretory cells in the nervous system of invertebrates and vertebrates (Scharrer and Scharrer, 1954).

The presence of neurosecretory cells in Mollusca was reported in some of the earliest investigations on neurosecretion in Invertebrata (B. Scharrer, 1935). In 1937, B. Scharrer described the neurosecretory cells in 20 species of molluscs belonging to the classes Polyplacophora, Gastropoda and Cephalopoda. Cells which were neurosecretory in nature could only be demonstrated by general histological methods in opisthobranch gastropods (*Aplysia, Pleurobranchaea, Tethys, Doris* and *Aeolis*). Our knowledge remained there for another 12 years. Since 1949, the presence of neurosecretory cells has been reported in a scaphopod (Gabe, 1949), polyplachophores (Martoja, 1967), in numerous gastropods (Gabe, 1951, 1953 *a, b*; Gorf, 1961; Herlant-Meewis and Van Mol, 1959; Krause, 1960; Lane, 1962; Lemche, 1955; Lever, 1957, 1958 *a*, 1958 *b*; Lever and Joosse, 1961; Pelluet and Lane, 1961; Sanchez, 1962; Nagabhushanam and Swarnamayye, 1963, 1964; Simpson *et al.*, 1966) and Lamellibranchs (Fahrmann, 1961; Gabe, 1955; Gabe and Rancurel, 1958; Lubet, 1955, 1956; Nagabhushanam, 1962, 1963, 1964). Actually, the Mollusca constitute one of the more highly developed branches of the animal kingdom in which our knowledge of neurosecretion is extremely poor. Most of the papers cited above give descriptions of neurosecretory cells, but in most cases the ultimate fate of the product has not been defined and there are very few valid indications of the functional significance of neurosecretion in Mollusca. The present paper gives a summary of what has been done so far on the morphology and physiology of neurosecretory system in molluscs.

I. MORPHOLOGY

Neurosecretory cells have been described in representatives of Scaphopoda, Polyplacophora, Gastropoda and Lamellibranchiata.

1. *Scaphopoda*

Of the four genera which form the class Scaphopoda, only *Dentalium entale* has been studied from the standpoint of neurosecretion (Gabe, 1949). Neurosecretory cells have been shown in the anterior buccal, cerebral and pleural ganglia. Each of the two anterior buccal ganglia contain a large pyriform cell measuring about 45 μ in its longest diameter; its nucleus is large and poorer in chromatin. The nucleolus is very large, centrally placed, markedly basophil and often shows some structure. The cytoplasm contains strongly acidophil oval masses of areolar structure; the hillock of large axon which runs from this perikaryon contains an appreciable number of acidophil granules; these granules are also found in the proximal part of the axonal path, but the secretory product disappears often after a fairly short course in the interior of the neuropile.

Small oval-shaped neurosecretory cells not exceeding 20 μ are seen in the cerebral and pleural ganglia. The cytoplasm, axon hillock and initial part of the axon path are rich in strongly acidophil secretory product which disappears from the axons, so that the ultimate destination of the processes of the neurosecretory perikaryons cannot be determined exactly.

2. *Polyplacophora*

The presence of neurosecretory cells in the buccal ganglia of three species of chitons, *Lepidochitona cinereus*, *Chiton olivaceus* and *Acanthochitona discrepans* has been reported for the first time by Martoja (1967).

3. *Gastropoda*

Prosobranchiata.—Gabe (1951, 1953 *a*) was able to demonstrate the presence of neurosecretory cells in 25 species of Monotocardia. Gabe (1954) also demonstrated the presence of secretory neurons in the ganglia of *Rhipidiglossa* and *Docoglossa* (Prosobranchiata: Diotocardia). Generally, the neurosecretory cells in prosobranchs are of small or medium size (greatest diameter 10–20 μ, rarely more). Their nuclei present no particular features, but the features of the cytoplasm render identification very easy. Ribonucleins are more poorly represented in these cells than in the ordinary neurons, and they sometimes contain a very acidophil product, most of which is dissolved by all alcoholic fixing agents but is very well preserved by aqueous fixatives. The affinity of the secretory product for eosin, acid fuchsin, and azocarmine explains why these elements are readily demonstrated by general histological methods. Iron haematoxylin likewise yields preparations in which neurosecretory cells are very distinct.

Just as in the case of the Scaphopoda, evidence of migration of neurosecretory product into the axons is rarely seen in the prosobranchs. In a large number of cases the secretory product is at first in the form of very fine granules, and these later fuse into more extensive pools the outlines of which become indefinite. However, there is clear indication of the migration of the neurosecretory product along the axons. Gabe (1953 *a*) noted in the abdominal ganglion of *Littorina* very rich neurosecretory cells; in the neuropile of this ganglion there are numerous moniliform, strongly acidophil tracts. However, this migration does not go beyond the limits of the neuropile, and no trace of acidophil secretory product can be detected in the communicating nerves or in the nerves which leave the ganglion.

With regard to the relative distribution of neurosecretory cells in the various ganglia, comparison of more primitive and more developed species reveals a progressive concentration of the neurosecretory cells in certain ganglia. In *Rhipidoglossa* and *Docoglossa*, all the ganglia and even the commencement of the pedal nerve cords contain cells with a fairly large quantity of acidophil secretory product in the cytoplasm. In *Stenoglossa* the cerebro-pleural ganglia and the supra-intestinal ganglion are the main sites of neurosecretory cells. In Heteropoda, the neurosecretory cells are located solely in the cerebral ganglion where they occupy a dorso-medial position. Their

morphological characteristics undergo considerable changes in the course of true secretory cycle. In *Firoloida*, Gabe (1951) reported that the secretory product first appears in the form of a few acidophil granules generally situated close to the nucleus. The granules increase in number and run together into a large homogeneous inclusion which pushes the nucleus towards the cell periphery. A ' signet ring ' cell is thus produced. At later stages in the secretory cycle the content of this ' pool ' loses its homogeneous appearance, becomes granular, dissolves steadily and the extensive homogeneous pool is replaced by a vacuole within which some cyanophil traces persists.

4. *Gastropoda*

Pulmonata.—Gabe (1954) described the neurosecretory cells in the nerve centres of 12 species of Pulmonata belonging to six genera: *Helix, Arion, Agriolimax, Oncidiella, Planorbis* and *Limnaea*. In all these cases the neurosecretory cells are very large neurons situated mainly in the cerebro-pleural ganglia and in the supra-intestinal ganglion. Their cytoplasm contains variable but often large quantities of a very acidophil secretory product, which retains its acidophilia despite permanganate oxidation of the sections so that only general histological methods are of any use in the examination of these perikaryons. The signs of migration in the axons are very indefinite and all traces of the acidophil product ends a short distance from the axon hillock, so that it is impossible to determine the ultimate fate of these fibres arising from the neurosecretory perikaryons. Lever (1957) describes the phenomena of secretion in the perikaryons of nerve centres in *Ferrissia* sp., according to this author, almost all the neurons in the central ganglia show signs of some form of ' glandular ' elaboration of substance. Lever consequently distinguished five types of secretory cells. In a subsequent paper the same author (Lever, 1958 *a*) describes a follicular gland situated in the lateral part of the cerebral ganglion close to the prominence formed by the ganglion where the nerves to the eyes and rhinophores leave. Lever *et al.* (1961) discovered the same structure in *Limnaea stagnalis* and were able to define the distribution of cells staining with Gomori's chrome-haematoxylin-phloxin (CHP) in the nerve centres of this species.

Neurosecretory cells have likewise been described in the Stylommatophora. Some paper, deal only with the morphology of the perikaryons and the migration of their secretory products whereas others discuss the possible relationship of neurosecretion to a follicular gland comparable to that of the Basommatophora, but which attrophies with the approach of sexual maturity.

Tuzet *et al.* (1957) reported the presence of large cells with cytoplasm containing granules which stain blue-black with Gomori's CHP in the tentacular ganglion of *Helix aspersa*. The secretory product leaves the cells, becomes scattered between the neurons and reaches the terminal distribution of the tentacular nerve, where it spreads out into pools. Herlant-Meewis and Van Mol (1959) described neurosecretory perikaryons in the postero-median region of the cellular cortex of the buccal ganglion in *Arion rufus* and *A. subfuscus*. Their perikaryons are of medium size with cytoplasm containing granules which stain with CHP after permanganate oxidation and with alcian blue. The neurosecretory product migrates along the axons. Van Mol (1960) reports the presence of quite large neurosecretory cells forming two dorsal caps in the mid-brain of the same species of *Arion*. The cytoplasm of these cells contains a secretory product the staining reactions of which are similar to those of the neurosecretory cells in the buccal ganglia. The products of neurosecretion can be traced along the nerves up to their terminations on the cerebral artery. Krause (1960) studied the neurosecretory cells of the perioesophageal ring of *Helix pomatia*. He suggests two types of secretory perikaryon: typical neurons (type I) and sac-like cells (type II) with a characteristic swelling opposite the cones of the axon; it is in this swelling that the neurosecretory product accumulates. According to Krause, there is no morphological evidence of migration of neurosecretion in the axons in *Helix*; it would appear probable that it is liberated into the neuroglial tissue from the entire surface of the cell. This tissue is rich in products which stain with CHP. This would mean that the product of neurosecretion leaves the nerve centres by travelling along neuroglia and not along axon. Nagabhushanam and Swarnamayye (1963, 1964) described two types of neurosecretory cells in the central nervous system of *Vaginulus* and *Ariophanta*,

They are designated as A and B cells. The type A-cells differ from B-cells in possessing polymorphic nuclei, several nucleoli and secretory material in the form of fine granules. In B-cells secretory material is in the form of large granules or colloids. Simpson *et al.* (1966) investigated for cytologic indications of neurosecretion by light and electron microscopy the central ganglia of the basommatophoran, *Helisoma tenue*. They observed fuchsinophilic neurons of possible neurosecretory significance which contained elementary granules, in the cerebral ganglia, the lateral lobes of the cerebral ganglia and the visceral ganglion. The structure of the follicle gland is not suggestive of an endocrine function. A new possible neurohaemal area is described: visceral fuchsinophilic cells apparently send processes to terminate on the anterior aorta and within the perineurium of the nervous intestinalis.

5. *Gastropoda*

Opisthobranchiata.—The first neurosecretory perikaryon to be discovered in Mollusca were in opisthobranchiates (Scharrer, 1935, 1937). Scharrer described two bilaterally symmetrical groups of nerve cells situated in the cellular cortex of the cerebral ganglion close to the dorsal surface in three species of *Aplysia*. The cytoplasm of the neurosecretory cells contains a considerable quantity of secretory products which is acidophil and siderophil when stained with iron haematoxylin. Cells of the same type are also present in the posterior visceral ganglia of *Aplysia*. Besides *Aplysia*, Scharrer described neurosecretory cells in *Pleurobranchaea*, *Tethys*, *Doris* and *Aeolis*. Gabe (1953 *b*) described neurosecretory cells in 25 genera of opisthobranchiates. There is considerable variation in the distribution of neurosecretory cells in various ganglia of different opisthobranchiates. This may be due to anatomical variability of the nerve centres in opisthobranchiate gastropods. The Nissl bodies of neurosecretory perikaryons in opisthobranchiates vary in position; in some species the basophil substance is marginal in the neurosecretory cells and in others the nucleus is surrounded by a strongly basophil cytoplasmic zone, the secretory products being, on the other hand, marginal. The secretory products may be present in the form of fine granules, lumps or pools. The signs of migration in the axons are more frequent and more striking than in prosobranchs extending up to central neuropile. In *Cylichna*, Lemche (1955) studied the distribution of the neurosecretory cells in the nerve centres using only preparations stained with Gomori's CHP.

6. *Lamellibranchiata*

Neurosecretory cells have been demonstrated in the nerve centres of 20 species of lamellibranchs (Gabe, 1955); their evolutionary cycle has been studied in detail in *Chlamys* and *Mytilus* (Lubet, 1955, 1956). Fahrmann (1961) described two types of neurosecretion in all the ganglia of the freshwater mussel, *Unio tumidus*, namely, grana I and grana II. The grana I has taken greyish colour with CHP while grana II stained dark blue with CHP. Both have reddish-violet colour with paraldehyde-fuchsin (PF) and strongly sudanophilic. Seasonal variations in secretion are found with grana I but not with grana II.

The cerebral and visceral ganglia of the oyster, *Crassostrea virginica*, possess two types of neurosecretory cells (Nagabhushanam, 1962). Both the cell types were recognized on the dorsal surface of the ganglia. *Cell type I*: The cytoplasm of these cells stains red with Mallory's and blue-black with CHP. These cells are somewhat pyriform in shape. The granules always appear as extremely fine particles. *Cell type II*: The cytoplasm stains weakly with CHP but strongly with Mallory's stain. The cell body is oval in shape and the secretory material appears as large colloidal droplets and in the perikaryon there are a number of vacuoles. In both the cell types axonal transport of secretory material was observed. A study of the developmental changes in the neurosecretory cells of the oysters, ranging from 10 mm. to about 100 mm. shell length was made. The nerve cells were small and uniform in appearance in the smallest specimen (10 mm. long). Each has a round nucleus and one nucleolus. The cytoplasm was very small and was indistinctly visible in usual preparations. The region where the neurosecretory cells would appear did not differ from the adjacent nervous tissue, and no sign of secretory activity was observed with

Mallory's stain. Granules were not detected in the neurons of most of the animals with a shell length of 11 to 15 mm. A cytochemical study of the secretory material in the Type I neurosecretory cells revealed the following features: the secretory substance is strongly sudanophilic; a positive PAS reaction may indicate the presence of polysaccharide. The Golgi substance of the neurosecretory cells which was demonstrated by Da Fano's method, was widely diverse from cell to cell (Nagabhushanam, 1963 *b*). Besides the oyster, the neurosecretory cells were observed in *Spisula* (Nagabhushanam, 1963 *c*), *Yoldia* (Nagabhushanam, 1963 *d*), *Branchiodontes* (Nagabhushanam, 1963 *e*), *Modiolus*, *Tegulus*, *Mulinia*, and *Diplothyra* (Nagabhushanam, 1964 *b*, *c*, *d*, *e*). *Modiolus*, *Branchiodontes* and *Yoldia* possess only one cell type while other bivalves investigated have two cell types similar to those described in the oyster. Antheunisse (1963) studied the role of neurosecretion in reproduction in the freshwater zebra mussel *Dreissena polymorpha*; the neurosecretory cells are present in the cerebropleural and visceroparietal ganglia. The author observed no relationship between secretory processes in the cerebropleural ganglia of *Dreissena* and the reproductive cycle. Baranyi and Salanki (1963) described three cell types, A, B and C, in the central nervous system of *Anodonta cygnea*; their distribution is similar in the three pairs of ganglia. Baranyi (1966) studied the alkaline and acid phosphatase activity in the central nervous system of *Anodonta* in connection with the periodical changes of neurosecretory activity.

7. *Cephalopoda*

It appears so far there is no description of authentic neurosecretory cells in this group. The two organs, *Corpus subpedunculatum* and *Corpus epistellare* are not actually neurosecretory in nature (Scharrer and Scharrer, 1954).

In conclusion, it may be mentioned that with the present knowledge, it is too early to establish homologies between these different cell categories described in Mollusca. Probably, a precise physiological data on the function of the different neurosecretory cell types will provide the necessary evidence in answer to this question.

II. Physiology

Neurosecretion has not been studied by the classical techniques of endocrinology. The neurosecretory cells are not sufficiently grouped together so that their selective removal cannot be contemplated, and the removal of the entire ganglia cannot provide very adequate indications of the function of the neurosecretory cells. There are also technical difficulties involved in surgical operations on molluscs. Some histophysiological findings on changes in the appearance of neurosecretory cells in the course of the normal evolutionary cycle of the animal or under special experimental conditions represent, then, the sum of the observations to be described here. Two physiological phenomena have been linked with neurosecretion in gastropods and lamellibranchs, namely, functioning of reproductive apparatus and osmoregulation.

Neurosecretion and reproductive cycle

Gastropoda.—Gabe (1935 *b*) observed in several opisthobranch snails some correlation between cerebral neurosecretion and reproduction. The first sign of neurosecretion appeared at the time of gametogenesis ; maximum accumulation of secretory products seen at the time of maturation of gonocytes and complete elimination of products at the time of spawning. Laviolette (1956) reported the secretion of a hormone by the gonads in the pulmonate, *Arion rufus* ; this gonadal hormone appears to play an important role in the maturation of the glands of the genital tract. However, he was unable to locate the precise cytological origin of the active substance.

In the pulmonate, *Limnaea stagnalis*, Hekstra and Lever (1960) made some interesting studies. After removal of the cerebral ganglia the animals were unable to copulate; also cerebral-

ectomized snails laid no eggs at all. On the other hand, extirpation of the left parietal ganglion had a stimulating effect on egg-laying. Pelluet and Lane (1961) noticed a relationship between neurosecretion and cell differentiation in the ovotestis of two slugs, *Arion subfuscus* and *A. ater*. The tentacles of the slugs were cut off and the animals maintained for different lengths of time before the ovotestis was examined. In both the species of slugs, the experimental animals showed a noticeable increase in the number of eggs as compared with the controls. Solutions of brain homogenate and tentacle homogenate were then injected separately into intact animals. The brain substance stimulated the production of eggs, while animals which received an injection of tentacular substance produced no increase in the number of eggs. Cytological study of the brain and tentacles reveals neurosecretory cells and a close association with the blood system of the animal, suggesting a basis for a hormonal system.

Lamellibranchiata

In *Mytilus edulis* and *Chlamys varia* the spawning corresponded to the period of evacuation of products from the cerebral neurosecretory cells (Lubet, 1955, 1956). Nagabhushanam (1963 *a*) working on the oyster, *Crassostrea virginica*, showed that the Type I neurosecretory cells of the central nervous system has a distinct annual cycle of secretory acivity. During October, November and December 1961 only a few cells contained granules as compared with the other months. The gonads of these oysters also showed a clear seasonal cycle; they were spent from October to December 1961. Nagabhushanam (1964 *a*) carried out operations on the oysters during their breeding season. Removal of the cerebral ganglia brought about immediate spawning in about 50% of the females and 22% of males while none of the controls responded.

Cephalopoda

The onset of sexual maturity in *Octopus* is determined by a secretion released into the blood stream by the optic glands; production of this secretion is regulated by an inhibitory nerve supply originating in the posterior part of the supra-oesophageal mass of the central nervous system (Wells and Wells, 1959). However, there is no evidence that these inhibitory nerves form part of a neurosecretory system (Wells, 1960).

A comparison of the neuroendocrine regulation of the reproductive cycle in the gastropods and lamellibranchs reveals that the cerebral neurosecretory material inhibits spawning in lamellibranchs while it stimulates egg-laying in gastropods.

Osmoregulation and neurosecretion

The influence of a 24-hour treatment with distilled water, tap-water or 0·05, 0·1, 0·2, 0·4 and 0·6% NaCl solutions on the neurosecretory cells of *Lymnaea* was studied (Lever *et al.*, 1961). Three cells in each lateral lobe of the cerebral ganglia lost their secretory material in the higher salt concentrations. Similar studies were conducted on two marine molluscs, *Patella* (Boddingius, 1960) and *Crassostrea* (Nagabhushanam, 1964 *a*). In both cases a change in the salt content of the medium brought about change in many neurosecretory cells.

Other probable functions of neurosecretion

Krause (1960) found accumulation of granules during hibernation period of *Helix* (winter) and discharge of the materials into blood at the end of hibernation.

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PHYSIOLOGY OF REPRODUCTION IN *CHITON GRANORADIATUS* LELOUP

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ABSTRACT

Observations were made on the spawning of *Chiton granoradiatus* in the laboratory at Waltair during June 1964 to July 1965. The animals spawned in the laboratory in the months of June and July 1964 and March to July 1965. Structure of the gonads and spawning behaviour were noted in both the sexes. Several factors appeared to control spawning in the laboratory. Association of both the sexes, temperature and salinity were strongest among these factors. The animals spawned in salinities between 32·60‰ and 35·53‰ and in temperature between 27° and 29·8° C. Animals were also observed to spawn at certain periods during daytime, *i.e.*, 10·45 A.M.–12·00 NOON. The possible mechanism of spawning at this time was not studied. There appears to be no lunar periodicity in spawning.

OBSERVATIONS on the spawning of chiton were made first by Clarke (1855), who worked on *Chiton cinereus* and found that a single female spawned in captivity. Later, Metcalf (1892) noticed that a single female each of *Chiton squamosus* and *C. marmoratus* spawned in the aquaria, while a number of males of both species shed their sperms profusely. Heath (1905) reported that *Ischnochiton magdalensis* seldom bred in captivity. Grave (1922) found that *Chaetopleura apiculata* spawned quite normally in the laboratory and made excellent observations on its spawning. He also correlated spawning in the laboratory with the phases of the moon. Studies on the reproductive cycle of chitons have drawn the attention of a group of workers from California (Giese *et al.*, 1959; Tucker and Giese, 1962; Giese and Araki, 1962; Nimitz and Giese, 1964) and a good deal of work has been done on this aspect.

There is no previous work concerning the reproduction of chitons from the Indian coast; and the present investigation was undertaken to study the physiology of spawning and the reproductive cycle of *Chiton granoradiatus*.

MATERIAL AND METHODS

Chitons were collected from Waltair point, Waltair, at low tide, during the period from June 1964 to July 1965. They were kept in the laboratory in enamel bowls holding 500 ml. of sea-water; sea-water was changed twice a day; small shingles with the encrusting algae were provided as the substratum which served as a feeding ground also. Fresh shingles were supplied as soon as the algal encrustation was eaten by chitons. Gonad maturity expressed in terms of gonad index was noted for each month for the entire period, following the method of Giese *et al.* (1959). Observations on spawning were made during the spawning season of 1965, during which time, spawning, whenever it took place in the laboratory, was noted along with the data on salinity, temperature and the time at which the animals spawned. Salinity and temperature of the sea-water in the laboratory were maintained as close to the values in nature as possible. Laboratory observations could not be checked against field observations because, the time of spawning in the laboratory did not coincide with the low tide periods fit for observation in the field. Spawning was also not noticed in the field at other times.

Spawning Behaviour

Spawning in the laboratory is usually initiated by males followed by females. The process of ejection is a combined effort of foot, mantle and gills. Prior to the release of genital products, the mantle is closely applied to the substratum, leaving a small gap at the posterior region through which the eggs or sperms escape. Contractions of the foot provide the necessary pressure for the ejection of gametes from the ripe gonads and the ciliary movements of the gills divert the genital products through the gap of the mantle.

Males remain flat on the substratum and seldom change their position during spawning. The milt is released in two white jets, very copious at the beginning and decreasing gradually. Male spawning duration varied from 20 to 35 minutes. Females assume a vertical position on the sides of the rock or the walls of the container and crawl about while laying the eggs. This forms an effective mechanism for the dispersal of benthic eggs. The eggs are released alternately from the two genital openings and remain independent, not forming strings as in *Ischnochiton magdalensis* (Heath, 1905) or enclosed in jelly. The egg is covered by spiny chorion the thickness of which varies from 45 μ to 50 μ. The diameter of the egg with the spiny chorion varies from 195 to 240 μ. A female lays 3,000 to 4,000 eggs at a time and spawns more than once during the breeding season. The duration of spawning of a female varies between 45 minutes and an hour.

Factors Controlling Spawning

Association

Association of one sex with the other is an essential factor for spawning in unisexual animals with external fertilization, to ensure minimum wastage of genital products. In several chitons, presence of males is necessary for spawning induction. Initiation of spawning by males was reported in *Chiton squamosus* and *C. marmoratus* by Metcalf (1892) and in *Ischnochiton magdalensis*, *I. mertensii* and *I. cooperi* by Heath (1905). However, Grave (1922) working on *Chaetopleura apiculata* stated that females and males spawned even when separated. In the case of *Chiton granoradiatus*, males usually initiated spawning and solitary females very rarely spawned.

Time of spawning

Chiton granoradiatus, commenced spawning in the laboratory between 10–45 A.M. and 12–00 NOON. Grave (1933) reported that *Chaetopleura apiculata* spawned in the laboratory between 7-30 P.M. and 10-30 P.M., but he also observed them spawning early in the morning during the peak of the breeding season. In the species under observation, the animals were not found spawning at any other time. Heath (1899) observed that in the field, *Ischnochiton magdalensis* spawned early in the morning on the days when the low tide prevailed at the same time. In their natural environment, *C. granoradiatus* was not observed to be spawning during the low tide periods, either in the morning or in the evening. Probably this might be due to the fact that these animals cease spawning at the least disturbance, without which it is impossible to reach them. Examination of water samples from the collection spot never revealed the presence of early developmental stages.

Salinity and Temperature

Spawning salinity and temperature varied within narrow limits, *i.e.*, 32·60‰–35·53‰, 27°–29·8° C. respectively. Incidentally, these are the highest ranges encountered during the summer months in the inshore waters of Walt[illegible]. An experiment concerning the independent influence of these two factors revealed that these ranges are optimal for spawning in the laboratory and are presumably so in the field also (Tables I and II).

TABLE I

Relationship between salinity and spawning in Chiton

(No. of animals in each grade = 10; Temperature = 29·3° C. ± 0·2° C.)

Salinity ‰	Set A No. of animals spawned		Set B No. of animals spawned	
	Males	Females	Males	Females
21·15	..	..	..	..
23·30	..	..	..	..
25·15	..	..	..	..
27·63	..	..	1	..
29·00	..	1	1	2
31·25	2	1	2	2
33·10	3	2	3	1
35·50	3	6	4	2

Set A were kept in different grades 1–2 hrs. prior to the spawning time.
Set B were acclimatized to each grade for 24 hrs. prior to spawning time.

TABLE II

Relationship between temperature and spawning in Chiton

(No. of animals in each grade = 10; Salinity = 35·5‰ ± 0·4‰)

Temperature ° C.	Set A No. of animals spawned		Set B No. of animals spawned	
	Males	Females	Males	Females
20·2	..	..	..	1
23·8	..	..	..	..
25·5	..	..	..	..
29·3	3	6	2	2
30·4	1	1	1	..
35·0	..	..	..	..
39·4	..	1	..	..

Other factors

Availability of food controlled spawning, when the animals were reared continually for about two months in the laboratory. In the initial stages, for about 2 or 3 weeks, they spawned irrespective of the food supply; but after one month, starving of the animals (by stopping the supply of their normal food and by keeping them in filtered sea-water) prevented spawning. When food supply was resumed, they spawned after one or two days. Delaying the renewal of sea-water was observed to delay spawning considerably. It was also noticed that animals kept in sea-water

containing organic matter in suspension spawned only after transferring them to clean, fresh sea-water.

Reproductive cycle

To determine the period the reproductive cycle, the gonad index method of Giese *et al.* (1959) was utilized. The gonad index was at its maximum in the month of June 1964, with a well-marked fall in the following month indicating the spawning as well as the spent animals. The gonad index was moderate in the month of October 1964, reaching a minimum in December 1964 and increasing gradually until May 1965. Thus, the data show two peaks of gonad maturity; a minor peak during October and major peak during May. Based on this data, *C. granoradiatus* may presume to be spawning in nature for two or three months following October 1964 and two or three months following May 1965 and hence, a semi-annual reproductive cycle.

Spawning in the laboratory was noticed during the months March–July, roughly coinciding with the major peak of gonad index, and spawning was not encountered at any other time during the period of investigation. In nature, spawning was not encountered at any time during the period of investigation. In the absence of direct observations of spawning in nature, establishment of the duration of the reproductive cycle, by spawning in the laboratory alone, may not be quite meaningful, though the conditions in the laboratory were kept as close to the natural conditions as possible.

SUMMARY

1. Observations were made on the spawning of *Chiton granoradiatus* in the Laboratory during June 1964 to July 1965. The animals spawned in the laboratory in the months of June and July 1964 and March to July 1965.

2. Several factors appeared to control spawning in the laboratory. Association of both the sexes, temperature and salinity were strongest among these factors. The animals spawned in salinities between 32·60‰ and 35·53‰ and in temperature between 27° and 29·8° C.

3. Animals were also observed to spawn at certain periods during daytime, *i.e.*, 10-45 A.M. to 12-00 NOON. The possible mechanism of spawning at this time was not studied.

4. The reproductive cycle, as measured by gonad index, is approximately of six months' duration. Laboratory spawning was noticed during the summer months. Spawning in the field was not observed at any time during the period of observation.

ACKNOWLEDGEMENT

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HAEMOGLOBIN OF MARINE BIVALVES *SCAPHARCA DEYROLLEI* (JOUSSEAUME, 1893) AND *CARDITA ANTIQUATA* LAMARCK

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ABSTRACT

This paper reports the occurrence of iron-linked respiratory protein Haemoglobin in an arcid clam *Scapharca deyrollei* and in a false cockle *Cardita antiquata* from the Bombay waters. In *Scapharca* the pigment occurs in erythrocytes, whereas in *Cardita* it is extracellular.

The spectrophotometric studies of the two oxyhaemoglobins and certain of their derivatives—haemoglobin, carboxyhaemoglobin and nitric oxide haemoglobin—show characteristic two-banded spectra, similar to corresponding derivatives prepared from the blood of other species. However, attempt to prepare a typical haemoglobin (methaemoglobin) from *Scapharca* blood failed, although the derivative from the blood of *Cardita* could be successfully prepared. This preparation shows a characteristic methaemoglobin spectrum with maximum absorption peaks at 500 and 630 mμ, similar to methaemoglobin preparation from the mammaliam blood.

Similarity in the prosthetic group is evidenced from the identity of the absorption spectra of haemin, acid porphyrin and pyridine haemochrome prepared from the two pigments and from the haemoglobins of other invertebrates and mammals.

INTRODUCTION

IRON-linked respiratory protein ' Haemoglobin ' has been found in many species of protozoa, annelida, crustacea, insecta and echinoderms (Vinogradov, 1953; Nicol, 1960). In these organisms haemoglobin occurs either in plasma, or in non-amoeboid corpuscles or in tissues. During our studies on the blood pigment of invertebrates, we observed erythrocytic blood in arcid clam *Scapharca deyrollei* (Jousseaume, 1893) and extra-cellular pigment in a heterodont false cockle *Cardita antiquata* Lam. from Bombay waters. The molecular weight of erythrocytic haemoglobin of arcid clam (*Anadara inflata*) is reported to be about 73,000 which is similar to vertebrate haemoglobin (Yagi *et al.*, 1957), whereas that of extracellular pigment of *Cardita floridana*, like many species of annaelida is of the order 3×10^6 (Manwell, 1963). Further *Cardita* lacks byssus and burrows partly in sand, whereas *Scapharca* is found attached to boulders and pier piles by means of byssus. Thus the two species showed marked divergence both in their habitat and in occurrence of blood pigments. In view of this, and since no attempts have been made to study these pigments from the species from Indian water, it was felt desirable to study the spectrophotometric differences between two haemoproteins, if any, and compare with the similar pigment found in other groups.

COLLECTION AND PURIFICATION OF HAEMOGLOBIN

The arcid clam *Scapharca* were collected from the Mahim Creek, and *Cardita* from the Chawpatty sands, Bombay. In *Scapharca*, like other species of genus *Anadara* and *Glyceymeridae* the haemoglobin was observed in cells; whereas in *Cardita* the pigment was found in plasma. Earlier Grieshbach (1891) reported the pigment in cells in *Cardita aculeata*. Manwell (1963), however, found the pigment in haemocoelic fluid of *Cardita floridana*; as observed during present studies on *Cardita antiquata* Lam.

Prior to bleeding the clams were washed thoroughly and kept in running sea-water for 2–3 hours. This conditioning was desired to remove trapped mud and foreign bodies to avoid possible contamination of the pigment. Blood was withdrawn from the extra-pallial sinus by hypodermic syringe and pooled. In case of *Scapharca* blood cells were washed with 0·9% NaCl solution, then taken in distilled water and haemolyzed using toluene. After centrifugation both the pigments were purified either by fractional precipitation with ammonium sulphate or by absorption on calcium phosphate gel. The gel was prepared according to Keilin and Hartree (1938).

In the initial stages the chromoproteins were purified by fractional precipitation with ammonium sulphate. During this procedure, however, it became obvious that precipitation using ammonium sulphate caused considerable change in the colour of the chromoproteins. Freshly withdrawn blood was bright red in colour and showed characteristic absorption spectrum of oxyhaemoglobin. On purification with ammonium sulphate, the pigment in M/15 phosphate buffer (pH 7·0), however, appeared considerably brown with occasional disappearance of both the peaks. The blood of *Cardita* was affected almost invariably and the product showed a peak at 630 mμ. This on further treatment with a pinch of Solid $Na_2S_2O_4$ changed its colour to cherry red and showed a characteristic band of reduced haemoglobin. Earlier, Patel and Spencer (1963) observed that the blood of *Arenicola marina* when purified by ammonium sulphate showed considerable browning and that it could not be reduced. They called the product of the reaction ' brown form '. In case of the blood of bivalves, however, especially the brown product of *Cardita* O_2Hb obtained either on purification with ammonium sulphate or on oxidation with potassium ferricyanide showed a peak at 630 mμ, and further this could be reduced back to haemoglobin. This then suggests that the brown product of bivalve haemoglobin was due to oxidation of Fe^{++} to Fe^{+++}, and that no denaturation of the protein had occurred as suspected in *Arenicola marina* haemoglobin (Patel and Spencer, 1963).

An alternative method of the purification by absorption on calcium phosphate gel was, therefore, tried. After adding the gel (1 ml. per 10 ml. blood) to the whole blood, the suspension was centrifuged and the pigment was eluted in M/15 phosphate buffer (pH 7·0). To the supernatant further quantity of gel was added and the procedure repeated till the supernatant became almost colourless. Repetition of this procedure provided a series of fractions. *Scapharca* blood thus yielded six fractions, whereas extra cellular haemoglobin of *Cardita* was rather unstable and yielded only three fractions. The ratio of optical density per 1 ml. cell at 540–42 and at 280 mμ for each fraction was calculated and expressed as ' Index of purification '. The browning or oxidation of pigment showed decrease in the absorption at 540–42 mμ and an increase at 510 mμ. The ratio of O.D. per 1 cm. cell at 510 and at 540–42 mμ for each fraction was, therefore, calculated and expressed as ' Index of Oxidation (browning)'. Table I records the purification and browning (Oxidation) indices of various fractions of oxyhaemoglobin of *Scapharca* and *Cardita*. It will be seen that fractions 2, 3 and 4 of *Scapharca* blood and fraction 2 of *Cardita* blood were the most pure and contained less of brown form. Fractions 2 and 3 were, therefore, pooled and used for further studies.

The oxy-haemoglobins, prosthetic groups and their derivatives were prepared as described by us earlier (Patel and Spencer, 1963; Patel and Patel, 1964). The absorption spectra were measured using either a Unicam SP 600 or Beckman DU-2 spectrophotometer. One centimeter cuvettes with reagent blank or distilled water as reference solutions were used.

OXY-HAEMOGLOBIN

The oxy-haemoglobins of *Cardita* and *Scapharca* show the characteristic absorption curves with a α peak at 576 and 578 mμ; β peak at 542 and 544 mμ and a Soret band at 409–10 and 416 mμ respectively (Table II, Fig. 1) and a band due to protein between 280 and 282 mμ. Though the peaks show slight shift, the ratios of $\alpha : \beta$; α : minimum, and β : minimum, were practically the same and agree well with those reported for the oxy-haemoglobins of other species (Table II).

TABLE I

Purification of oxy-haemoglobin of Scapharca *and* Cardita *by calcium phosphate gel*

	Scapharca							*Cardita*			
	Whole blood	Fraction I	II	III	IV	V	VI	Whole blood	Fraction I	II	III
Purification Index O.D. 540–41 : O.D. 280–82	0·288	0·244	0·364	0·323	0·333	0·279	0·210	0·188	0·200	0·215	0·197
Browning (Oxidation) Index O.D. 510: O.D. 540–42	0·551	0·679	0·532	0·497	0·640	0·672	0·756	0·739	0·624	0·634	0·674

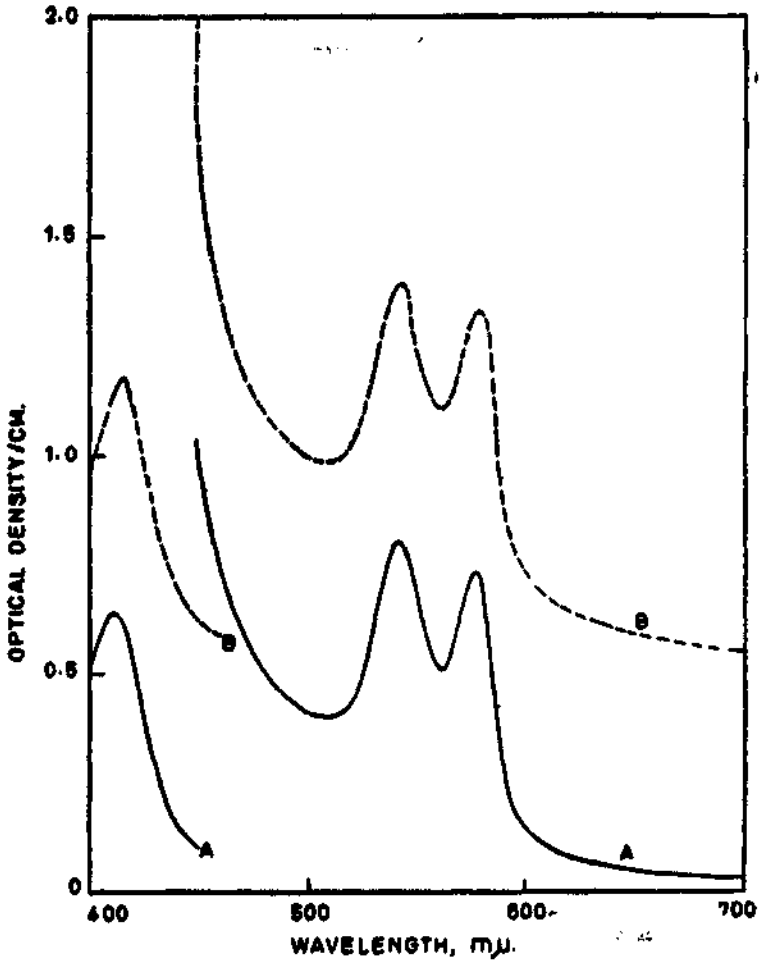

FIG. 1. Absorption Spectra of Oxy-haemoglobin (in M/15 Phosphate buffer pH 7·0) of (A) *Cardita* and (B) *Scapharca*. For measurement in Soret region solutions were conveniently diluted.

The close similarity in ratios of optical densities at various wavelengths suggests that oxy-haemoglobins of both the species of clams though not related exhibit a typical curve of same order and magnitude irrespective of the location of pigment either intracellular or extracellular.

Haemoglobin

Table III records the position of the absorption maxima of reduced haemoglobin (reduced by an addition of Na-dithionite). Both the de-oxygenated haemoglobins show a single broad absorption peak between 558 and 562 mμ, and a Soret peak at 428–30 mμ. These were very similar to those obtained for reduced haemoglobins of other species. Vlés (1923) and Kobayashi (1936), on the other hand, reported two banded curve for reduced haemoglobin of polychaete worms *Arenicola piscatorum, Marphysa sanguinea, Pheretima communis* and *Pheretima hilgendorfi.* However, haemoglobins of worms *Arenicola marina* (Patel and Spencer, 1963), *Marphysa sanguinea* (Chew *et al.*, 1965) and *Marphysa mossambica* (Patel and Patel—unpublished) and arcid clams *Anadara rhombea* and *A. granosa* (Patel and Patel, 1964) do not show the second peak. The second peak observed by Vlés (1923) and Kobayashi (1936) may well be due to impurities and the influence of reducing agent used. The shift in absorption peak of reduced haemoglobin of *Arenicola marina* has been explained by Patel and Spencer (1963) in terms of reducing agent used and the method of purification.

Table II

Positions of absorption maxima (mμ) of oxyhaemoglobin of Cardita antiquata, Scapharca deyrolleti, *and other species*

Species	Alpha	Beta	Gamma (soret)	Minimum	Alpha/ Beta	Alpha/ min.	Beta/ min.
Cardita antiquata ..	576	542	409–10	560–62	0·935	1·33	1·57
Scapharca deyrollei ..	578	544	416	562	0·935	1·34	1·45
Marphysa sanguinea	576	540	414	..	..	..	..
Anadara rhombea ..	576	540–42	412	562	0·96	1·40	1·50
Anadara granosa ..	574–76	540–42	412	562	0·95	1·39	1·46
Arenicola marina ..	574	538	412–15	560	0·95	1·63	1·67
OX ..	574	540	411–12	560	0·97	1·45	1·50

Carboxyhaemoglobin

The oxy-haemoglobins of *Cardita* and *Scapharca* when treated with carbon monoxide formed a typical carboxyhaemoglobin. Table IV shows the absorption maxima of two CoHbs, which show close similarity to those reported for the corresponding derivatives from the blood of various species. The ratios of α : minimum, and β : minimum, also indicate that the preparation exhibit typical curves of same nature for both the species, though the peaks show slight shifts.

Nitricoxide Haemoglobin

The two oxy-haemoglobins readily formed nitricoxide haemoglobin similar in spectral behaviour to those reported for various species (Table V, Fig. 2). The spectra follow the pattern of O_2 Hb spectrum, but for the peaks were broad and minima not well defined.

TABLE III

Absorption maxima (mμ) of reduced haemoglobin (reduced with $Na_2S_2O_4$) of Cardita antiquata, Scapharca deyrollei, *and other species*

Species	Alpha	Gamma (Soret)	Minimum	Alpha/min.
Cardita antiquata ..	562	428–30	490	2·26
Scapharca deyrollei ..	558	430	488	2·96
Marphysa sanguinea ..	553	428	..	..
Anadara rhombea ..	556	428	490	2·46
Anadara granosa ..	556	428	490	3·30
Arenicola marina ..	560	427–30	480	2·70
OX ..	555	..	490	2·68

TABLE IV

Positions of absorption maxima (mμ) of carboxyhaemoglobin of Cardita antiquata, Scapharca deyrollei, *and other species*

Species	Alpha	Beta	Soret (Gamma)	Minimum	Alpha/min.	Beta-min.
Cardita antiquata ..	572–74	540	418	562	1·29	1·67
Scapharca deyrollei ..	570–72	537	419	560	1·099	1·224
Marphysa sanguinea	570	537	417	..	..	..
Anadara rhombea ..	570–72	540	414–16	558–60	1·10	1·240
Anadara granosa ..	572	540–42	418	558–60	1·15	1·230
Arenicola marina ..	568	538	..	555–58	1·13	1·270
OX ..	565	537·38	..	555	1·11	1·20

TABLE V

Absorption maxima (mμ) of nitric oxide haemoglobin of Cardita antiquata, Scapharca deyrollei, *and other species*

Species	Alpha	Beta	Soret	Minimum	Alpha/min.	Beta/min.
Cardita antiquata ..	570–74	544	416	564	1·03	1·12
Scapharca deyrollei ..	570	542	415	566	1·02	1·06
Anadara rhombea ..	572	546–48	416	564–66	1·01	1·06
Anadara granosa ..	570	544–46	416	564	1·006	1·05
Arenicola marina ..	572	540	..	560	1·04	1·07
OX ..	570	542	..	..	..	..

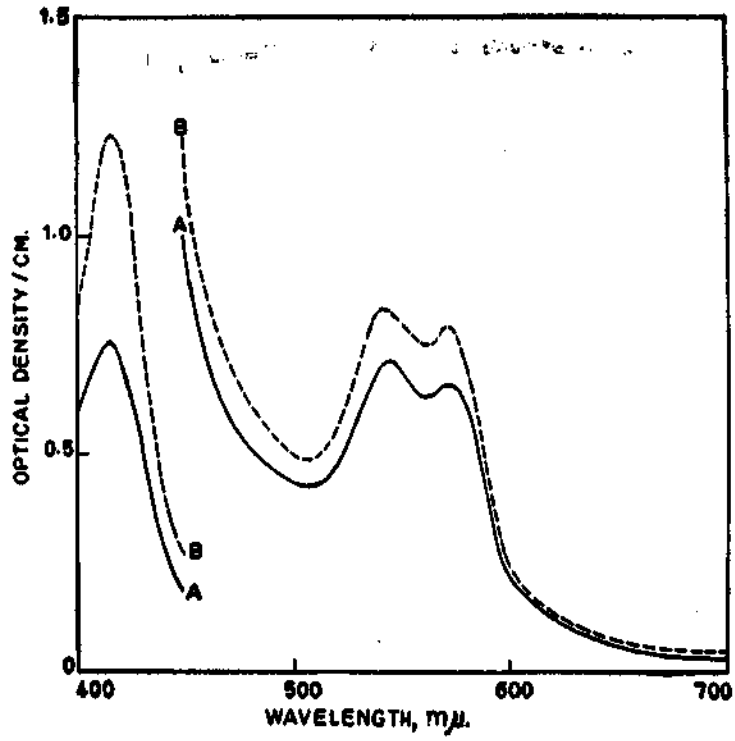

FIG. 2. Absorption spectra of nitric oxide haemoglobin of (A) *Cardita* and (B) *Scapharca*.

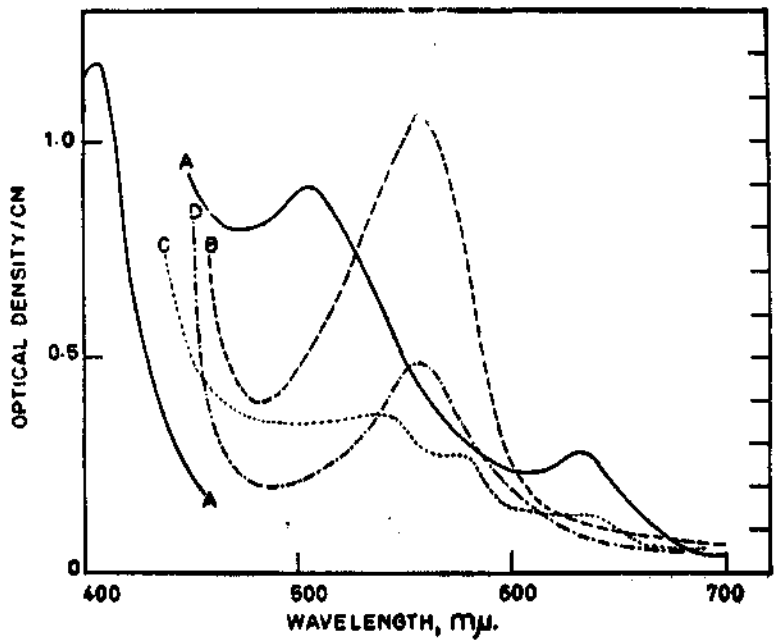

FIG. 3. Absorption spectra of haemiglobin-haemoglobin (A, B) of *Cardita* and (C, D) of *Scapharca*.

HAEMIGLOBIN-HAEMOGLOBIN

The oxy-haemoglobin of *Cardita antiquata* when treated with potassium ferricyanide changed its colour from bright red to brown and formed a typical haemiglobin (methaemoglobin) with maximum absorption at 500 and 630 mμ (Fig. 3). On reduction with Na-dithionite the product changed its colour from brown to cherry red, both the bands disappeared and a single broad band with a peak at 556–560 mμ appeared (Fig. 3). The brown form thus obtained seemed to be an oxidative product obtained as result of oxidation of haemoglobin to haemiglobin. The oxy-haemoglobin of *Scapharca*, on the other hand, did not form a typical haemiglobin, though its colour changed from red to brown. However, there was some indication of greater absorption in region between 620 and 630 mμ (Fig. 3) similar to brown form prepared from the blood of *Anadara rhombea* and *A. granosa* (Patel and Patel, 1964).

CHOLEGLOBIN

The mechanism of choleglobin formation necessitates the formation of a typical haemiglobin which could be reduced to haemoglobin (Patel and Spencer, 1963). Since *Cardita* haemoglobin

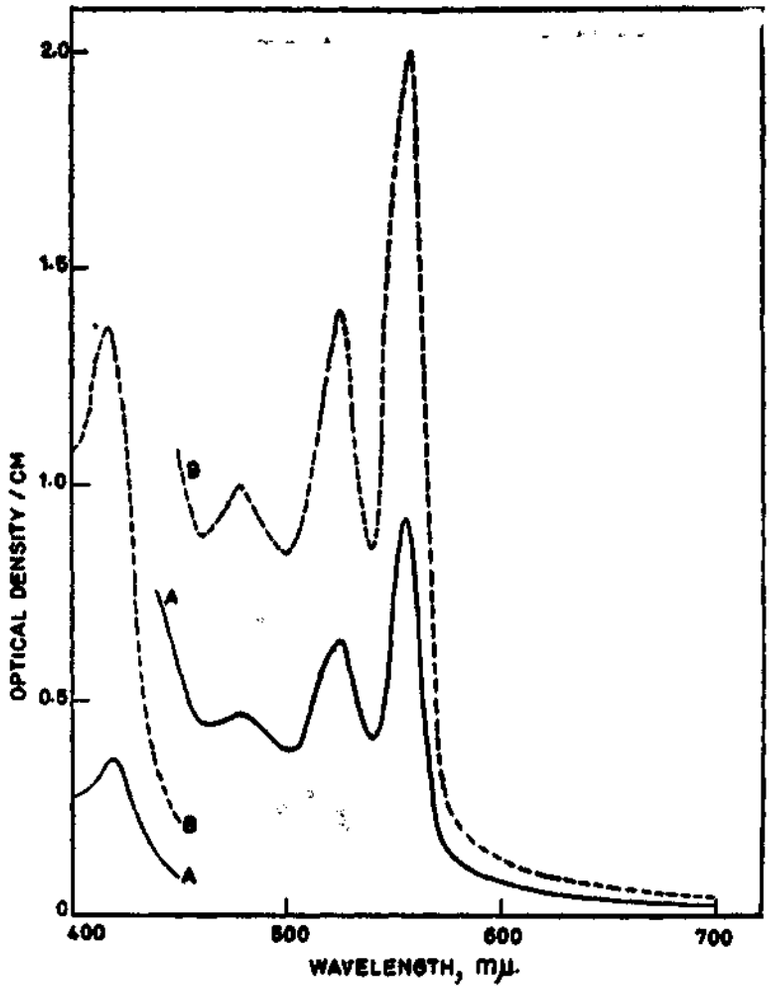

FIG. 4. Adsorption spectra of pyridine haemocrome of (A) *Cardita* and (B) *Scapharca*.

formed a typical haemiglobin, an attempt was made to form a choleglobin from *Cardita* haemoglobin. Haemiglobin was treated with a small quantity of Na-dithionite followed by a pinch of ascorbic acid and a few drops of 3% hydrogen peroxide. The colour of the product changed from brown to cherry red and then to green. The absorption spectrum, however, did not show a choleglobin band at 618–20 mμ, though there was some indication of higher absorption in this region.

Prosthetic Group

The prosthetic group was separated by treatment with acid acetone. The haemin in acid acetone from two haemoproteins show similarity in absorption curves and the ratios of optical densities at various wavelengths (Table VI). This similarity also extends to the corresponding derivatives from the blood of other species, and further show that the same type of haemin is linked with various haemoproteins.

Table VI

Absorption maxima (mμ) of acid porphyrin and haemin of Cardita antiquata, Scapharca deyrollei, *and other species*

Species	Acid Porphyrin			Haemin in acid Acetone					
	Alpha	Beta	Soret	*a*	*b*	*c*	soret	*a/b*	*b/c*
Cardita antiquata ..	598	558	409	508	540	640	380	1·02	2·11
Scapharca deyrollei	600	557	409	510	540	638	381–82	1·028	2·09
Anadara rhombea ..	599	557	408	510	538–40	638	383	1·02	1·80
Anadara granosa ..	600	556	409	510	538	638–40	382–83	1·01	1·90
Arenicola marina ..	598	557	407	510	538–40	638-40	381–83	1·02	1·82
OX ..	..	..	..	508–510	538	638	..	1·00	..

The pyridine haemochrome prepared from *Cardita* and *Scapharca* haemins also show identical spectra (Fig. 4, Table VII). It is interesting to note that both the haemochromes show an additional band with a peak at 480 mμ, similar to those reported for *Anadara rhombea* and *A. granosa* (Patel and Patel, 1964), *Arenicola marina* (Patel and Spencer, 1963), *Marphysa sanguinea* (Chew *et al.*, 1965), *Marphysa mossambica* (unpublished) and *Busycon canaliculatum* (Person *et al.*, 1962) haemoproteins.

Table VII

Absorption maxima (mμ) of pyridine haemochrome of Cardita antiquata, Scapharca deyrollei, *and other species*

Species	Alpha	Beta	Additional Peak	Gamma (soret)
Cardita antiquata ..	556–58	524	480	418–19
Scapharca deyrollei ..	557	524	478–80	417
Marphysa sanguinea ..	557	525	480	..
Anadara rhombea ..	554	522	480	418
Anadara granosa ..	554	522	476–80	418–19
Arenicola marina ..	555	522–24	480	417
Busycon canaliculatum ..	554–55	524–25	480	417

The acid porphyrin derivatives prepared from the blood of two species also show similar curves. The absorption maxima further show close similarity to those prepared from other species (Table VI).

Haemin crystals prepared by Teichmann's and Nippe's techniques (Hawlk *et al.*, 1954) from the two oxy-haemoglobins agree well in crystalline form with those prepared from other species (Pyrega, 1912; Kobayashi, 1936; Sato, 1931; Patel and Patel, 1964).

It will be seen from the results presented that the haemoglobins of two unrelated species of bivalves *Cardita* and *Scapharca* irrespective of location—extracellular *vs.* intracellular and high *vs.* low molecular weight show similar spectral characteristics. The absorption maxima of both the haemoglobins and derivatives, however, show slight changes, but not significant enough to elucidate species specificity. Earlier workers, on the contrary, held the view that shifts in peaks show the species specificity. The same order of ratios of optical densities observed at various wavelengths further indicate that various haemoglobins follow the similar spectral pattern. The shift in peaks may very well be due to impurities, temperature effect and experimental errors. Similarly, Manwell (1963) also reported that in spite of intracellular *vs.* extracellular location, and the presence of positive *vs.* negative haeme-haeme interactions, the haemoglobins of an arcid clam *Noetia ponderosa* and heterodont cockle *Cardita floridana* are quite similar in general non-sigmoid shape of the oxygen equilibrium curve, in moderately low oxygen affinity and in the absence of a Bhor effect.

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RESISTANCE TO DESICCATION AND OXYGEN DEBT IN WEDGE CLAMS

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ABSTRACT

The wedge clams, *Donax cuneatus* Linnaeus and *Donax faba* Gmelin acclimated to about 30° C. and exposed to air, indicated that at room temperature (average 30° C) *Donax faba* have a longer survival time (94 hours) than *D. cuneatus* (69 hours) as suggested by their 50% survival time. Survival of *D. cuneatus* exposed to air was studied at two lower temperatures (17 and 12° C) as well. While the survival of *D. cuneatus* at 17° C does not appear to be markedly different from that at room temperature, at 12° C the 50% survival time of *D. cuneatus* was considerably reduced (29 hours).

The amount of mantle cavity fluid in both *D. cuneatus* and *D. faba* exposed to air declined gradually with increase in the duration of exposure. The relative amount of the mantle cavity fluid was considerably higher in *D. faba* than in *D. cuneatus*.

Oxygen debt estimated from measurements of oxygen consumption of animals exposed to air upto 48 hours, indicated that in *D. cuneatus* and *D. faba* the post-exposure oxygen consumption increased 3·3 and 1·7 times their pre-exposure levels respectively. It is possible that *D. faba* has better anaerobic abilities with more capacity to withstand desiccation which may explain the longer survival time of *D. faba* than that of *D. cuneatus* when exposed to air under the conditions described.

INTRODUCTION

THE capacity of certain intertidal molluscs to withstand extreme conditions such as prolonged exposure and extremes of temperature has been investigated by many workers (*see* Newell, 1964). However, systematic studies on such aspects on molluscs occurring along the Indian coasts are few. *Donax cuneatus* Linnaeus and *Donax faba* Gmelin are the most common among wedge clams occurring in the sandy intertidal zone in the Gulf of Mannar Coast of Mandapam area. These clams are economically important for their meat, which is of food value and is also used as fish bait, and the shells which are used for making lime locally, and hence the present study may be of interest. But for some work on their taxonomy and some studies on their biology by Nayar (1955), Alargar swami (1967) and Krishnamurty *et al.* (1967) these animals have not received much attention. In the present study certain physiological aspects of survival of these two clams, such as the influence of exposure to air and conequent desiccation and oxygen debt, have been investigated.

MATERIAL AND METHODS

The experimental animals were collected from the sandy intertidal area at Vedalai, near Mandapam, and immediately brought to the laboratory in a bucket ¾-full of sea-water. Within an hour after the collection they were transferred to a wooden aquarium tank (60 cm. × 38 cm. × 23 cm.) containing sand (7 cm. depth), which served as the acclimation tank. The tank was supplied with running sea-water at a salinity of 33 ± 1‰, a temperature of 30 ± 1° C and at dissolved oxygen concentration near air saturation.

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Experimental Procedure

For experiments on survival the animals were removed from the acclimation tank, after they had remained in the tank for two to forty-eight hours, and were transferred to aerated sea-water (4 cm. depth) in a large enamel tray. All the animals which showed activity by extending the foot or the siphon were taken out and wiped with a piece of cloth and filter-paper for removing the traces of water on the shell of the animals, and they were kept exposed to air inside the laboratory in lots of five or six animals in dry petri dishes (3–4" diameter). At intervals of six hours or, as indicated, single groups were removed, and each animal in the lot was kept separately in a beaker containing aerated sea-water. The animals were watched at close intervals and if they again showed any activity such as extending either the foot or the siphon (*cf.* Morton *et al.*, 1957) within six hours after their transfer to the beaker, they were taken as living on counter-checking that the animals still retained the ability to withdraw the extended part.

As an index of desiccation the change in the amount of water trapped inside the closed shells was measured. This trapped water for all purposes can be assumed to be the mantle cavity fluid, as indicated by Dugal (1939), and is referred to as such in this paper. Dugal points out that though the mantle cavity fluid is not a true body fluid it becomes definitely altered when enclosed in the clam (*Venus mercenaria*) and that the total carbon dioxide contents were about the same in the mantle cavity fluid and in the fluid drawn from the pericardial cavity of both freshly collected and exposed clams. For this reason, it is felt that it is better to use the term 'mantle cavity fluid' for the water trapped inside the clam. Animals were wiped to remove traces of water as indicated earlier, and their total weights were taken. Then the weight of shells without meat was taken. The animal, removed from the shells by cutting only the adductor muscles, was put on a filter-paper and the fluid inside the mantle cavity absorbed out by gently pressing and rolling the meat on the filter-paper. Subsequently, the wet weight of meat was taken. From the above data the weight of the mantle cavity fluid was calculated for unexposed animals and also for those exposed to air upto 72–96 hours, at intervals of 12–24 hours. Data so obtained are included in Appendices I and II.

In experiments on post-exposure oxygen consumption the animals were exposed to air in separate lots in petri dishes, as in the case of the survival tests, upto 48 hours. At intervals of 12 hours single lots were removed and reimmersed in sea-water, and their metabolic rates were measured as explained below. The apparatus described by Kutty (1966) was used. The animals were kept one each in 300 ml. conical flasks. These flasks or respirometers remained submerged under sea-water inside a 'metabolism chamber' (Job, 1955, 1959). The water flowed through the respirometers without any apparent disturbance to the animals, and the flow was continuous except during the period of the actual determination of the metabolic rate, when the vessels were closed off. The respirometers were closed within 5–10 minutes after the animals were transferred to sea-water and remained closed for the next four hours. From water samples, taken before and after the period of closure, the rate of oxygen consumption was calculated.

The experiments on exposed animals were done at room temperature (average: 30° C; range: 26–33° C) except for a few performed at low temperatures of 12° C and 17° C. The latter experiments were done in a cold room where air temperture could be controlled with fair accuracy ($\pm$ 1° C), the maximum period of the experiments being 3 days. During the experiments on exposure the relative humidity ranged between 75 and 100%, except for the experiment at 17° C. The experiments for determining the oxygen debt were done in sea-water at a temperature of 30 $\pm$ 1° C

Results

Results of experiments on the survival of clams exposed to air are presented in Fig. 1, A-E. Time to 50% survival indicated by cross-marks on the curves drawn in Fig. 1, referred to hereafter only as 'survival time', is taken here as an index of the resistance of the clams to air exposure. The survival time of *D. cuneatus* (average length 1-3 cm.) exposed to air at room temperature

(30° C), was found to be 69 hours (Fig. 1 A). Smaller sized *D. cuneatus* (average length–2 cm.), also exposed to air at room temperature were found to have a shorter survival period. The survival time of the arger *D. cuneatus*, exposed to air at 17° C, was 69 hours (Fig. 1, C) and of those exposed to air at still lower temperature of 12° C was 29 hours (Fig. 1, D). While survival in these

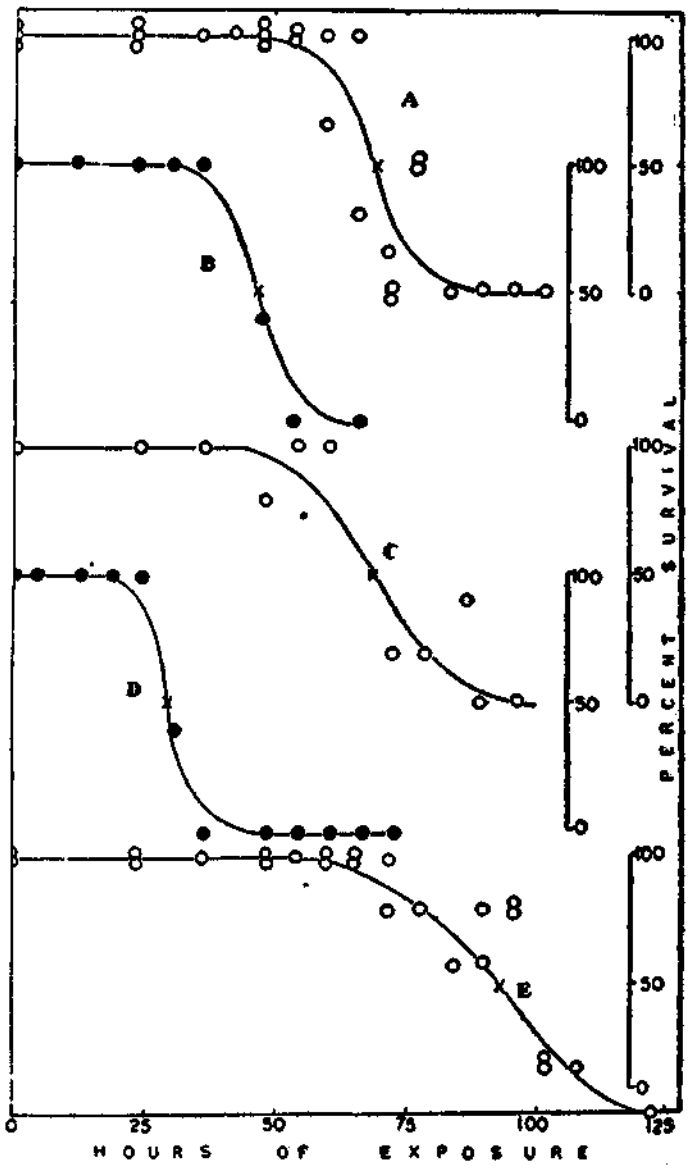

FIG. 1. (A–E). Survival of *Donax cuneatus* and *Donax faba* exposed to air. (A) *Donax cuneatus*—large size (avera length—3 cm.) exposed to air at room temperature (average 30° C; range 26–33° C); relative humidity 85–100%. (B) *Donax cuneatus*—small size (average length—2 cm.) exposed to air at room temperature (average 30° C: Range—26–32° C); relative humidity 75–90%. (C) *Donax cuneatus* (size same as for A) exposed to air at 17 ± 1° C; relative humidity 27–65%. (D) *Donax cuneatus* (size: same as for A) exposed to air at 12 ± 1° C; relative humidity 76–89%. (E) *Donax faba* (average length—2·5 cm.) exposed to air at room temperature (average 30° C; range 24–32° C); relative humidity 80–100%. All curves in the figure are fitted by the eye.

clams at 17° C does not appear to be different from that at room temperature, at 12° C temperature appears to be a prime factor in causing death as evident from the shift in the survival time.

In *D. faba* (average length—2·5 cm.) exposed to air at room temperature the survival time was 94 hours (Fig. 1, E). It is, however, to be noted that even though the time to 50% survival was longer for *D. faba* than *D. cuneatus* the survival curve of the latter appears much steeper (*see* curves A and E in Fig. 1). which may be due to the basic difference in the nature of mortification in the two species under the conditions of the tests.

Results of experiments on desiccation on exposure to air, as indicated by the change in the amount of mantle cavity fluid in *D. cuneatus* and *D. faba*, are given in Tables I A and I B and Fig. 2. In *D. cuneatus* exposed to room temperature the mantle cavity fluid (*see* methods) declined more or less gradually from an initial value (unexposed animals) of 0·41 to 0·34 g./g. of anima weight[1] after 48 hours of exposure (Table I A and Fig. 2, open circles). Determinations made after 72 hours indicated a value of 0·29 g./g. animal weight, but this value may have included some individuals which might have been dead (*see* survival curve A in Fig. 1) and hence cannot be strictly compared. It is also noted that there is no significant difference between the values of the mantle cavity fluid in unexposed animals and those exposed to 24 hours, the mean value being slightly higher in latter case. It is possible that this is because the animals did not lose any water in the first day of exposure, or due to some experimental error, but this cannot be verified further with the available information.

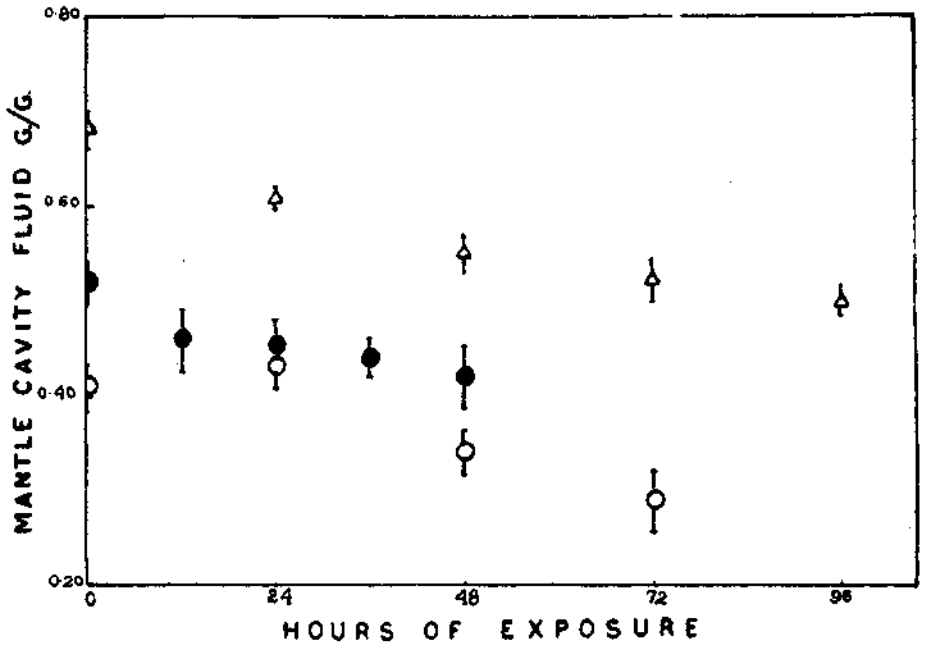

FIG. 2. Decline in the amount of mantle cavity fluid in relation to duration of exposure to air in *Donax cuneatus* and *Donax faba*. The open circles denote values obtained from *Donax cuneatus* exposed to air at room temperature. The closed circles denote values obtained from *Donax cuneatus* exposed to air at 17° C and the triangles represent *Donax faba* exposed to air at room temperature. The values plotted are taken from Tables I A and I B. The vertical lines indicated in the figure denote one standard error from the mean values.

[1] 'Weight of animals' indicates the total weight of the animal excluding the shell weight (*see* Appendices I & II).

TABLE I A

Decline in the amount of mantle cavity fluid (g./g. animal weight) in relation to duration of exposure in Donax cuneatus *exposed to air at room temperature (average : 30° C; range: 28–34° C) and relative humidity of 90–95%; lower column shows values of* Donax cuneatus *exposed to air at 17 ± 1° C and relative humidity of 27–69%. Each value is a mean of 4–6 separate determinations. The figures given after the sign ± indicate standard error. Gram animal weight indicated includes that af mantle cavity fluid*

Experimental temperature	Hours of exposure					
	0	12	24	36	48	72
30° C	0·41 ±0·024	..	0·43 ±0·027	..	0·34 ±0·025	0·29 ±0·032
17° C	0·52 ±0·025	0·46 ±0·033	0·45 ±0·020	0·44 ±0·020	0·42 ±0·031	..

TABLE I B

Decline in the amount of mantle cavity fluid (g/g animal weight) in relation to duration of exposure in Donax faba *exposed to air at room temperature (average: 30° C; range 26–32° C) and a relative humidity of 75–85%. Each value is a mean of 4–6 separate determinations. The figures after the sign ± indicate standard error. Gram animal weight indicated includes that of mantle cavity fluid*

Experimental temperature	Hours of exposure				
	0	24	48	72	96
30° C	0·68 ±0·021	0·61 ±0·012	0·55 ±0·020	0·52 ±0·022	0·49 ±0·016

In *D. cuneatus* exposed to 17° C the mantle cavity fluid declined from an initial value of 0·52 to 0·42 g./g. of animal weight after an exposure of 48 hours (Table I A and Fig. 2, closed circles). While the mean values from clams exposed to 17° C are higher than those exposed to 30° C at all levels of exposures, the difference appears to be insignificant at most levels. It is noted that in animals exposed to 30° C all or most of the water was lost between 24 and 48 hours, whereas in those exposed to 17° C most of the loss was between 0 and 24 hours (*see* Table II). It may be pointed out that *D. cuneatus* kept exposed to air at room temperature appeared to have remained tightly closed especially during the first half of the experimental period, while those exposed to 17° C kept their shells open from the beginning of the experiment and almost throughout the period of exposure. Difference in the experimental conditions other than temperature (relative humidity was markedly low at 17° C *see* legend for Fig. 1) might have influenced the results, but the extent of this influence cannot be known.

In *D. faba* exposed to room temperature the mantle cavity fluid declined gradually from an initial value of 0·68 to 0·55 g./g. of animal weight in animals exposed for 48 hours (Table I B and Fig. 2, triangles). Values of mantle cavity fluid have been determined in animals exposed to 72 and 96 hours as well, which also show a gradual decline with the duration of exposure to air even though loss of water appears to be much less towards the later part (48 to 96 hours). Here again the last two values may have included determinations made on groups in which some animals might have been dead, since mortality in *D. faba*, though to a lesser extent, begins at about the 60th hour of exposure (Fig. 1, E). It appears that in *D. faba* desiccation was at about the same rate in the first two days of exposure (Table II). It is interesting to note that in the three groups of animals tested desiccation, as indicated by the loss of mantle cavity fluid, at the end of the second day of exposure appears to be about the same magnitude (17 to 19%—*see* Table II).

Table I and Fig. 2 show clearly that the relative amount of mantle cavity fluid is more in *D. faba* (about 70% for unexposed animals) than in *D. cuneatus* (both test groups at 30° and 17° C) (40–50% for unexposed animals at all levels of exposure). *D. faba* used are smaller than *D. cuneatus* and there is the possibility that the difference in the relative amount of mantle cavity fluid in the two groups is due to the size difference, but it is felt that size difference will not fully account for the difference in the relative amount of fluid.

TABLE II

Percentage deviation in the amount of mantle cavity fluid from the initial values (unexposed animals) in Donax cuneatus *and* Donax faba *(values calculated from data presented in Tables I A and I B)*

Species	Experimental temperature	Period of exposure in hours		
		0–24	24–48	0–48
Donax cuneatus ..	30° C	+4·9?	−22·0	−17·1
	17° C	−13·4	− 5·8	−19·2
Donax faba ..	30° C	−10·3	− 8·8	−19·1

Results of experiments on the oxygen consumption of *D. cuneatus* and *D. faba* subsequent to specific periods, up to 48 hours, of exposure to air are given in Tables III A and III B and are graphically shown in Fig. 3. In view of the observations of earlier authors (*cf.* van Dam, 1935) that the oxygen debt accumulated during the period of exposure in other bivalves is paid back in the first few hours of reimmersal in water, the present estimate of oxygen debt is made from values obtained during the first four hours of reimmersal in sea-water. The tendency to increase oxygen consumption with an increase in duration of exposure appears to be similar in both species (Fig. 3).

TABLE III A

Oxygen consumption in Donax cuneatus *on reimmersal in sea-water (30° C) subsequent to different periods of exposure to air at room temperature (average: 30° C). Values of the mean ± one standard error are indicated. Each mean is calculated from 4 separate determinations. Metabolic rate shown is per gram wet weight of meat*

	Hours of exposure				
	0	12	24	36	48
Oxygen consumption mg./ g. hour	0·071 ±0·003	0·162 ±0·024	0·170 ±0·015	0·239 ±0·141	0·234 ±0·123

TABLE III B

Oxygen consumption in Donax faba *on reimmersal in sea-water (30° C) subsequent to different periods of exposure to air at room temperature (average 30° C). Values of the mean ± one standard error are indicated. Each mean is calculated from 4 separate determinations, except for the value at 24 hours (n = 3). Metabolic rate shown is per gram wet weight of meat*

	Hours of exposure				
	0	12	24	36	48
Oxygen consumption mg./g./hour	0·413 ±0·107	0·517 ±0·050	0·455 ±0·060	0·623 ±0·079	0·607 ±0·075

The 48 hour-exposed (the longest period tested in this series) groups in both species accumulate maximum oxygen debt as can be known from the enhanced oxygen consumption. Values given in Table III indicate that the oxygen consumption of *D. cuneatus* and *D. faba* increased 3·3 and 1·7 times, respectively, the level of oxygen consumption of unexposed animals. It appears that the higher metabolic rate of *D. faba* than that of *D. cuneatus*, as shown in Fig. 3, is due to the smaller size of *D. faba* tested (Zeuthen, 1953). If one assumes a '*b*' value of unity, which is rather high for molluscs (Zeuthen, 1953) in the weight-length relation of the molluscs investigated for a weight correction, most of the corrected values so obtained from *D. cuneatus* do not appear to be significantly different from those of *D. faba*. Increased oxygen consumption in molluscs consequent to exposure to air has been observed by several authors (Mitchell, 1912; van Dam, 1935; Nagabhushanam, 1966 and others) and has been taken as due to the oxygen debt accumulated during the period of exposure and subsequently rapid on reimmersal to water. It has also been observed that certain molluscs do not accumulate an oxygen debt (Morton *et al.*, 1957). As evident both species included in the present study accumulate an oxygen debt.

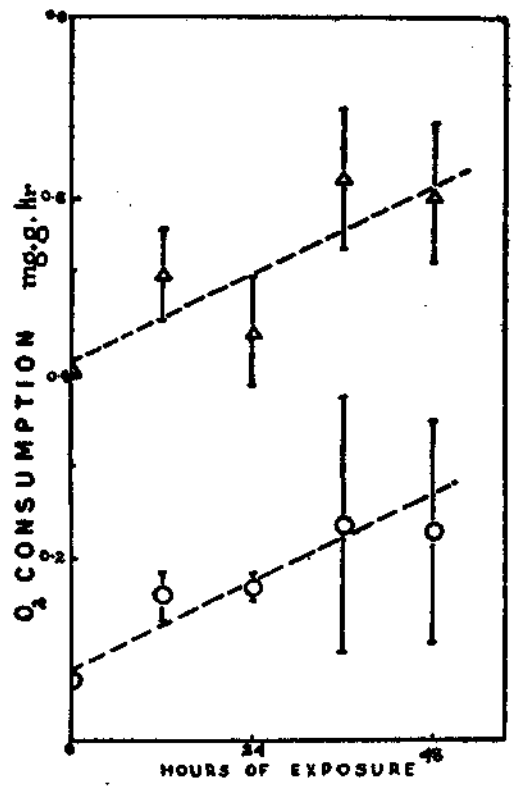

FIG. 3. Oxygen consumption in *Donax cuneatus* (circles) and *Donax faba* (triangles) on reimmersal in sea-water (30° C) subsequent to different periods of exposure to air at room temperature (30° C). The broken lines indicate the trend in increase in oxygen consumption with the increase in the duration of exposure. The vertical lines indicated in the figure denote one standard error from the mean values. The metabolic rates indicated are per gram wet weight of meat.

DISCUSSION

From comparisons of the survival times it would appear, as already pointed out, that at 12° C, temperature does act as one of the major factors causing death in *D. cuneatus* exposed to air as

indicated by the shift of survival curve to the left (compare curves A and D in Fig. 1). The survival time of *D. cuneatus* exposed to air at 17° C appears to be same as that at 30° C. Whether temperature was of more or less consequence in causing mortality at 17° C than at 30° C cannot be clearly known, because of the difference especially in the humidity conditions of the two sets of experiments. The trend in water loss at these two temperatures as indicated by the loss of the mantle cavity fluid does not seem to be markedly different. In both groups (17 and 30° C) animals began dying after about the 2nd day of exposure, when the amount of mantle cavity fluid had declined by about 20% from the initial value.

Low temperature deaths in molluscs have been studied by various authors (Newell, 1964). Kanwisher (1955) has observed that certain molluscs (*Crassostrea virginica, Mytilus edulis, Modiolus modiolus, Littorina littorea,* and *L. rudis*) can withstand even freezing temperatures for long periods. *Mytilus edulis* has been known to revive after 6 to 8 months at −20° C or below in solid ice at Labrador (Kanwisher, 1959). Kanwisher is of the opinion that resistance to freezing runs parallel with ability to withstand desiccation. Within the limited scope of the present experiments such aspects of temperature death cannot be further discussed.

Death at higher temperature has not been included in the present study. *Ostera cucullata* from the tropics of the Indo-Pacific region have been known to tolerate temperatures as high as 45° C (Stephenson, 1924). Such studies will indeed be of much interest as there are only a few observations on the temperature death of molluscs in the tropics.

It may be pertinent to point out that in the present studies ability to survive and withstand exposure have been investigated only at a single level of temperature acclimation in both species. It is needless to stress that a full picture of the adaptational abilities of these animals with regard to temperature can only be known by further studies on animals acclimated to different levels of temperature.

Some field observations made on the clams studied presently indicate that both the species have more or less overlapping distribution in the vertical gradient, but there are possibly demarcations of zones along the intertidal area where only one species (*D. faba*) occurs, which may be taken to suggest that *D. faba* have a wider range of tolerance of the various ecological factors than *D. cuneatus*. This aspect needs further study to arrive at firm conclusions.

Ability to withstand desiccation has been closely correlated with the order of occurrence of certain molluscs in the intertidal areas (Broekhuysen, 1940; Newell, 1964). Morton *et al.* (1967) studied water loss in the lamellibranch *Lasaea rubra* by following the change in weight of animals exposed for 12 hours and found that low neap *Lasaea* lost much more water than the high tidal forms. There does not appear to be much difference in the pattern of water loss between the two species tested presently, but it does appear as already pointed out that *D. faba* has a relatively larger amount of mantle cavity fluid than *D. cuneatus* and this can possibly enable it to withstand desiccation to a better extent and may explain at least partly the longer survival time of *D. faba* exposed to air (Fig. 1). Size difference cannot be the reason for the longer period of survival of *D. faba* as it would appear from the results of tests on the influence of size on survival in *D. cuneatus* exposed to air. If *D. faba* tested were as large as *D. cuneatus*, one may expect the survival period of the former to be longer than that indicated by the presently observed value, thereby suggesting a wider disparity between the two species of clams than is apparently noted.

It also appears that *D. faba* accumulates relatively much less oxygen debt than *D. cuneatus* (Fig. 3). While it is recognized that relative inactivity to a great extent allows the animals to stay exposed for considerable time without accumulating a large oxygen debt, it is possible that the anaerobic abilities of the two clams are different (Cole, 1921; Dugal, 1939; von Brand, 1946, 1951) and that *D. faba* is able to function better anaerobically than *D. cuneatus* possibly by obtaining energy through more unconventional methods (Blazka, 1957; Saz and Weil, 1960). From the present study it can only be concluded that *D. faba* is better adapted than *D. cuneatus* to survive when exposed to air, being subject to less desiccation and probably having better anaerobic abilities.

Acknowledgements

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APPENDIX I A

Basic data on Donax cuneatus *used in experiments at room temperature (average: 30° C; range: 26–33° C)*
(The experimental details are briefly indicated in the remarks column)

Serial No.	Shell length in cm.	Total weight in g.	Shell weight in g.	Meat weight including mantle cavity fluid in g.	Meat weight excluding mantle cavity fluid in g.	Weight of mantle cavity fluid in g.	Percentage weight of mantle cavity fluid in values given in Column 5	Remarks
1	2	3	4	5	6	7	8	9
1	3·2	5·2025	3·4518	1·7507	1·0361	0·7146	40·81	Unexposed animals
2	3·2	5·0062	3·1428	1·8634	1·2229	0·6405	34·37	,,
3	3·1	4·5601	2·8274	1·7327	1·0721	0·6606	38·12	,,
4	3·1	4·5032	2·8500	1·6532	0·8884	0·7648	46·26	,,
5	3·0	3·8247	2·3980	1·4267	0·7445	0·6822	47·81	,,
6	3·2	5·3203	3·6804	1·6399	0·8613	0·7786	47·47	24 hours exposed
7	3·2	4·9709	3·1951	1·7758	1·0505	0·7253	40·84	,,
8	3·0	3·9291	2·5963	1·3328	0·7112	0·6216	46·63	,,
9	3·1	4·5775	3·0807	1·4968	0·8187	0·6781	45·30	,,
10	3·1	4·4074	2·9560	1·4514	1·0093	0·4421	30·46	,,
11	3·3	4·9914	3·4756	1·5158	0·8437	0·6921	45·65	,,
12	3·1	4·2223	3·0073	1·2150	0·8101	0·4049	33·32	48 hours exposed
13	3·1	4·2312	3·2924	0·9388	0·7049	0·2339	24·91	,,
14	3·2	2·5083	3·0702	1·4381	0·8684	0·5697	39·61	,,
15	3·2	4·3155	2·9782	1·3373	0·9040	0·4333	32·40	,,
16	2·9	3·4915	2·4126	1·0789	0·6670	0·4119	38·17	,,
17	2·8	2·7935	2·1307	0·6628	0·4899	0·1729	26·08	72 hours exposed
18	3·0	3·5249	2·5711	0·9538	0·5918	0·3620	37·95	,,
19	2·9	3·2740	2·3258	0·9482	0·6667	0·2815	29·68	,,
20	3·0	3·4242	2·6106	0·8226	0·6496	0·1730	21·03	,,
21	3·0	3·6048	2·5496	1·0552	0·6385	0·4167	39·49	,,
22	2·9	2·8665	2·1923	0·6742	0·5110	0·1632	24·20	,,

APPENDIX I B

Basic data on Donax cuneatus *used in experiments at 17° C*
(The experimental details are briefly indicated in the remarks column)

Serial No.	Shell length in cm.	Total weight in g.	Shell weight in g.	Meat weight including mantle cavity fluid in g.	Meat weight excluding mantle cavity fluid in g.	Weight of mantle cavity fluid in g.	Percentage weight of mantle cavity fluid in values given in Column 5	Remarks
1	2	3	4	5	6	7	8	9
1	3·3	5·7052	3·8093	1·8959	0·7652	1·1307	59·64	Unexposed animals
2	3·1	4·8850	3·0892	1·7958	0·9376	0·8582	47·79	"
3	2·9	4·4458	2·7519	1·6939	0·7446	0·9493	56·04	"
4	3·1	5·0488	2·9757	2·0731	1·1081	0·9650	46·55	"
5	3·2	5·2954	3·3030	1·9924	0·9820	1·0104	50·71	"
6	3·3	5·4146	3·5333	1·8813	0·9591	0·9222	49·02	12 hours exposed
7	3·2	4·9108	3·2035	1·7073	0·7493	0·9580	56·11	"
8	3·1	4·5035	2·9238	1·5797	0·9661	0·6136	38·84	"
9	3·2	4·3270	2·6651	1·6619	0·8514	0·8105	48·77	"
10	3·2	4·8570	3·0526	1·8044	1·0907	0·7137	39·55	"
11	3·1	4·8432	3·1381	1·7051	0·8916	0·8135	47·71	24 hours expose
12	3·3	5·4070	3·7189	1·6881	1·0080	0·6801	40·29	"
13	3·1	4·5162	2·9410	1·5752	0·6969	0·8783	55·76	"
14	3·0	4·1372	2·8180	1·3192	0·8019	0·5173	39·21	"
15	3·0	3·7903	2·4518	1·3385	0·7444	0·5941	44·39	36 hours expo
16	3·0	4·4111	2·9372	1·4739	0·8180	0·6559	44·50	"
17	3·1	4·0331	2·6855	1·3476	0·8420	0·5056	37·52	"
18	3·0	4·0853	2·6560	1·4298	0·7808	0·6490	45·39	"
19	3·0	4·0534	2·7922	1·2612	0·7049	0·5563	44·11	"
20	3·2	4·6945	3·3190	1·3755	0·6845	0·6910	50·24	"
21	3·2	5·0478	3·6594	1·3884	0·8880	0·5004	36·04	48 hours exposed
22	3·2	4·2814	3·1050	1·1764	0·6532	0·5232	44·47	"
23	3·0	3·7830	2·6060	1·1770	0·5900	0·5870	49·87	"
24	2·9	3·3350	2·3402	0·9948	0·6678	0·3270	32·87	"
25	2·9	3·6228	2·5631	1·0597	0·5892	0·4705	44·40	"

APPENDIX II

Basic data on Donax faba *used in experiments at room temperature* (*average* = 30° *C*; *range*: 26-33° *C*)
(The experimental details are briefly indicated in the remarks column)

Serial No.	Shell length in cm.	Total weight in g.	Shell weight in g.	Meat weight including mantle cavity fluid in g.	Meat weight excluding mantle cavity fluid in g.	Weight of mantle cavity fluid in g.	Percentage weight of mantle cavity fluid in values given in Column 5	Remarks
1	2	3	4	5	6	7	8	9
1	2·7	3·4099	2·3829	1·0270	0·2766	0·7504	73·07	Unexposed animals
2	2·7	3·7539	2·4967	1·2572	0·3179	0·9393	74·71	,,
3	2·4	2·7673	1·8522	0·9151	0·3213	0·5938	64·89	,,
4	2·5	2·7048	1·8869	0·8179	0·2981	0·5198	63·55	,,
5	2·6	2·8287	1·9489	0·8798	0·3032	0·5766	65·54	,,
6	2·5	2·4054	1·7595	0·6459	0·2608	0·3851	59·52	24 hours exposed
7	2·6	2·6920	2·0437	0·6483	0·2607	0·3876	59·79	,,
8	2·5	2·5821	1·9540	0·6281	0·2397	0·3884	61·84	,,
9	2·5	2·4820	1·8884	0·5936	0·2377	0·3559	59·96	,,
10	2·7	3·2965	2·3850	0·9115	0·3110	0·6005	65·88	,,
11	2·7	2·9858	2·3674	0·6184	0·2757	0·3427	55·42	48 hours exposed)
12	2·7	3·3740	2·6574	0·7166	0·3038	0·4128	57·61	,,
13	2·7	3·0722	2·4815	0·5907	0·3084	0·2823	47·79	,,
14	2·5	3·0010	2·2678	0·7332	0·2916	0·4416	60·23	,,
15	2·4	2·4088	1·8661	0·5427	0·2540	0·2887	53·20	,,
16	2·5	2·7300	2·1617	0·5683	0·2987	0·2696	47·44	72 hours exposed)
17	2·8	2·7530	2·2390	0·5140	0·2286	0·2854	55·53	,,
18	2·7	3·2470	2·6336	0·6134	0·2722	0·3412	55·62	,,
19	2·5	2·5218	2·0560	0·4658	0·2068	0·2590	55·60	,,
20	2·6	2·5288	2·0788	0·4500	0·2469	0·2031	45·13	,,
21	2·6	2·7704	2·3450	0·4254	0·2166	0·2088	49·08	96 hours exposed
22	2·5	2·7900	2·3535	0·4365	0·2390	0·1975	45·25	,,
23	2·7	2·7500	2·2266	0·5234	0·2436	0·2798	53·46	,,
24	2·7	2·4817	1·9815	0·5002	0·2413	0·2589	51·76	,,
25	2·4	2·1610	1·7425	0·4185	0·2287	0·1898	45·35	,,

STUDIES ON THE CHROMATOPHOROTROPIC PRINCIPLES IN THE NERVE RING AND OPTIC TENTACLES OF THE PULMONATE GASTROPOD *ONCIDIUM VERRACULATUM*

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ABSTRACT

Previous investigations revealed that certain principles capable of evoking pigment dispersion and/or concentration in crustacean chromatophores were present in the nerve ganglia of some molluscs. However, there has been no report concerning the nature and properties of the chromatophorotropic principles in the nerve ganglia of molluscs. The present study was designed to determine the presence, distribution and properties of the chromatophorotropic principles in the nerve ganglia of *Oncidium verraculatum*. To determine the chromatophorotropic activity, extracts of the nerve ring, medio-dorsal bodies and optic tentacles were prepared and injected into intact specimens of the crab, *Ocypode macrocera*, adapted to a white background and eyestalkless *Ocypode macrocera*. In the crabs adapted to a white background the black and red pigments in the chromatosomes were concentrated while the white pigment was maximally dispersed. In eyestalkless individuals the black and white pigments were concentrated while the red pigment was maximally dispersed. The saline extracts of nerve ring and medio-dorsal bodies of *Oncidium* evoked concentration of red and white pigments in the chromatosomes of *Ocypode*. The saline extracts of optic tentacles of *Oncidium* evoked dispersion of black, red and white pigments of *Ocypode* and concentration of red pigment. The principles evoking concentration of red and white pigments are soluble in ethanol and acetone. In contrast, the principles provoking dispersion of black, red and white pigments are insoluble in ethanol and acetone. The chromatophorotropic principles in the optic tentacles of *Oncidium* are resistant to heat. In contrast, the white pigment concentrating principle present in the nerve ring and mediodorsal bodies of *Oncidium* was destroyed by heat. Trypsin inactivated the chromatophorotropic principles. An electrophoretic analysis revealed that at pH 7·5 the red pigment-concentrating principle of the nerve ring was electro-positive and in this respect was similar to the red pigment-concentrating harmone in the eyestalk ganglia of *Ocypode*. The crustacean chromatophorotropins are neurosecretory products. It is known that neurosecretory cells are present in the nerve ganglia and optic tentacles of pulmonate gastropods. It is interesting to find a correlation between neurosecretion and chromatophorotropic activity in the gastropod, *Oncidium verraculatum*. The electrophoretic and solubility properties of the chromactive principles from *Oncidium* and *Ocypode* were compared and their interrelationships discussed. There are no chromatophores in *Oncidium*, but it has principles with chromatophorotropic activity. Probably these principles have other physiological activities in the mollusc.

A SURVEY of the literature on control of chromatophores (Fingerman, 1963) reveals that a variety of invertebrates which lack chromatophores possess in their nervous tissues certain principles that are capable of provoking pigment migrations in the chromatophores of crustaceans. The crustacean chromatophorotropins are products of neurosecretion. The neurosecretory cells have been found in several molluscs (Simpson, Bern, and Nishioka, 1966). McVay (1942) was the first to report on the presence of a chromatophorotropic principle in the ganglia of molluscs. She found that the nerve ganglia of *Venus* contain a principle that evokes dispersion of white pigment in the chromatophores of *Cambarus*. The extracts of the visceral ganglia of the oyster, *Crassostrea*, concentrated the pigments in the chromatophores of *Orconectes* (Nagabhushanam, 1962). The extracts of visceral ganglia of *Meretrix* concentrated the black pigment in the chromatophores of the crab, *Uca annulipes* (Nagabhushanam, 1964). So far nothing is known about the properties of these chromactive principles or their relationship with the crustacean chromatophorotropic principles. In the present investigation the chromatophorotropic activities of extracts of the nerve ring, medio-dorsal bodies, and optic tentacles of the pulmonate gastropod, *Oncidium verraculatum*, were determined by assaying them on the crab, *Ocypode macrocera*. The stability, solubility, electrophoretic mobility, and enzyme inactivation of these principles were investigated.

Material and Methods

Specimens of *Oncidium verraculatum* collected from the Southern Lighter Channel of the Visakhapatnam Harbour, and *Ocypode macrocera* collected from the Visakhapatnam beach were used in the present investigation. In the laboratory specimens were placed in the aquaria containing sea-water and maintained under normal day-night conditions. The chromatophorotropic activities of extracts of the following tissues of *Oncidium* were determined: (1) nerve ring including all the central nerve ganglia, (2) medio-dorsal bodies which lie in close association with the cerebral ganglia, and (3) optic tentacles. Eyestalkless *Ocypode* and *Ocypode* adapted to a white background were used as assay animals to determine the chromatophorotropic activity of the extracts. The bilateral ablation of the eyestalks was performed 18 hours before the use of specimens in the experiment. In the eyestalkless *Ocypode* the red pigment was maximally dispersed while the white and black pigments were maximally concentrated. To obtain the second type of assay animals, the crabs were adapted to a white background for two hours prior to use in the experiment. In the white adapted *Ocypode* the red and black pigments were concentrated while the white pigment was maximally dispersed. Thus, using these two types of assay crabs it was possible to identify the principles with the following chromatophorotropic activities: black pigment dispersion, white pigment dispersion, red pigment dispersion, white pigment concentration, and red pigment concentration.

The tissues to be extracted were dissected fresh from *Oncidium*, placed in an embryological dish with a ground glass bottom, triturated with a glass rod, and extracted with filtered sea-water. The extract was centrifuged at 1,000 × g. for ten minutes and the supernatant was injected into assay crabs in a dose of 0·05 ml./crab. Different methods of fractionation were used and these are described at appropriate places in the text. In each set of the experiments one group was injected with the extract to be tested while the other group received injections of sea-water as a control in a dose of 0·05 ml./crab. The different extracts were tested in the following concentrations: 2 optic tentacles/0·05 ml./crab, nerve ring of one specimen/0·05 ml./crab, and medio-dorsal bodies from one specimen/0·05 ml./crab.

The chromatophores were staged following the method of Hogben and Slome (1931). According to this scheme stage 1 represents maximal concentration, stage 5 represents maximal dispersion, and stages 2, 3 and 4 intermediate conditions. The activity of each extract was expressed in terms of activity values following the method of Sandeen (1950). In each experiment the average stage of each type of chromatophores was recorded at the start of the experiment and 15-30 minutes following the injection of the extract and at 30-minute intervals thereafter for the duration of the effect. When pigment dispersion occurred the sum of the average chromatophore stages recorded throughout the experiment for the control group was subtracted from the sum for the experimental group. While calculating the pigment concentrating activities the sum for the experimental group was subtracted from the sum for the control group. To calculate the pigment concentrating activities the data obtained 5 minutes after the injection of the extract were also included in addition to the usual data collected at time intervals specified above. The differences obtained between the experimental and control groups represent the activity values which are a measure of both the intensity and duration of the response.

Experiments and Results

Chromatophorotropic principles and their resistance to heat

The effect of heat on the chromatophorotropic principles was determined by comparing the activities of sea-water extracts of optic tentacles, medio-dorsal bodies, and nerve rings dried for three hours at 100° C. with those of extracts prepared from fresh, unheated optic tentacles, medio-dorsal bodies, and nerve rings. In each experiment tissues from 20 animals were used, for extraction. Each of the extracts was assayed on 5 eyestalkless crabs and 5 crabs adapted

to a white background. A control group of 5 white adapted crabs and 5 eyestalkless crabs received injections of sea-water in a dose of 0·05 ml./crab. The experiment was repeated twice.

The results are shown in Table I. The saline extracts of freshly dissected nerve ring and medio-dorsal bodies evoked white pigment concentration and red pigment concentration. In contrast, the saline extract of optic tentacles evoked dispersion of black, red, and white pigments and concentration of the red pigment. The red pigment concentrating principle present in the optic tentacles, medio-dorsal bodies, and nerve ring was resistant to heat. Similarly, the pigment dispersing principles present in the optic tentacles were resistant to heat. In contrast the white pigment concentrating principle present in the medio-dorsal bodies and the nerve ring was not resistant to heat.

TABLE I

The chromatophorotropic activities of sea-water extracts of freshly dissected and oven-dried nerve ring, medio-dorsal bodies and optic tentacles of the gastropod, Oncidium verraculatum

Material	Activity values*				
	BPDH	WPDH	RPDH	RPCH	WPCH
Nerve ring (fresh) ..	-0-	-0-	-0-	10·2	6·1
Nerve ring (dried) ..	-0-	-0-	-0-	8·8	0·2
Medio-dorsal bodies (fresh) ..	-0-	-0-	-0-	9·6	4·2
Medio-dorsal bodies (dried) ..	-0-	-0-	-0-	9·2	0·1
Optic tentacles (fresh) ..	11·9	14·1	5·0	2·3	
Optic tentacles (dried) ..	10·8	14·4	4·6	2·4	-0-

*BPDH: Black pigment dispersion; WPDH: white pigment dispersion; RPDH: red pigment dispersion; RPCH: red pigment concentration; WPCH: white pigment concentration.

Differential solubility analysis of the chromatophorotropins

The nerve rings dissected fresh from 20 specimens of *Oncidium* were placed in an embryological dish. After preliminary drying at the room temperature the ganglia were triturated with a glass rod and extracted with 10 ml. of 100% ethyl alcohol. The extract was centrifuged and the supernatant was decanted into a watch-glass. After allowing the alcohol to evaporate the alcohol-soluble fraction was dissolved in 1 ml. sea-water. The alcohol-insoluble residue was extracted in 1 ml. sea-water. Each of these extracts was injected into 5 eyestalkless *Ocypode* and 5 white-adapted *Ocypode*, in a dose of 0·05 ml./crab. Following the above procedure alcohol soluble and insoluble fractions of the optic tentacles and medio-dorsal bodies were prepared and assayed. Furthermore, acetone soluble and insoluble fractions of the nerve ring, medio-dorsal bodies, and optic tentacles were prepared and assayed. Each experiment was repeated twice.

The results are shown in Fig. 1. For comparison the chromatophorotropic activities of sea-water extracts of the different tissues tested were also represented in the figure. The white pigment-concentrating substance present in the nerve ring and medio-dorsal bodies was soluble in alcohol and acetone. The white pigment-concentrating activities of the saline extract, alcohol-soluble fraction, and the acetone-soluble fraction of the nerve ring were nearly identical to each other. In contrast, the white pigment-concentrating activities of the acetone-soluble and alcohol-soluble fractions of the medio-dorsal bodies were more than the saline extract of the medio-dorsal bodies. The red pigment-concentrating principle in the medio-dorsal bodies was soluble

in alcohol and acetone. However, the red pigment concentrating substance in the nerve ring was only partially soluble in alcohol while being more soluble in acetone. The principles in the optic tentacles of *Oncidium* which evoke dispersion of black, red, and white pigments of *Ocypode* were insoluble in alcohol and acetone.

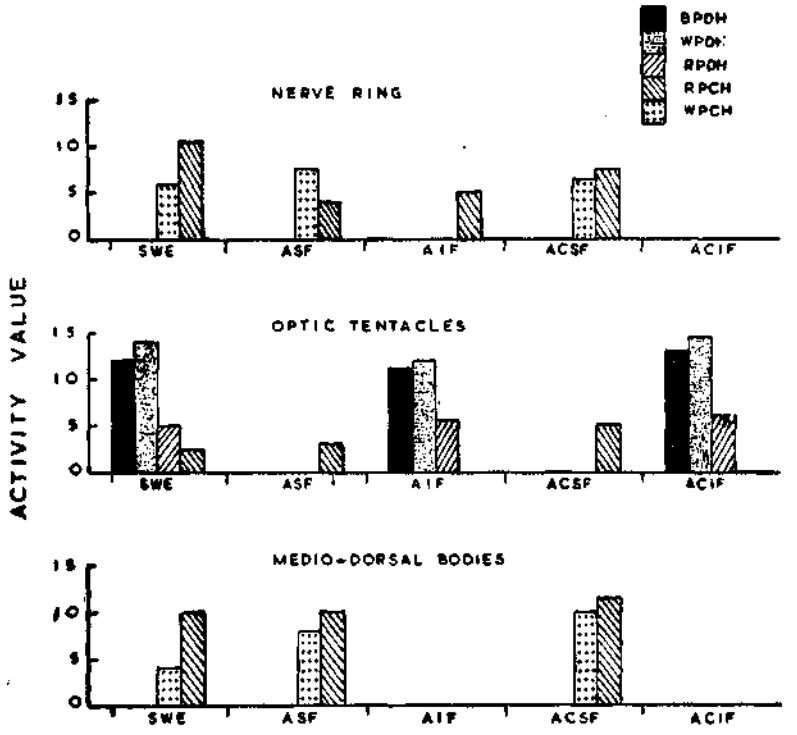

FIG. 1. Chromatophorotropic activities of extracts of the nerve ring, optic tentacles, and medio-dorsal bodies of the gastropod, *Oncidium verraculatum*, as assayed on the chromatophores of the crab, *Ocypode macrocera*. SWE: sea-water extracts; ASF: alcohol-soluble fraction; AIF: alcohol-insoluble fraction; ACSF: acetone-soluble fraction; ACIF: acetone-insoluble fraction; BPDH: black pigment dispersing activity; RPDH: red pigment dispersing activity; WPDH: white pigment dispersing activity; RPCH: red pigment concentrating activity; WPCH: white pigment-concentrating activity.

Electrophoretic analysis of the chromatophorotropins

The electrophoresis was carried out on Whatmann No. 1 filter-paper in the horizontal type of electrophoresis apparatus. The paper strips six of which could be used simultaneously were stretched horizontally. From the power supply unit a stabilized voltage was obtained in the range from 0 to 500 volts. It was not possible to stabilize voltage and current at the same time. In all the runs only the voltage was stabilized. In the present study phosphate buffer (N/15) was used. Filter-paper strips of 28 cm. length and 3 cm. width were used. The extract to be separated by electrophoresis was applied to the central point of the paper, midway between the electrodes. An air blower was used to evaporate the water as the extract was applied thereby preventing the spread of the extract over a band of more than 0·5 cm. width. A spot of DL-leucine was added at the edge of the paper to serve as a reference substance. After electrophoresis a thin piece of paper was cut lengthwise from the dried paper and stained with ninhydrin, and this gave a measure of the constancy of conditions from one run to the other.

After electrophoresis the paper was dried in air and later cut into sections. The band extending 0·5 cm. on either side of the origin was cut and numbered zero. The remaining portion of the paper was cut transversely into five equal parts, 2·7 cm. wide on each side of the origin and numbered −1 to −5 at the cathode and + 1 to + 5 at the anode. Each of these bands was eluted with 0·5 ml. sea-water for a period of 30 minutes in a watch-glass and the eluate injected into 5 eyestalkless *Ocypode* and 5 *Ocypode* adapted to a white background in a dose of 0·05 ml./crab. Each experiment was repeated once.

The optic tentacles were dissected fresh from 10 specimens of *Oncidium* and extracted in 0·1 ml. distilled water. The extract was subjected to electrophoresis at pH 7·5, 360 V, 0·2–0·4 mA/cm. and 22° C. for 18 hours. The results are shown in Fig. 2. The pigment-dispersing principles did not move from the point of application. In contrast, the red pigment-concentrating principle moved towards the cathode (Fig. 2, A).

The nerve rings were dissected fresh from 20 specimens of *Oncidium*. Half of them were extracted directly in 0·1 ml. distilled water and the rest were dried for 3o hurs at 100° C. and then extracted in 0·1 ml. distilled water. Both these extracts were subjected to electrophoresis at pH 7·5, 360 V, 0·2–0·4 mA/cm. and 22° C. for 16 hours. The results are shown in Fig. 2. In the analysis of extracts of fresh nerve rings it was found that the zero portion of the paper strip had white pigment-concentrating and red pigment-concentrating activities while the −1 portion had only red pigment-concentrating activity (Fig. 2, C). As noted earlier the extract of heated nerve rings was devoid of white pigment-concentrating activity. The red pigment-concentrating principle in this extract moved towards the cathode and in this respect resembled the red-pigment concentrating principle present in the optic tentacles.

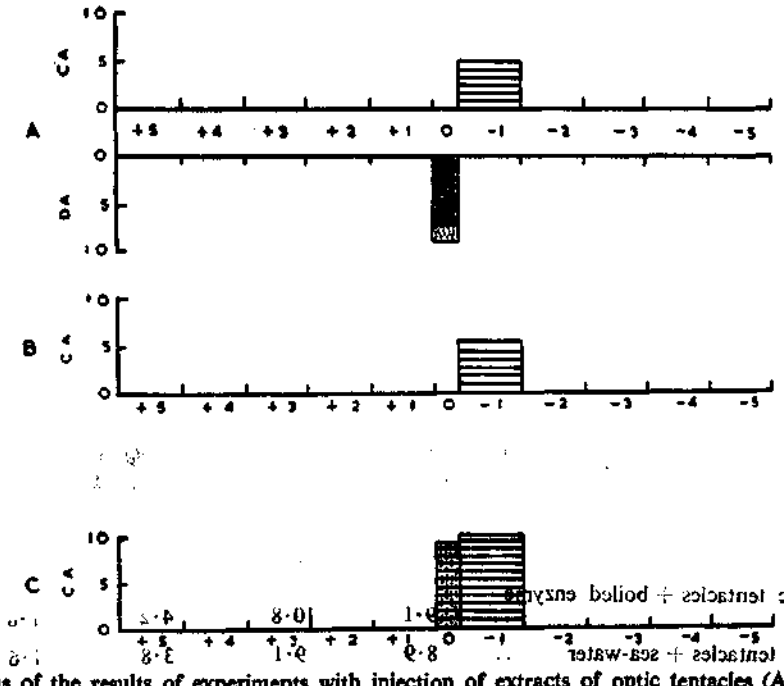

FIG. 2. Diagrams of the results of experiments with injection of extracts of optic tentacles (A), nerve rings dried at 100° C. for 3 hosur (B), and freshly dissected nerve rings of the gastropod, *Oncidium veraculatum* into the crab, *Ocypode macrocera* after electrophoresis. CA: pigment-concentrating activities; DA: pigment-dispersing activities; the area with horizontal bars represents the response of red pigment, area with circles represents the response of black pigment, and the area with dots represents the response of white pigment.

Inactivation of the chromatophorotropins by trypsin

An enzyme preparation was made using a fresh supply of Matheson Coleman and Bell-trypsin powder in a concentration of 1 part per 10,000 parts sea-water. A portion of this enzyme solution was inactivated by keeping in a water-bath at 100° C. for 15 minutes. The following six mixtures were prepared :

(A) 1 ml. of an extract of the nerve ring (1 nerve ring/0·05 ml.) + 0·5 ml. enzyme solution.

(B) 1 ml. of an extract of the nerve ring (1 nerve ring/0·05 ml.) + 0·5 ml. of the boiled preparation of the enzyme.

(C) 1 ml. of an extract of the nerve ring (1 nerve ring/0·05 ml.) + 0·5 ml. sea-water.

(D) 1 ml. of an extract of the optic tentacles (2 tentacles/0·05 ml.) + 0·05 ml. enzyme solution.

(E) 1 ml. of an extract of the optic tentacles (2 tentacles/0·05 ml.) + 0·5 ml. of the boiled preparation of the enzyme.

(F) 1 ml. of an extract of the optic tentacles (2 tentacles/0·05 ml.) + 0·5 ml. of sea-water.

The above mixtures were taken separately into centrifuge tubes and incubated at 37° C. for 15 minutes. Then the tubes were immersed in a boiling water-bath for 3 to 5 minutes, allowed to cool and centrifuged. The supernatant of each sample was injected into 5 eyestalkless animals and 5 white adapted crabs in a dose of 0·05 ml./crab. The experiment was repeated once. The results are shown in Table II. The incubation of extracts of the nerve ring with the solution of trypsin in sea-water resulted in the inactivation of red pigment-concentrating and white pigment-concentrating principles. However, when the extract was incubated with a boiled preparation of the enzyme the chromatophorotropins were not inactivated. This is true with the chromatophorotropins in the optic tentacles of *Oncidium*.

TABLE II

Effect of trypsin on the chromatophorotropic principles in the nerve ring and optic tentacles of the gastropod, Oncidium verraculatum

Material*	Activity values†				
	BPDH	WPDH	RPDH	RPCH	WPCH
Extract of the nerve ring + enzyme solution ..	-0-	-0-	0	-0-	-0
Extract of the nerve ring + boiled enzyme solution ..	-0-	-0-	-0	9·0	5·3
Extract of the nerve ring + sea-water ..	-0	-0-	-0-	8·5	5·1
Extract of the optic tentacles + enzyme solution ..	-0-	-0-	-0-	-0-	-0-
Extract of the optic tentacles + boiled enzyme solution ..	9·1	10·8	4·2	1·8	-0-
Extract of the optic tentacles + sea-water ..	8·9	9·1	3·8	1·6	-0-

* Details given in the text.

† BPDH: black pigment dispersion; WPDH: white pigment dispersion; RPDH: red pigment dispersion RPC H: red pigment concentration; WPCH: white pigment concentration.

Discussion

As observed in the bivalve molluscs (McVay, 1942; Nagabhushanam, 1962 and 1964) the nerve ganglia of the gastropod, *Oncidium*, contained certain principles with chromatophorotropic activity on crustaceans. It was also noted that chromatophorotropins are present in the medio-dorsal bodies and optic tentacles of *Oncidium*. The nerve ring and medio-dorsal bodies had principles with qualitatively similar chromatophorotropic activity. The extracts of these tissues evoked concentration of red and white pigments in the chromatophores of *Ocypode*. However, the qualitative nature of the principles present in the optic tentacles was different from the nature of principles present in the nerve ring and medio-dorsal bodies. The optic tentacles had principles which evoked dispersion of black, red, and white pigments in the chromatophores of *Ocypode*, and a red pigment-concentrating principle. In contrast to the nerve ring and the medio-dorsal bodies the optic tentacles are devoid of a white pigment-concentrating principle. It is known that neurosecretory cells are present in the optic tentacles of gastropod molluscs (Lane, 1962). The staining properties of the neurosecretory material of the cells in the optic tentacles were different from those of the neurosecretory material in the nerve ganglia of *Arion* (Pelluet and Lane, 1961). It is not known whether such a difference exists between the neurosecretory materials of the optic tentacles and nerve ganglia of *Oncidium*. However, it is interesting to note the differences between the chromatophorotropic activities of extracts of the optic tentacles and nerve ring of *Oncidium*.

The role of medio-dorsal bodies and their association with the cerebral ganglia of some molluscs were discussed by Lever (1958). He observed that under the medio-dorsal bodies large A_8 cells of the cerebral ganglia showed many signs of neurosecretory activity. Both the C cells of the medio-dorsal bodies and the A_8 cells of cerebral ganglia send their offshoots to the central part of the boundary between the medio-dorsal bodies and cerebral ganglia. Since these two systems had structural connections Lever (1958) suggested that they possibly constitute a neurosecretory system. Later, Joosse (1964) produced further evidence to support the endocrine nature of the medio-dorsal bodies and found that they regulate ovulation and oviposition in *Lymnaea*. In the present study it was found that the extracts of medio-dorsal bodies and nerve ring had qualitatively identical chromatophorotropic activity. These results indicate that (1) structural connections may exist between the medio-dorsal bodies and the cerebral ganglia in which case a free interflow of the secretory products into these organs may be expected or (2) cells in the medio-dorsal bodies and the nerve ganglia may elaborate secretory products of identical nature.

The principles present in the optic tentacles of *Oncidium* are resistant to heat. The melanin-dispersing principle and the red pigment-concentrating principle present in the crustacean eyestalk are resistant to heat (Kleinholz, 1966). The white pigment-concentrating substance in the nerve ring and medio-dorsal bodies of *Oncidium* was not resistant to heat. The white pigment-concentrating principle in the eyestalk of *Ocypode* was also not resistant to heat (Rao, 1965).

Although the relative solubility of the red pigment-concentrating principle of *Oncidium* in alcohol varied with the tissue tested, in general the red pigment-concentrating and white pigment-concentrating principles are soluble in alcohol and acetone. In contrast, the black pigment-dispersing, white pigment-dispersing, and red pigment-dispersing principles in the optic tentacles of *Oncidium* are insoluble in acetone and alcohol. The solubilility properties of the pigment-concentrating principles of *Oncidium* are similar to those of the pigment-concentrating principles in the eyestalks of *Ocypode* (Nagabhushanam and Rao, 1964; Rao, 1967). In contrast, the pigment-dispersing hormones in the eyestalks of mature *Ocypode* are soluble in alcohol and insoluble in acetone (Rao, 1967). The pigment-dispersing hormones in the eyestalks of juvenile *Ocypode* are relatively insoluble in alcohol and acetone (Rao, 1965) and in this respect resemble the pigment-dispersing principles present in the optic tentacles of *Oncidium*.

In the crabs, *Ocypode platytarsis* (Nagabhushanam and Rao, 1964) and *Ocypode macrocera* (Rao, 1967), it was not possible to evoke white pigment concentration using saline

extracts of eyestalks or alcohol-soluble fraction of the eyestalk. This appears to be due to the presence in the extract of a white pigment-dispersing principle that antagonizes the action of the white pigment-concentrating principle. The white pigment-dispersing principle was absent in the acetone-soluble fraction of the eyestalk. Since the white pigment-concentrating principle was soluble in acetone, the acetone-soluble fraction of the eyestalk had white pigment-concentrating activity. In *Oncidium* the white pigment-dispersing principle was absent in the nerve ring and medio-dorsal bodies. The saline extracts of these tissues evoked white pigment concentration. Although the integrated response of the white pigment concentration evoked by the different extracts of the nerve ring are nearly identical to each other, the white pigment-concentrating activity evoked by the acetone-soluble fraction of the medio-dorsal bodies was more than the saline extract. This indicates that the white pigment-concentrating principle in the medio-dorsal bodies may not be readily soluble in water and that the acetone treatment renders it soluble in water.

The electrophoretic analysis indicated that the red pigment-concentrating principle in the tissues of *Oncidium* was electropositive at pH 7·5 and in this respect was similar to the red pigment-concentrating principle of *Palaemon* (Knowles, Carlisle, and Raabe, 1955) and *Ocypode* (Rao, 1965). The pigment-dispersing principles in the aqueous extracts of eyestalks of juvenile *Ocypode* did not move from the point of application on the filter-paper after electrophoresis at pH 7·5 (Rao, 1965). Interestingly, the pigment-dispersing principles of the optic tentacles of *Oncidium* responded in a similar way.

The crustacean chromatophorotropins are believed to be peptides (Kleinholz, 1966) since they were inactivated by trypsin. The chromatophorotropins of *Oncidium* were also inactivated trypsin indicating the possible peptide nature of the substances. Although functional chromatophores are absent in *Oncidium* chromatophorotropic principles are present in the nerve ganglia, medio-dorsal bodies, and optic tentacles of the animal. In crustaceans the colour changes are controlled by neurosecretory hormones. It is interesting to find a correlation between the chromatophorotropic activity and neurosecretion in molluscs. These studies indicate that some of the neurosecretory products of *Oncidium* may be closely related to some of the neurosecretory hormones of Crustacea. It may not be imprudent to assume that these principles serve other endocrine functions in molluscs, such as ovulation and oviposition. It would be interesting to test whether the crustacean chromatophorotropins have any effect on the processes that are under neurosecretory control in molluscs. So far none of the neurosecretory hormones of molluscs were isolated in a pure form and characterized. When this goal is achieved it would be possible to determine their relationship with the crustacean hormones.

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Summary

In the present study extracts of the nerve ring, medio-dorsal bodies, and optic tentacles of the gastropod, *Oncidium verraculatum*, were injected into specimens of the crab, *Ocypode macrocera*, to determine the chromatophorotropic activity.

The saline extracts of the optic tentacles of *Oncidium* evoked dispersion of black, red, and white pigments and concentration of red pigment in the chromatosomes of *Ocypode*. In contrast, the extracts of medio-dorsal bodies and nerve ring of *Oncidium* concentrated the red and white pigments in the chromatosomes of *Ocypode*.

The principles evoking concentration of red and white pigments are soluble in ethyl alcohol and acetone. In contrast, the pigment-dispersing principles are insoluble in ethyl alcohol and acetone.

The chromatophorotropic principles in the optic tentacles are resistant to heat. The white pigment-concentrating principle in the nerve ring and medio-dorsal bodies was destroyed by heat.

Trypsin inactivated the chromatophorotropic principles indicating the possible peptide nature of the principles.

An electrophoretic analysis revealed that the red pigment concentrating principle was electropositive at pH 7·5 while the pigment-dispersing principles did not move from the point of application on the filter-paper.

The properties of the principles of *Oncidium* were compared with those of the crustacean chromatophorotropins and their possible interrelationship was discussed.

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CYTOLOGICAL STUDIES OF PACIFIC LAND SNAILS[1]

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ABSTRACT

Land snail species have been studied cytologically from the following Pacific areas: Japan, Taiwan (Formosa), Australia, Papua, the Solomon Islands, the New Hebrides, Fiji, Hawaii and the Galapagos Islands. Although such investigations are just beginning on the molluscan fauna of the Pacific islands and adjacent land masses, preliminary observations already give indications that cytotaxonomic studies will prove helpful in showing relationships among the species within this vast area, and relationships of species of this area to those of other geographic regions.

The chromosomes of 65 species of 17 Pacific land snail families have now been investigated. These families are: Helicinidae (Archaeogastropoda); Pupinidae and Assimineidae (Mesogastropoda); Ellobiidae (Basommatophora); Succineidae, Athoracophoridae, Achatinellidae, Partulidae, Enidae, Clausiliidae, Philomycidae, Zonitidae, Helicarionidae, Bulimulidae, Camaenidae, Bradybaenidae and Subulinidae (Stylommatophora).

Where information is available, chromosome numbers of Pacific land snails usually fall within the ranges already determined for related snails elsewhere in the world. Haploid chromosome numbers of land snails of the Pacific gastropod Streptoneura range from 13 to 18, and the Euthyneura range from 5 to 44.

INTRODUCTION

BECAUSE of their isolation and endemism, the faunas of the Pacific islands have always been intriguing to zoogeographers. Pacific faunas have been especially interesting to students of malacology, because Pilsbry (1900 *a*) reorganized pulmonate land snail taxonomy after studying *Partula* and *Achatinella*, both endemic to the Pacific islands, and, in a paper directly following it (Pilsbry, 1900 *b*), gave strong evidence to support theories of a former large mid-Pacific land mass. He concluded that the present islands are now superposed on the sunken heights of this ancient land mass, and that the current land snails are the descendants of an archaic fauna, which has no admixture of modern families on the continents. Because of my own work on mollusk chromosomes, and their bearing on speciation, systematics and evolution, I have been interested in extending my cytological studies to the land snails of the Pacific.

The usefulness of chromosome number surveys to systematics have been shown by Burch (1965, 1967 *a*) and Patterson (1967, 1969). The purpose of the present paper is to review cytological information, meager as it is, on Pacific land snails, to relate this information to what is known about chromosomes of land snails generally, and to discuss systematics as it relates to cytological information of Pacific land snails.

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CHROMOSOMES OF PACIFIC LAND SNAILS

Chromosomes of 65 land snail species of 17 families in the Pacific area now have been studied. These are found in the papers of Inaba (1945, 1959), Kawabe (1947), Koyama (1955), Burch (1964 *b*, 1965, 1967 *a*, *b*), Laws (1965), Patterson (1969), and unpublished observations from my own laboratory. Chromosome numbers of Pacific land snails are listed in Tables I-III. Chromosomes have been studied of snail species from the following Pacific areas: Japan, Taiwan (Formosa), Australia, Papua, the Solomon Islands, the New Hebrides, Fiji, Hawaii and the Galapagos Islands.

TABLE I

*Chromosome numbers of land Streptoneura from Pacific Islands**

Species	Chromosome Number (*n*)	Locality
Order *ARCHAEOGASTROPODA*		
Family HELICINIDAE		
Palaeohelicina sp.	18	Solomon Is.
Pleuropoma sp.	18	Solomon Is
Pleuropoma sp.	18	Solomon Is.
Order *MESOGASTROPODA*		
Family PUPINIDAE		
Pupina sp.	13	Solomon Is.
Family ASSIMINEIDAE		
Assiminea sp.	15	Solomon Is.
Adelomorpha sp.	17	Solomon Is.

* From Burch, 1967 *a*.

In the streptoneuran Archaeogastropoda, the 3 species of the Helicinidae studied of the genera *Palaeohelicina* and *Pleuropoma* from the Solomon Islands have 18 pairs of chromosomes (Table I). This is the chromosome number that has most commonly been found in the Archaeogastropoda, although the total known range of chromosome numbers in this prosobranch order is $n = 9$ to $n = 21$ (Fig. 1). No other members of this large and widely distributed family have been studied

In the Mesogastropoda, 3 species have been studied, all from the Solomon Islands. They have fewer than the modal haploid number for the order, but they are still well within the chromosome number range of the mesogastropods, $n = 7$–20. *Pupina* has 13 pairs of chromosomes. Two non-Pacific species have been studied in its superfamily (*Cyclophoroidea*), *Cochlostoma septemspirale* ($n = 13$) and *Cyclophorus aurantiacus* ($n = 14$), both of the family Cyclophoridae. Seven species have been studied in the Assimineidae (Rissoidea), 4 species from Japan ($n = 12$ and $n = 15$), 2 from the Solomon Islands ($n = 15$ and $n = 17$), and, 1 from Europe ($n = 12$). Only the Solomon Island species have terrestrial habits, the other 5 species are aquatic. One would assume that the species with 12 pairs of chromosomes are related, as are those with 15 pairs of chromosomes. If so, the Solomon Island species of *Assiminea* is probably related to the Japanese subgenus *Angustassiminea* which contains 2 other species with 15 pairs of chromosomes. *Adelomorpha* with 17 pairs of chromosomes is probably only distantly related. A careful anatomical study of the Assimineidae on a world-wide basis is desirable.

The mainly aquatic euthyneuran Basommatophora are very conservative in regard to change in chromosome numbers. If the polyploid numbers are discounted, then the haploid chromosome number range for 129 species is $n = 15$–19. The great majority of the species have 18 pairs of chromosomes, the number found in the only land basommatophoran studied in the Pacific, *Pythia scarabaeus*.

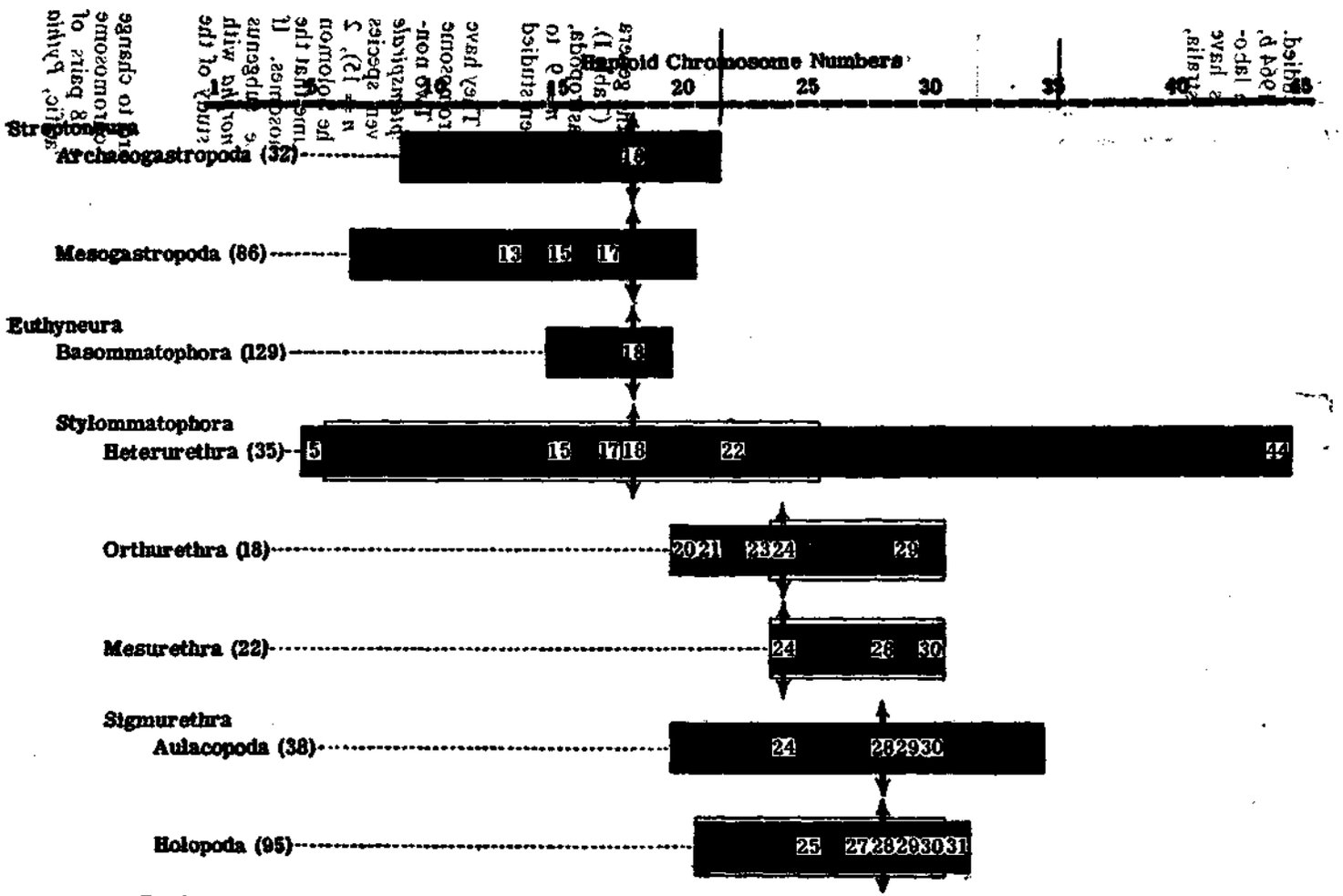

FIG. 1. Ranges of chromosome numbers in higher taxa of the Gastropoda which have land snail species in the Pacific area. Total ranges of chromosome numbers are shown by black bars. Insets refer to chromosome numbers found in Pacific area land snails. White bars mark the chromosome number ranges in non-Pacific snails in those groups that the extreme chromosome numbers are found in Pacific area snails. The arrows mark the empirical modal chromosome numbers for each group. The numbers following the group name refer to the total number of species and subspecies that have been studied. Data from present paper and Patterson, 1969

The widest range in chromosome numbers known in the mollusks (Fig. 1) occurs in the stylommatophoran suborder Heterurethra (Patterson, 1969). The highest and lowest numbers found in this group, $n = 44$ and $n = 5$, occur in the Pacific area. *Catinella rotundata*, which has the lowest chromosome number ($n = 5$) reported in mollusks, has only 1 less pair of chromosomes than does *C. gabbi*, *C. texana* and *C. vermeta* of the U.S.A., and *C. arenaria* of Europe. The close relationships of these species are therefore shown cytologically, as well as by morphology. The chromosome number of *Oxyloma japonica* ($n = 15$) has not been encountered in any other succineid species, but that of *O. hirasei* and *O. h. kwansae* ($n = 17$) is the same as that reported for *O. sarsi* from Europe by Butot and Kiauta (1964). *Succinea strubelli* of Papua and *S. kuntziana* of the New Hebrides have the same chromosome numbers ($n = 18$) as *S. campestris*, *S. concordialis*, *S. greeri*, *S. luteola* and *S. urbana* of the U.S.A. (Natarajan, Hubricht and Burch, 1966; Patterson, 1968). *Succinea lauta* and *S. l. sphaerica* have the same chromosome numbers ($n = 22$) as does *S. putris* of Europe (Perrot, 1938; Burch, 1965). The high chromosome number ($n = 44$, Patterson, 1969) of the Athoracophoridae is as anomalous as is the morphology of these strange slugs. A cytological study of the other species of this family should be interesting indeed. Although the Heterurethra exhibits a wide range of chromosome numbers, it is interesting to note that its most common chromosome number is $n = 18$, the same as that of the Basommatophora.

The Orthurethra ($n = 20$–30) are somewhat more conservative than are the Heterurethra, but their modal chromosome number ($n = 24$) is considerably higher. The lower numbers of the Hawaiian Achatinellidae correspond to their primitive position, but that of the New Hebridean *Partula turneri* ($n = 29$) of the closely related Partulidae is unexpectedly high. The 2 species of Enidae ($n = 24$) from Japan have an intermediate number of chromosomes.

In the mesurethran family Clausiliidae 17 species have been studied: 11 species from Europe and Japan with 24 pairs of chromosomes, 3 species from Japan and Taiwan with 28 pairs, 1 species from Japan with 30 pairs, and 2 species from Europe with 'about' 30 pairs of chromosomes. The unsatisfactory taxonomy of the family and its need for further cytological and morphological studies has been mentioned previously (Burch, 1965, 1967*a*; Patterson, 1969).

The modal chromosome number ($n = 28$) of the Sigmurethra, the most advanced order of the Stylommatophora, is higher than that of the Orthurethra and Mesurethra (Fig. 1). In the sigmurathran infraorder Aulacopoda, chromosome numbers range from $n = 20$ to $n = 34$, but only 4 numbers have been found in Pacific area snails, $n = 24$, 28, 29 and 30. In the infraorder Holopoda, chromosome numbers range from $n = 21$ to $n = 31$. Five chromosome numbers have been reported from this group from the Pacific, $n = 25$, 27, 28, 29 and 31. The general conservativeness in molluscan chromosome numbers can easily be noted in the holopod families Camaenidae and Bradybaenidae (Table III).

Discussion

Cytological studies of Pacific land snails have hardly begun. However, cytological investigations are currently under way, and the usefulness of such studies will increase as more information is compiled.

Where information is available, chromosome numbers of Pacific land snails generally fall within the ranges already determined for chromosome numbers of related snails elsewhere in the world. A comparison of chromosome numbers at the higher taxa level is shown in Fig. 1. At this level, the notable exceptions to correspondence of chromosome numbers are due to chromosome numbers of 2 families endemic to the Pacific area: Athoracophoridae (Heterurethra) and Achatinellidae (Orthurethra). In the world-wide Succineidae, another family of the Heterurethra, chromosome numbers of non-Pacific members range from $n = 6$ to $n = 25$. Pacific island species range from $n = 5$ to $n = 22$. In the Orthurethra, the relatively low numbers $n = 21$–23 occur in *Achatinella* and *Auriculella* of the primitive family Achatinellidae.

TABLE II

*Chromosome numbers of Basommatophora and lower Stylommatophora from Pacific Islands**

Species	Chromosome Number (*n*)	Locality
Order *BASOMMATOPHORA*		
Pythia scarabaeus	18	Solomon Is.
Order *STYLOMMATOPHORA*		
Suborder ORTHURETHRA		
Family ACHATINELLIDAE		
Achatinella mustelina	20	Hawaii, U.S.A.
Achatinella bellula	20	Hawaii, U.S.A.
Achatinella producta stewartii	21	Hawaii, U.S.A.
Auriculella auricula	23	Hawaii, U.S.A.
Family PARTULIDAE		
Partula turneri	29	New Hebrides
Family ENIDAE		
Ena andersoniana	24	Japan
Ena japonica	24	Japan
Suborder MESURETHRA		
Family CLAUSILIIDAE		
Megalophaedusa martensii	24	Japan
Phaedusa subaculus	24	Japan
Euphaedusa tau	28	Japan
Euphaedusa pseudosheridani	28	Taiwan
Hemiphaedusa similaris	28	Taiwan
Stereophaedusa japonica	30	Japan
Suborder HETERURETHRA		
Family SUCCINEIDAE		
Catinella rotundata	5	Hawaii, U.S.A.
Oxyloma japonica	15	Japan
Oxyloma hirasei	17	Japan
Oxyloma h. kwansae	17	Japan
Succinea horticola	17	Japan
Succinea kuntziana	18	New Hebrides
Succinea strubelli	18	Papua
Succinea lauta	22	Japan
Succinea l. sphaerica	22	Japan
Family ATHORACOPHORIDAE		
Aneitea sp.	44	New Hebrides

* From Inaba, 1945, 1959; Koyama, 1955; Burch, 1964 *b*, 1965, 1967 *a*, and unpublished observations; Burch and Natarajan (unpublished); Patterson (1969).

Before concluding this discussion on chromosome numbers and systematics of land snails, I would like to comment on the recent contention, based partly on chromosome numbers, that the Succineidae should be removed from the land pulmonate order Stylommatophora and established as an order of its own (Butot and Kiauta, 1967), the Succineoidea.[3] In doing so, the authors raise

[3] Butot and Kiauta say they rejected using the older names Elasmognatha and Heterurethra because they prefer to follow Taylor and Sohl (1962). However, Taylor and Sohl use Pilsbry's suborder Heterurethra, in which they subordinate the superfamilies Succineacea and Athoracophoracea.

Pilsbry's suborder Heterurethra (1900 *a*) to ordinal rank, but retain Baker's (1956) superfamilial name Succineoidea.

TABLE III

*Chromosome numbers of Sigmurethra from Pacific Islands**

Species	Chromosome Number (*n*)	Locality
Division Aulacopoda		
Family PHILOMYCIDAE		
Incilaria confusum	24	Japan
Incilaria fruhstorferi	24	Japan
Family ZONITIDAE		
Vitrinopsis sp.	28	Solomon Is
Trochomorpha sp.	28	Solomon Is.
Trochomorpha sp.	30	Solomon Is.
Dyakia striata	29	Fiji
Family HELICARIONIDAE		
Helicarion sp.	28	Solomon Is.
Division Holopoda		
Family BULIMULIDAE		
Bulimulus nux	29	Galapagos Is.
Placostylus miltochellus	30	Solomon Is.
Diplomorpha sp.	30	New Hebrides
Family CAMAENIDAE		
? *Draparnaudia singularis*	27	New Hebrides
Papuina sp.	28	Papua
Chloritobadistes sp.	29	Australia
Hadra sp.	29	Australia
Meracomelon sp.	29	Australia
Pleuroxia sp.	29	Australia
Sinumelon sp.	29	Australia
Xanthomelon sp.	29	Australia
Satsuma myomphala	29	Japan
Family BRADYBAENIDAE		
Bradybaena similaris	28	Japan
Bradybaena gainesi	29	Japan
Euhadra callizona	28	Japan
Euhadra congenita	28	Japan
Euhadra eoa	28	Japan
Euhadra grata	28	Japan
Euhadra peliomphala	28	Japan
Euhadra sadoensis	28	Japan
Euhadra sandai	28	Japan
Euhadra senckenbergiana	28	Japan
Euhadra awaensis	29	Japan
Euhadra idzumonis	29	Japan
Euhadra quaesita	29	Japan
Euhadra scaevola	29	Japan
Acuta despecta	29	Japan
Aegista vatheleti	29	Japan
Family SUBULINIDAE		
Lamellaxis mauritianus	25	Solomon Is.
Subulina octona	31	Solomon Is., Australia

* From Kawabe, 1947; Inaba, 1959; Laws, 1965; Burch, 1967 *a* and unpublished observations; Burch and Natarajan (unpublished).

Chromosome numbers of the Succineidae, except for the genus *Catinella*, form an almost unbroken series from $n = 11$ to $n = 25$ (*see* Patterson, 1969). One species of the related heterurethran family Athoracophoridae has the chromosome number $n = 44$. In the other stylommatophoran orders, chromosome numbers, as presently known, range from $n = 20$ to $n = 34$ (the low number $n = 20$ occurs in both the Orthurethra and Sigmurethra). Even without considering the Athoracophoridae, there is an overlap in chromosome numbers from $n = 20$ to $n = 25$. The generally lower chromosome numbers of the Succineidae are what would be expected if they were primitive members of the Stylommatophora, regardless of whether or not the order descended from opisthobranch or basommatophoran ancestors. Both of the latter orders are characterized by haploid chromosome numbers less than 20. The exceptionally low chromosome numbers of the succineid genus *Catinella* are certainly an anomaly, as was pointed out when they were first discovered (Burch, 1964 *a*, *b*), and have since been discussed several times (Burch, 1965, 1967 *a*). But in view of the higher chromosome numbers such as $n = 25$ and $n = 44$ in the Heterurethra, the low numbers of *Catinella* do not in themselves warrant removing the Succineidae from the Stylommatophora and forming a separate order for the family, *unless* morphological evidence strongly supports such a procedure. Therefore, we need to review the morphological basis for Butot's and Kiauta's action.

The following characters are mentioned by Butot and Kiauta: "[1] They [Succineidae] resemble basommatophorans in the broader bases of the tentacles, [2] the discrete prostate, [3] the occurrence of a posterior gastric coecum, [4] their egg capsules bound together in the spawn, [5] and a caudal vesicle not being visible during the embryonic period (from Rigby, 1965), [6] the reproductive system differs from that of the Basommatophora and agrees with the pattern of opisthobranch systems.... [7] *Succinea putris* resembles the Basommatophora in the presence of a group of chrome-hematoxylin positive cells lying under the medio dorsal bodies (from Cook, 1966), the presence of a lateral lobe, the structure of the medio-dorsal bodies and the lack of cerebral commissure nerves....Because of the Succineidae are pulmonates with the eyes at the tips of the dorsal tentacles, it would seem logical to regard them as Stylommatophora, [but] [8] other stylommatophoran 'pulmonates' with the eyes at the tips of the dorsal tentacles: the families Onchidiidae, Rathouisiidae and Veronicellidae have been removed from Stylommatophora as a separate order Soleolifera within Euthyneura... . The position at the base of Stylommatophora seems improbable, as the family, especially in the [9] elasmognathous jaw, shows an homogeneity which prevents any phylogenetical relationship to the remainder of Stylommatophora". The above 9 points, used to support chromosome numbers for creating a separate order for the Succineidae, will be discussed individually below.

(1) The broadened bases of the tentacles is a superficial character of no validity for comparing Succineidae to Basommatophora. The tentacles of these 2 taxa are quite fundamentally different both structurally and functionally, and there can be no doubt that the tentacles of the succineids are basically stylommatophoran, *e.g.*, there are 4 instead of 2, they are introvertible rather than contractile, the posterior pair bear eyes at their tips and are hollow tubes through which the tentacular retractor muscles and tentacular and optic nerves pass. That succineid tentacles may have slightly broadened bases does not liken them to those of the Basommatophora, but to the tracheopulmonate Athoracophoridae and perhaps to some of the stylommatophoran Orthurethra (e.g., *Auriculella cerea*).

(2) A discrete "prostate" gland not only occurs in some opisthobranch and basommatophoran snails, but also in many Stylommatophora (*e.g.*, the orthurethran family Achatinellidae).

(4) Although the gelatinous eggs of *Succinea putris* usually appear in masses, some other Succineidae lay their eggs singly, similar to other Stylommatophora, and, for some succineid species the fact that the eggs adhere in a mass is due to the stickiness of the egg surfaces. Even when they are laid in masses, the egg masses of succineid snails differ basically from those of the Basommatophora.

(6) Rigby (1965) places special emphasis on the functional division of *three* channels in the hermaphroditic reproductive tract of opisthobranchs, *i.e.*, a male tract, a female tract, and the path taken by allosperm received during copulation. In some opisthobranchs all 3 tracts may not be separated by distinct ducts, but nevertheless, apparently there is a functional separation. Rigby considers *Succinea* to have the opisthobranch type reproductive tract, because, in addition to having separate

male and female ducts, *Succinea putris* also has a functional ciliated groove for allosperm, whereas she is of the opinion that the Stylommatophora and Basommatophora do not have a functional or structurally separate tract for allosperm. She says, "Both spawn and these sperm [allosperm] traverse the mucous gland, for the ciliated groove which may guide ingoing sperm in *Lymnaea*... and *Physa*... is not functionally separated from the remainder of the mucous gland." However, Walter (1959) has quite clearly shown a morphological and functional ciliated fold in *Lymnaea emarginata*, evident both externally and internally along the "uterus" ("mucous gland" of Rigby). Therefore the 3-ducted reproductive tract of *S. putris* occurs sometimes in both opisthobranchs and basommatophorans (Hygrophila). Since so many of the stylommatophoran groups have not been studied in this regard, it is not known whether similar conditions also occur in some taxa of that order. We should know the condition in the Orthurethra, especially in the lower families such as Amastridae, Achatinellidae and Partulidae.

(7) Cook (1966) points out some resemblances to the Basommatophora in the central nervous system of *Succinea putris*, but concludes that in most respects the central nervous system of this species is typically stylommatophoran in character. Additionally, he says, "The morphology of the head...would confirm this opinion... . The Succineidae are unique because of their low degree of complexity. One can suppose that they are primitive stylommatophorans, and therefore a similarity between this family and the Opisthobranchia is not surprising. This would also explain the resemblance of *S. putris* to the Basommatophora". Although I have no information on similarities (3) and (5) above of *Succinea* to Basommatophora, Cook's latter statement would seem to pertain as well in regard to those characters.

Van Mol (1967) considers the Succineidae to be a natural group of the most primitive living Stylommatophora, as evidenced by their central nervous system. He considers it erroneous to place them with the opisthobranchs, for they possess the typical cerebral nerves of the Stylommatophora. He shows that the central nervous system of the Athoracophoridae deviates from that of the Succineidae, but that the 2 families share enough common features to justify placing them in the same stylommatophoran suborder.

(8) Justifying the removal of Succineidae from the Stylommatophora because the Soleolifera also have eyes at the tips of their tentacles and their removal from the Stylommatophora has been advocated by several malacologists, is negative reasoning. One must take into account the many fundamentally different morphological characters possessed by the Onchidiidae, Rathouisiidae and Veronicellidae when compared to the Stylommatophora. Since the Soleolifera are quite different in body plan from other "pulmonates", it is easier to remove them than to try to force them into a system in which they do not fit easily. Nevertheless, there is still disagreement about the placement of the Soleolifera, some authors, *e.g.*, Van Mol (1967), advocate retaining the Soleolifera within the Pulmonata. Regarding tentacles, I am not familiar with those of the Rathouisiidae, but those of the Veronicellidae, although having eyes at their tips are in other respects quite different from those of the Stylommatophora. (Admittedly, the single pair of tentacles of the Onchidiidae are introvertible, similar to those of the Stylommatophora.)

(9) The jaw in the Succineidae is basically like that of the majority of the Stylommatophora, except for the possession of a basal flange. A similar condition is found in the Athoracophoridae. This is an interesting modification of the jaw, and certainly shows affinities between the Succineidae and Athoracophoridae, but it is hardly a character to be used to separate ordinal taxa. The jaws of some other land snails are just as different when compared to the majority of the Stylommatophora. A much greater diversity of jaw structure is found within some of the basommatophoran families (*e.g.*, the Planorbidae) than is evidenced between the Heterurethra and the other Stylommatophora. The elasmognathous flange of the heterurethran jaw does not in itself indicate a separate origin for the Succineidae; perhaps it is an ancestral condition that has not been retained in the more advanced Stylommatophora. Or perhaps it has been secondarily derived.

From the above discussion it appears evident that there is no adequate morphological basis for separating the Succineidae from the Stylommatophora as a distinct euthyneuran order of equal rank. The use of chromosome numbers as a predominant character to separate higher taxa should be applied with great caution, and then only when gross morphological characters support such a taxonomic separation. The chromosome numbers of the Heterurethra *per se* do not indicate that a major systematic separation at the ordinal level is justified, especially at this stage in our knowledge about chromosome numbers in euthyneuran snails. Less than 3% of the recent species of Euthyneura have been studied, there is still no information on 59% of the families, and not a single species has been studied for 5 of the orders.

The chromosome numbers of the heterurethran Succineidae have the widest aneuploid range ($n = 5$–25) known for any taxon of the Mollusca. If the Athoracophoridae ($n = 44$) are included in the Heterurethra, then the range in chromosome numbers of this order is wide indeed. Such a wide range in chromosome numbers may be the result of the ancient age of the Heterurethra, and the long intervening time period for chromosomal change in certain groups to take place.

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RNA-ACTIVITY DURING THE DEVELOPMENT OF SLUG OOCYTES

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ABSTRACT

The employment of RNA-staining methods has shown that the secondary nucleoli are always less deeply stained than primary nucleoli in the slug, *Vaginulus*. During initial growth of the oocyte, there is an increase of nucleolar size. The cytoplasmic and nucleolar RNA also increases. In the intermediate growth phase of the oocyte cytoplasmic and nucleolar concentrations of RNA decrease and protein yolk synthesis is initiated. The fully mature oocyte filled with yolk material is characterized by the absence of cytoplasmic RNA which undergoes chemical transformation during active yolk synthesis, but the nucleoli are still RNA positive.

INTRODUCTION

ANALYSIS of the oogenesis is facilitated by the fact that all the developmental stages of oocyte in molluscs especially in the slugs (Bridgeford and Pelluet, 1952; Cowden, 1962 *a*) can be found side by side in the hermaphrodite gonad all around the year. In addition to this, certain special structural aspects of oocytes of some of the gastropods and the slugs have attracted particular attention. The oocyte nucleoli, for example of the gastropod, *Patella coerula* (Jörgensen, 1913; Ludford, 1921; Bolognari, 1959 *a*, *b*) and the slugs, *Deroceras reticulatum* (Bridgeford and Pelluet, 1952; Cowden, 1958, 1962 *a*), *Arion* (Cowden, 1962 *a*), and *Vaginulus* (Gupta, 1967) are of two types, *viz.*, (1) primary nucleolus and (2) secondary nucleolus or nucleoli, which have different behavior and chemistry during the oocyte growth.

Rebhun (1956 *a*, *b* and 1961) has described in snails and clams a special structure with electron microscope, the 'periodic lamellae' which he called yolk nucleus. Cowden (1962 *a*) in young oocytes of the slugs, has also shown the nucleoprotein rich fibres or membranes oriented parallel to the nuclear membrane by the cytochemical techniques.

In the present piece of work the author has paid more attention to studying the RNA containing cell organelles, *viz.*, nucleoli and yolknucleus rather than the conventional process of egg formation and yolk deposition in the slug, *Vaginulus* sp.

MATERIAL AND METHODS

The garden slug, *Vaginulus* sp., was collected from the damp grassy grounds near Chandigarh during the months of June to September. The ovotestes were dissected from the live specimens in normal physiological saline, and then the ovotestes were cut into small pieces by a sharp blade. Zenker (3 h.) and Carnoy (½ h.) fixatives were employed for the fixations of the material. For general morphological studies Mallory's phosphotungstic acid-haematoxylin method was used. Methyl green/pyronin G, azure B, toluidine blue/methyl green/orange G techniques along with extraction of RNA in perchloric acid were employed for staining RNA. Mercuric bromphenol blue and acetone/Sudan black B were used for demonstration of proteins and lipoproteins respectively (*see* Barka and Anderson, 1963).

OBSERVATIONS

The youngest oocytes are differentiated from the germinal epithelium of a follicle and remain in the vicinity of it throughout their growth. The oocyte can be distinguished from the germinal epithelium and spermatocytes by their slightly larger size and by the presence of a distinct basophilic nucleolus. To begin with, there is a large single nucleolus in the big rounded nucleus (Fig. 1). Later as growth proceeds, there are usually two nucleoli present in the nucleus, one being larger than the other (Figs. 2 and 3). The staining behavior of these two nucleoli differs considerably. The primary nucleolus is always smaller in size and stains more deeply with pyronin G, azure B, toluidine blue, Sudan black B (in acetone) and mercuric bromphenol blue than the secondary nucleolus. In a few sections the secondary nucleolus is seen migrating from the nucleus to the ooplasm during growth phase of the oocytes (Fig. 4). Initially, the primary and the secondary nucleoli are stained homogeneously with haematoxylin, azure B, toluidine blue, pyronin, Sudan black B and bromphenol blue dyes (Figs. 2 and 3), but as growth proceeds the secondary nucleoli show vacuolization (Fig. 2).

The nucleoplasm is negative for basic dyes like azure B, pyronin, etc., in the initial growth phase (Figs. 1 and 3), but in the late growth phase it starts showing colouration in the RNA staining techniques (Figs. 2 and 5) and with Berenbaum's technique for lipoproteins (Fig. 6). In the vitellogenic oocytes, besides the nucleolus in the necleoplasm, some small RNA and protein rich granules are also seen (Fig. 7). There is no evidence that during the oogenesis of *Vaginulus* the nucleolar material passes out from the nucleus to the ooplasm except for the secondary nucleolus which migrates as a whole from the nucleus to the ooplasm in some oocytes. However, throughout the ooplasm in the young oocytes there are large, distinct RNA rich granules present (Fig. 1). In slightly advanced oocytes the ooplasm does not show any granulation, and the concentration of ooplasmic RNA decreases progressively.

A careful examination of the ooplasm of the partially grown oocytes indicates that cytoplasmic basophilia is associated with definite membranous components which are especially heavy around the nucleus (Figs. 2, 3, 8 and 9). This component is termed here as yolk nucleus. The yolk nucleus in the present material is rich in RNA, proteins, and lipoproteins (Figs. 2, 7–9). In phosphotungstic acid-haematoxylin preparations, the mitochondria and the yolk nucleus stain blue with haematoxylin (Fig. 3). The mitochondria are granular in form and are thickly associated with yolk nucleus (Fig. 3). Throughout the growth phase, the yolk nucleus is attached to the nuclear membrane. With the attenuation of the membranous component of the yolk nucleus the vitellogenesis starts (Fig. 7). The thinning out of the yolk nucleus is proportional to the amount of yolk deposition.

DISCUSSION

The aspect of slug oocyte growth which has proven to be most interesting and which is undoubtedly shared by some other gastropod molluscs, particularly *Patella coerula*, is the presence of two distinct types of nucleoli in the germinal vesicle. This condition was first investigated in *Patella* by Jörgensen (1913). Later Ludford (1921), Bolognari (1959 *a*, *b*) and Cowden (1962 *a*) in the slugs, *Deroceras* and *Arion* gave morphological and cytochemical account of both the types of the nucleoli with light and electron microscope. Two nucleoli have been observed in the oocytes of the slug, *Vaginulus*, but the primary nucleolus which always stains deeply with azure B, pyronin G and Sudan black is always smaller in size than that of secondary nucleolus. On the contrary, Cowden (1962 *a*) has shown that the primary nucleolus is always bigger in size. The number of secondary nucleoli reaches up to five in the case of *Arion* (Cowden, 1962 *a*). In the oocytes of the slug examined presently, it is the primary nucleolus which maintains normal form, position, and tinctorial behavior, while the secondary nucleolus migrates to the ooplasm, at least in some oocytes if not in all.

The primary nucleolus in the present material is rich in RNA, proteins, and lipoproteins and behaves as true nucleolus in the oocytes of various animal species. The secondary nucleolus is poor in RNA and lipoproteins, but in mercuric bromphenol blue it stains deeply. These observations suggest that both the nucleoli differ in their ribonucleoprotein constituents. Brown and Ris (1959) in amphibian oocytes have shown two types of nucleoli, one which stains deeply in RNA staining methods and the other which does not. These observations are further confirmed by the cytochemical studies of Cowden (1962 *a*) who has shown the absence of 'nucleolonema' in secondary nucleoli of the slug species.

The existence of a distinct fibrous or membranous system in the ooplasm of the growing oocytes has been described with light microscope in ascidians (Cowden, 1962 *b*) and in slugs (Cowden, 1962 *a*). With electron microscope Rebhun (1956 *a*, *b* and 1961) described these components in some molluscs. The reality of the membranous component of the ooplasm is supported by its presence in material fixed in Zenker for 3 *h*. and Carnoy for ½ *h*. during the present investigations. The membranous components are highly basophilic in nature. Rebhun has also shown the basophilic nature of these components in *Spisula* by employing azure B staining after Carnoy. The membranous component (yolknucleus) is also rich in proteins and lipoproteins. Gupta (1966) has clearly shown in insect oocytes that RNA rich structures are also rich in lipoproteins.

During vitellogenesis the membranes gradually become attenuated. This shows their close relationship with yolk formation and, therefore, the whole membranous system of the early oocytes is termed here as 'yolknucleus' (*cf.* Nath, 1968). With lack of direct electron microscopic evidence, it is impossible to associate the basophilic ooplasmic membranous components with the endoplasmic reticulum.

In his Fig. 1 Cohen (1966) has drawn the sequence of reactions required for the translation of the sequence of nucleotides in DNA into protein structure. He has shown that DNA is transcribed to give ribosomal, soluble and messenger RNA. The ribosomal RNA combines with proteins to form ribosomes, which can then attach to messenger RNA to form polysomes. In the polysomes the information contained in the messenger RNA is translated so as to govern the formation of specific polypeptides. The oocyte filled with yolk material in *Vaginulus* is characterized by the disappearance of the cytoplasmic RNA which might have been converted into polypeptides through an intermediate stage of polysomes.

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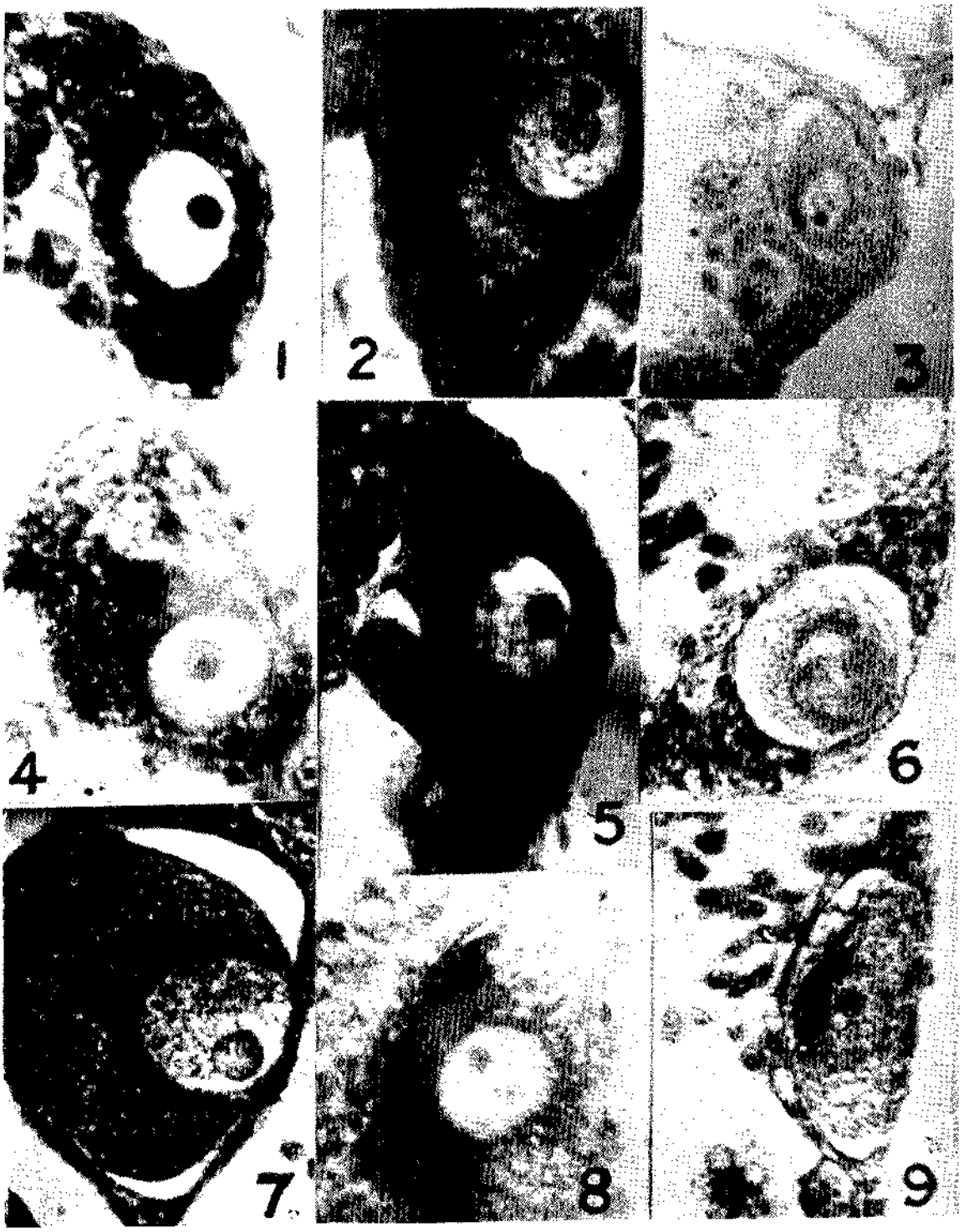

FIGS. 1–9

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LEGEND TO PLATE I

FIGS. 1–9. Fig. 1. Oocyte showing primary nucleolus and RNA-rich material in the very young oocyte. Azure B, ×4,000 approx. Fig. 2. Two nucleoli are present in the germinal vesicle, primary nucleolus is small and secondary is big. The nucleoplasm is also stained, and membranous components are present near the nuclear membrane, Azure, B, ×1,600. Fig. 3. Secondary nucleoli is stained homogeneously in a small oocyte adjacent to larger one in which the secondary nucleolus shows vacuolization. Phosphotungstic acid-haematoxylin, ×1,600. Fig. 4. Secondary nucleolus passing from the germinal vesicle to ooplasm (Arrow). Azure B, ×5,000, Fig. 5. Primary nucleolus, nucleoplasm and ooplasm are stained with Azure B, ×1,600. Fig. 6. Nucleoplasm showing bound lipids in Berenbaum's technique, ×1,600. Fig. 7. Vitellogenic oocyte showing RNA granules in the nucleoplasm. A few membranes are seen attached with the nuclear membrane. Azure B, ×1,600. Fig. 8. Membranous component of the yolk nucleus arranged parallel to the nuclear membrane, previtellogenic oocyte. Azure B, ×4,000. Fig. 9. Previtellogenic oocyte having membranous saucer-shaped yolk nucleus near the nuclear membrane. Berenbaum's technique, × 1,600.

MUCOPOLYSACCHARIDES IN THE SALIVARY GLAND CELLS OF CERTAIN GASTROPOD MOLLUSCS

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ABSTRACT

Four gastropods, namely, *Pila globosa* (Swainson), *Cryptozona* (*Xestina*) *semirugata* (Beck), *Laevicualis alte* (Ferrusac) and *Oncidium verraculatum* (Cuvier) were selected for studies. Various histochemical stains and techniques like PAS, AB, AB-PAS, Toluidine blue (0·003%), Azure A, pyridine extraction and methylation—demethylation procedures were applied to investigate mucopolysaccharides in the gland cells.

Histologically, the gland cells are of two types but, histochemical investigations suggest that probably there are more than two types of cells.

The gland cells contain carbohydrates and acid mucopolysaccharides in them. Sulphomucopolysaccharides form major and oxidizable vicinal hydroxy group minor secretory components.

INTRODUCTION

OUR knowledge of gastropod salivary glands is limited. It is mostly centered round their histology, physiology, and cytology (Pacau and Vigier, 1905–06 ; Frankenberger ; 1923 ; Bowen, 1926; Müller, 1957). To seek more information, an attempt has been made to study the presence of mucopolysaccharides in the gland cells.

MATERIAL AND METHODS

Four gastropods, namely, *Pila globosa* (Swainson), *Cryptozona* (*Xestina*) *semirugata* (Beck), *Laevicaulis alte* (Ferrusac), and *Oncidium verraculatum* (Cuvier) were selected for study. Animals were dissected alive and tissues were fixed in Bouin's fluid (18 hours) and calcium acetate formalin (Lillie, 1954) for histological and histochemical investigations respectively. Blocks were prepared and cut at 8 micra by usual methods. For histological studies Delafield's haematoxylin and alcoholic eosin were used. For histochemical investigations techniques used were PAS (Pearse, 1960), AB (Mowry, 1956), AB-PAS (Mowry, 1956), and Toluidine blue (0·003%) aqueous, Azure A at pH 1·5 and pH 4·5 (Spicer, 1960). The techniques were followed by routine control methods like pyridine extraction (Baker, 1946), methylation-demethylation (Spicer, 1960), and acid hydrolysis (Quintarelli, 1963).

OBSERVATIONS

Histological

The gland in each animal is composed of two types of cells, cell type-A and cell type-B. The cell type-A is round in *Pila* and *Cryptozona* and conical in *Laevicaulis* and *Oncidium*. It does not possess much secretion. In sections it always looks empty (Plate I, Figs. 1, 2, 3, *a*). The cell type-B is also round in *Pila* and *Cryptozona* and conical in *Laevicaulis* and *Oncidium*. It is rich in secretion and stains intensely with haematoxylin (Plate I, Figs. 1, 2, 3, *b*).

Histochemical

PAS.—Both cell types were PAS positive. They demonstrated light-pink staining. Among them, cell type-B was brightly stained (Plate I, Figs. 4, 5 *b*). Pyridine extraction did not affect the staining.

AB.—Both cell types were AB-positive, but cell type-B was intensely stained (Plate I, Figs. 6–9).

AB-PAS.—The cell type-A demonstrated light bluish-green staining, while the cell type-B in all cases particularly in *Cryptozona* showed different shades ranging from deep blue to purple and pink.

Methylation-demethylation.— Methylation at 60° C. for four hours reduced AB staining completely from cell type-A which, subsequent demethylation failed to restore. In cell type-B, methylation at 60° C. for four hours reduced AB staining considerably but not completely. Subsequent demethylation failed to restore the staining that was lost during methylation.

Acid hydrolysis.—The hydrolysis did not affect AB staining.

Toluidine blue.—Cell type-A was orthochromatic. Cell type-B was metachromatic. It demonstrated moderate metachromasia.

Azure A.—Cell type-A remained orthochromatic at both pHs., pH 1·5 and pH 4·5. Cell type-B was metachromatic at both pHs. With pH 1·5 it demonstrated light metachromasia; with pH 4·5 it showed moderate metachromasia.

Conclusions

The findings throw light on two important aspects: (1) They indicate that though histologically the salivary gland cells are made up of two cell types, AB-PAS sequence suggests that histochemically they are formed of more than two cell types; (2) They indicate that carbohydrates and mucopolysaccharides are present in them. AB-PAS sequence probably suggests the presence of oxidisable vicinal hydroxy group (Mowry, 1963) and acid mucopolysaccharides. The methylation-demethylation procedure suggests that the mucopolysaccharides are of two types: one resistant to methylation and non-resistant demethylation, while the other is lost during methylation itself. The former may be due to sulphated or Sialic acid groups, while the latter may be due to unknown chemical material. The result obtained with acid hydrolysis suggests absence of sialic acid. Results with Toluidine blue and Azure A at pH 1·5 support the presence of a sulphated group.

In the opinion of Dr. Spicer (Personal communication, 1966) there is a possibility of one type of secretion rather than two. In four hours methylation at 50° C. this substance may be only partially desulphated; and, if glucosidic bonds are susceptible to subsequent alcoholic KOH, then the latter step would complete the removal of acid groups. Considering this view I feel that methylation-demethylation procedure indicates presence of two types of material. It is possible that both materials are sulphated, but definitely they are of different types.

Acknowledgement

I am grateful to Dr. Spicer for his suggestion. My thanks are due to Dr. Kamat, Head of Zoology Department, Shivaji University, for guidance and authorities of N. Wadia College for facilities.

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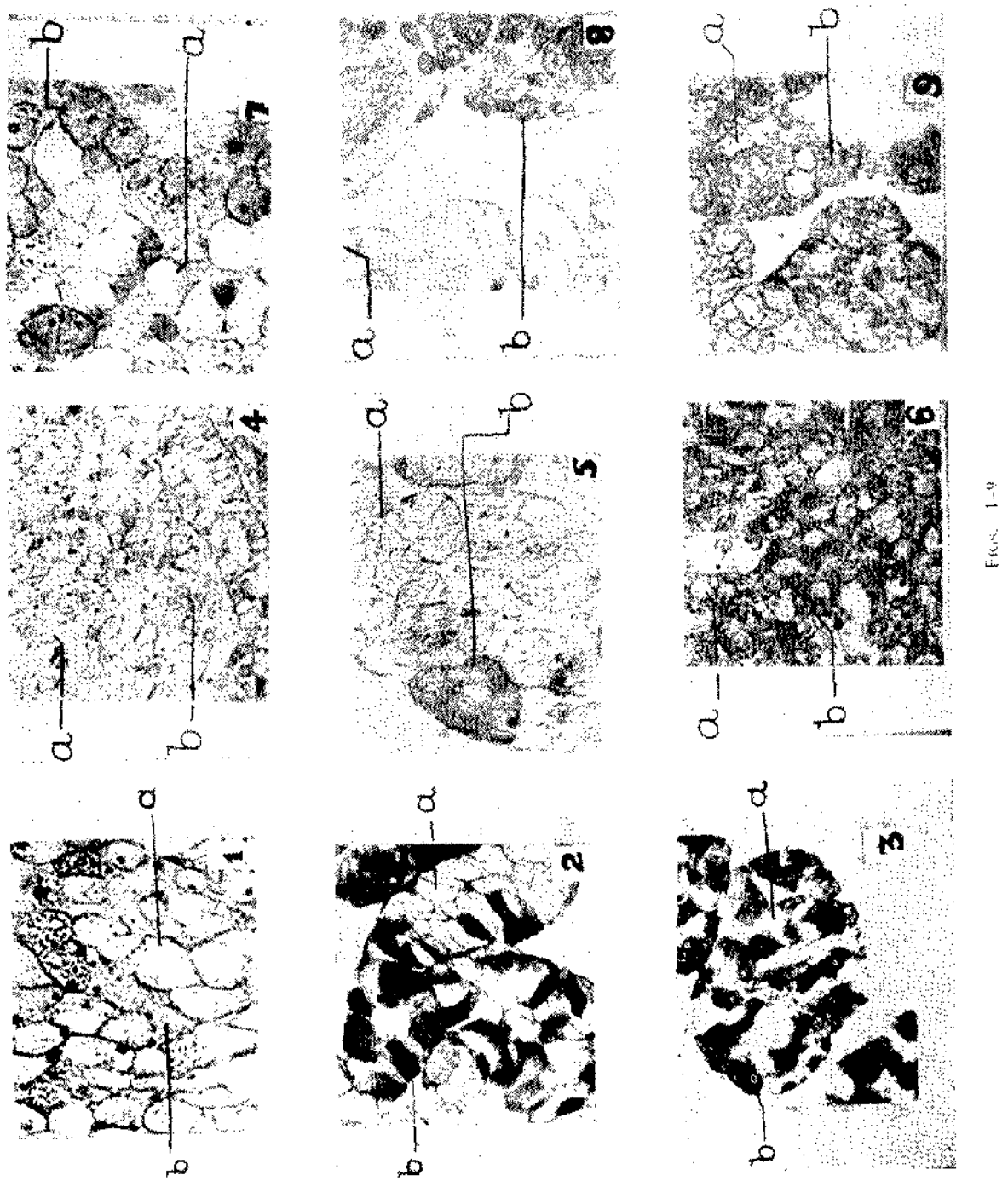
Legend to Plate I

Figs. 1–9. Figs. 1–3. Photomicrographs showing histology of salivary gland cells. Fig. 1. T.S. of salivary gland of *Cryptozona* (*Xestina*) *semirugata*, ×500. Fig. 2. T.S. of salivary gland of *Laevicaulis alte*, ×500. Fig. 3. T.S. of salivary gland of *Oncidium verraculatum*, ×500. Figs. 4–9. Photomicrographs showing presence of mucopolysaccharides in salivary gland cells. Fig. 4. T.S. of salivary gland of *Pila globosa*, PAS staining, ×400. Fig. 5. T.S. of salivary gland of *Laevicaulis alte*, PAS staining, ×400. Fig. 6. T.S. of salivary gland of *Pila globosa*, AB staining ×350. Fig. 7. T.S. of salivary gland of *Cryptozona* (*Xestina*) *semirugata*, AB staining, ×320. Fig. 8. T.S. of salivary gland of *Laevicaulis alte*, AB staining, ×400. Fig. 9. T.S. of salivary gland of *Oncidium verraculatum*, AB staining, × 400.



FIGS. 1–9

A CYTOLOGICAL AND CYTOCHEMICAL STUDY OF THE NEUROSECRETORY CELLS IN THE BIVALVE, *MERETRIX CASTA*

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ABSTRACT

In *Meretrix casta* two types of neurosecretory cells could be found with Gomori's chrome-haematoxylin-phloxin method (CHP): (1) Gomori positive cells with blue-black neurosecretory material (NSM) and (2) phloxinophilic cells with red NSM. Transport of this NSM along the axons could be observed in both the cell types. When paraffin sections of neurosecretory cells were stained with Sudan Black B, they were found to be strongly sudanophilic. The NSM had disappeared after treating the sections with alcohol. The Gomori positive material also stained strongly with aldehyde-fuchsin (AF). With P.A.S. positive reaction was observed. It is interesting that, in addition to the neurosecretory materials mentioned, almost all nerve cells showed limited numbers of fuchsinophilic granules (with AF), differing from the real AF positive granules in size and staining properties.

THE presence of neurosecretory cells in molluscs was first reported by Scharrer (1935). She observed cells filled with secretory droplets in the cerebral and visceral ganglia of the gastropod, *Aplysia*. Since then the neurosecretory cells have been reported in several gastropods [*see* Nagabhushanam (1966) for other literature on gastropods]. Despite the considerable amount of research that has been done on the neurosecretory system of gastropods, little attention seems to have been paid to the class, Lamellibranchiata. Gabe (1955) in a short note mentioned the presence of neurosecretory cells in 20 species of lamellibranchs. Lubet (1956) described the relationship between neurosecretion and sexual cycle in two marine bivalves, *Mytilus* and *Chlamys*. Nagabhushanam (1962 *a*) distinguished two cell types in the oyster, *Crassostrea virginica*.

The present study was undertaken with the aim of extending our knowledge on neurosecretion in the lamellibranchs.

MATERIAL AND METHODS

Adult specimens of *Meretrix* were collected in the vicinity of the Marine Biological Station, Porto Novo. As soon as the specimens were brought to the laboratory, the shells were opened and the soft parts were fixed in bouin's fluid. The various ganglia were then dissected out, dehydrated in alcohol, cleaned in xylol, and embedded in paraffin. Serial sections were cut 6 to 10 μ in thickness and stained with chromalum-haematoxylin-phloxin (Gomori, 1941) and Mallory's triple stain.

RESULTS AND DISCUSSION

The neurosecretory cells were found to be distributed along the dorsal surface of the cerebral, pedal, and visceral ganglia. On the basis of the difference in size and staining reactions, two kinds of neurosecretory cells are recognized in the cerebral and visceral ganglia and one type in the pedal ganglia. The main characters of the two cell types are as follows:

Cell Type I.—The cell bodies are pyriform in some sections but are often irregular in shape. These cells measure about 25 to 30 μ in length and 10 to 15 μ across the broadest part. The nucleus is round in shape, has little chromatin, and has one large nucleolus; the nucleus may be either central or eccentric in position. The secretory material stains blue-black with Gomori's stain and red with Mallory's stain. These cells are present in all the ganglia.

This cell type bears a close resemblance to the pyriform-shaped cells of *Tegulus* (Nagabhushanam, 1964).

Cell Type II.—This cell type, restricted to the cerebral and the visceral ganglia, is oval or round and has a diameter of about 20 to 25 μ. Their nuclei are similar to those of Type I cells. Some cells contain a few large vacuoles that almost completely fill the cytoplasm. The secretory material stains red with Gomori's and Mallory's stains. These cells appear to be abundant in the visceral ganglia where they are arranged in some follicles along the dorsal side. They resemble in shape and tinctorial properties the Type II neurosecretory cells of *Bankia* (Nagabhushanam, 1962 *b*) and the Cell Type II of *Tegulus* (Nagabhushanam, 1964).

Axonal transport

In both the neurosecretory cell types the secretory material is observed near the proximal end of the axon. Occasionally, the secretory droplets of different sizes are observed in the neuropile of all the ganglia. The granules are also found inside all nerves.

Cytochemistry

In order to find out the chemical nature of the neurosecretory material a few cytochemical techniques were applied on the Type I neurosecretory cells, as they are larger in size, for observations. In sections fixed in Bouin's fluid and stained with Einarson's gallocyaninchromalum (Pearse, 1960), the cytoplasm of the neurosecretory cells showed blue coloration indicating a concentration of RNA. A positive test for PAS was obtained in diastase-digested sections. The neurosecretory material has disappeared from paraffin sections fixed in 80% ethanol. This could be due to the lipid-solvent action of alcohol. A study of the adjacent sections of the ganglia, one stained with Mallory's and the other with Sudan Black B, demonstrated the sudanophilic nature of the neurosecretory material. These various observations reveal that the cytoplasm of the neurosecretory cells contains a high concentration of RNA, and the positive tests for PAS and lipid seem to be due to the presence of glycolipid.

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CHROMOSOMES OF MOLLUSCS

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ABSTRACT

Recent chromosome studies of molluscs have encompassed cytotaxonomy, cytogenetics and medical zoology. Early reports of chromosome numbers in molluscs date from the late nineteenth and early twentieth centuries, but these older reports need verification.

Chromosome numbers have been reliably reported for representatives of the molluscan classes Gastropoda, Pelecypoda, and Cephalopoda. Haploid chromosome numbers range from $n = 10$ to $n = 23$ among the 22 species of pelecypods studied. Two species of cephalopods have 28 pairs of chromosomes. Cytological information is more extensive for the Gastropoda. Chromosome numbers in the gastropod subclass Streptoneura range from $n = 7$ to $n = 36$ in the 140 species studied excluding possible polyploidy in the Cerithiacea. In the streptoneuran order Archaeogastropoda, 32 species representing 8 families have haploid chromosome numbers ranging from $n = 9$ to $n = 21$. Eighty-six species from 17 families of the Mesogastropoda have a chromosome number range of $n = 7$ to $n = 20$ (excluding polyploidy). In the order Neogastropoda, 23 species (6 families) have haploid chromosome numbers ranging from $n = 28$ to $n = 36$, except for *Purpura lapillus*, which has races with 13 and 18 pairs of chromosomes.

In the gastropod subclass Euthyneura, haploid chromosome numbers range from $n = 5$ to $n = 44$, excluding polyploidy, among the more than 400 species studied. The various opisthobranchiate orders have chromosome numbers that range from $n = 7$ to $n = 18$ in the 81 species from the 30 families that have been investigated. In the order Basommatophora, 129 species comprising 10 families have haploid chromosome numbers ranging from $n = 15$ to $n = 19$ (excluding polyploidy). Haploid chromosome numbers in the order Stylommatophora range from $n = 5$ to $n = 44$ for 216 species and subspecies (32 families).

Members of both the Streptoneura and Euthyneura exhibit a conservativeness in regard to chromosomal change. Variation in haploid chromosome numbers seldom exceeds ± 2 bivalents from the basic numbers characterizing the lower taxa. Existing variation in chromosome numbers have most likely been derived through aneuploidy, except for the few demonstrated cases of polyploidy. Many of the euthyneuran groups considered "advanced" or "specialized" also have higher haploid chromosome numbers, reflecting aneuploid changes involving additions of chromosomes to the complement. However, such a cytotaxonomic correlation is not clearly evident in the Streptoneura, except perhaps in the Viviparacea. Cytological information has been shown to be helpful in species discrimination in both the Streptoneura and Euthyneura.

Cytogenetic studies of molluscs include those concerned with hybrid studies, polyploidy, chromosome morphology and behavior, karyotype analyses, sex chromosomes and supernumerary chromosomes.

Cytological studies have been important in aspects of medical zoology, especially in regard to clarifying systematics of the intermediate hosts of *Schistosoma haematobium* and *S. japonica*. Cytology has also been useful in dealing with intermediate hosts of paragonimiasis.

INTRODUCTION

AN increasing number of studies on molluscan chromosomes have been conducted in recent years. The relationships of chromosome numbers and systematics in certain gastropod taxa have been previously summarized by Burch (1965 *b*, 1967 *d*), Inaba (1961 *b*) and Patterson (1967 *b*). The purpose of this paper is to (1) consolidate the information presented in the previous summaries; (2) add

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the results of recent research; (3) review available cytological information for the Pelecypoda and Cephalopoda; and (4) to review cytological studies other than cytotaxonomy.

Studies on chromosomes of molluscs date from the late 1800's, but due to inferior optical equipment and methods many of the early reports were inaccurate. The paraffin section technique, used in all of the early studies, compresses the chromosomes from 1/3 to 1/2 of their size as seen in acetic-orcein squash preparations, the technique used by most of the more recent researchers. It is difficult to make accurate chromosome number determinations from paraffin sections, and karyotype analyses are virtually impossible from such slides. Information contained in the old reports are not included in the following discussion. The pertinent and reliable papers on which this current review is based, and other papers concerned with molluscan chromosomes, are indicated by an asterisk in the literature cited.

Review of Chromosome Numbers

There have been a number of studies specifically intended to elucidate chromosome numbers of molluscs. In addition to papers on cytotaxonomy, incidental reports of chromosome numbers have been included in studies on hybridity, polyploidy, sex determination, parthenogenicity, supernumerary chromosomes, karyotype analyses and medical zoology. Chromosome numbers are known for less than 0·5% of the species of recent molluscs. They have been accurately determined for 22 species of the Pelecypoda. Chromosomes of the classes Amphineura and Scaphopoda have not yet been studied.

Chromosome numbers of the pelecypods range from 10–23 haploid (Table I). In the order Filibranchia, thirteen species of oysters have haploid chromosome numbers of $n = 10$ or 12 while haploid chromosome numbers range from $n = 12$ to $n = 23$ in the order Eulamellibranchia. *Sphaerium corneum* ($n = 18$) (order Telodesmacea, family Sphaeriidae) is the only freshwater pelecypod with a reliably determined chromosome number.

In the class Cephalopoda, chromosome numbers have been determined for only 2 species, *Octopus vulgaris* and *O. variabilis*, each with the haploid number $n = 28$ (Table I). With such dearth of chromosomal information in the Pelecypoda and Cephalopoda, little can be concluded concerning cytological relationships among their taxa. They may exhibit the conservativeness of chromosome numbers that is evident in many gastropod groups. For example, most American oysters have 10 pairs of chromosomes, and both octopods have the most number $n = 28$.

In the class Gastropoda, Taylor and Sohl (1962) list 121 recent families for the subclass Streptoneura and 174 for the subclass Euthyneura. Chromosome numbers have been determined for species of 23 streptoneuran families (*ca.* 19%) and 72 euthyneuran families (*ca.* 41%). The haploid chromosome numbers reliably reported in the various superfamilies of the Streptoneura are shown in Fig. 2.

The streptoneuran order Archaeogastropoda contains 21 families and has chromosome numbers that range from $n = 9$ to $n = 21$ in the 8 families investigated. Seventeen of the 81 families of the Mesogastropoda have chromosome numbers ranging from $n = 7$ to $n = 20$ (excluding polyploidy). In the more advanced Neogastropoda, chromosome numbers range from $n = 28$ to $n = 36$ (except for 1 species) in 6 of the 19 recent families.

Reliable information concerning chromosome numbers is available for 32 species of the order Archaeogastropoda (Table II). The superfamily Pleurotomariacea contains 3 recent families with only 1 species, *Haliotis japonica* ($n = 17$) (Haliotidae), having been studied cytologically. The Fissurellacea contains 1 family, the Fissurellidae, of which 3 species have been investigated:

TABLE I
Chromosome numbers of Mollusca

Species	Haploid No.	Source	Reference
Class GASTROPODA (Tables II–XX)			
Subclass STREPTONEURA (Tables II–VI)			
Order *ARCHAEOGASTROPODA* (Table II)	9–21		
Order *MESOGASTROPODA* (Tables III–V)	7–60		
Order *NEOGASTROPODA* (Table VI)	13–36		
Subclass EUTHYNEURA (Tables VII–XX)			
Order *NOTASPIDEA* (Table VII)	12		
Order *NUDIBRANCHIA* (Table VIII)	13		
Order *CEPHALASPIDEA* (Table VII)	17–18		
Order *SACOGLOSSA* (Table VII)	7–17		
Order *ENTOMOTAENIATA* (Table VII)	17		
Order *ANASPIDEA* (Table IX)	17		
Order *SOLEOLIFERA* (Table IX)	16–18		
Order *BASOMMATOPHORA* (Tables X–XIV)	15–72		
Order *STYLOMMATOPHORA* (Tables XV–XX)	5–44		
Class PELECYPODA			
Order *FILIBRANCHIA*			
Family MYTILIDAE			
Mytilus edulis	12	U.S.A.	Menzel, 1968 (*see* addendum)
Family OSTREIDAE			
Crassostrea amasa	10	Australia	Menzel, 1968
C. angulata	10	England	Menzel, 1968
C. commercialis	10	Australia	Menzel, 1968
C. iredalei	10	Philippines	Menzel, 1968
C. gigas	10	U.S.A.	Menzel, 1968
C. rhizophorae	10	Puerto Rico Canal Zone	Menzel, 1968
C. virginica	10	U.S.A.	Longwell *et al.*, 1967 (*see* addendum); Menzel, 1968
Ostrea edulis	10	Europe	Longwell *et al.*, 1967
O. equestris	10	U.S.A.	Menzel, 1968
O. lurida	10	U.S.A.	Ahmed and Sparks, 1967 (*see* addendum)
O. ariakensis	12	Japan	Kobayashi, 1954
O. laperousi	12	Japan	Kobayashi, 1954
Order *EULAMELLIBRANCHIA*			
Family CARDIIADE			
Dinocardium robustum	12	U.S.A.	Menzel, 1968
Family PHOLODAIEDAE			
Cyrtopleura costata	17	U.S.A.	Menzel, 1968
Barnea truncata	17	U.S.A.	Menzel, 1968
Family MACTRIDAE			
Labiosa plicatella	18	U.S.A.	Menzel, 1968
Mulinia lateralis	18	U.S.A.	Menzel, 1968
Family VENERIDAE			
Chione cancellata	19	U.S.A.	Menzel, 1968
Mercenaria mercanaria	19	U.S.A.	Menzel and Menzel, 1965
M. campechiensis	19	U.S.A.	Menzel and Menzel, 1965
Family PETRICOLIDAE			
Petricola pholadiformis	23	U.S.A.	Menzel, 1968
Order *TELODESMACEA*			
Family SPHAERIIDAE			
Sphaerium corneum	18	Europe	Keyl, 1956
Class CEPHALOPODA			
Order *OCTOPODA*			
Family OCTOPODIDAE			
Octopus vulgaris	28	Japan	Inaba, 1959 *d*
O. variabilis	28	Japan	Inaba, 1959 *d*

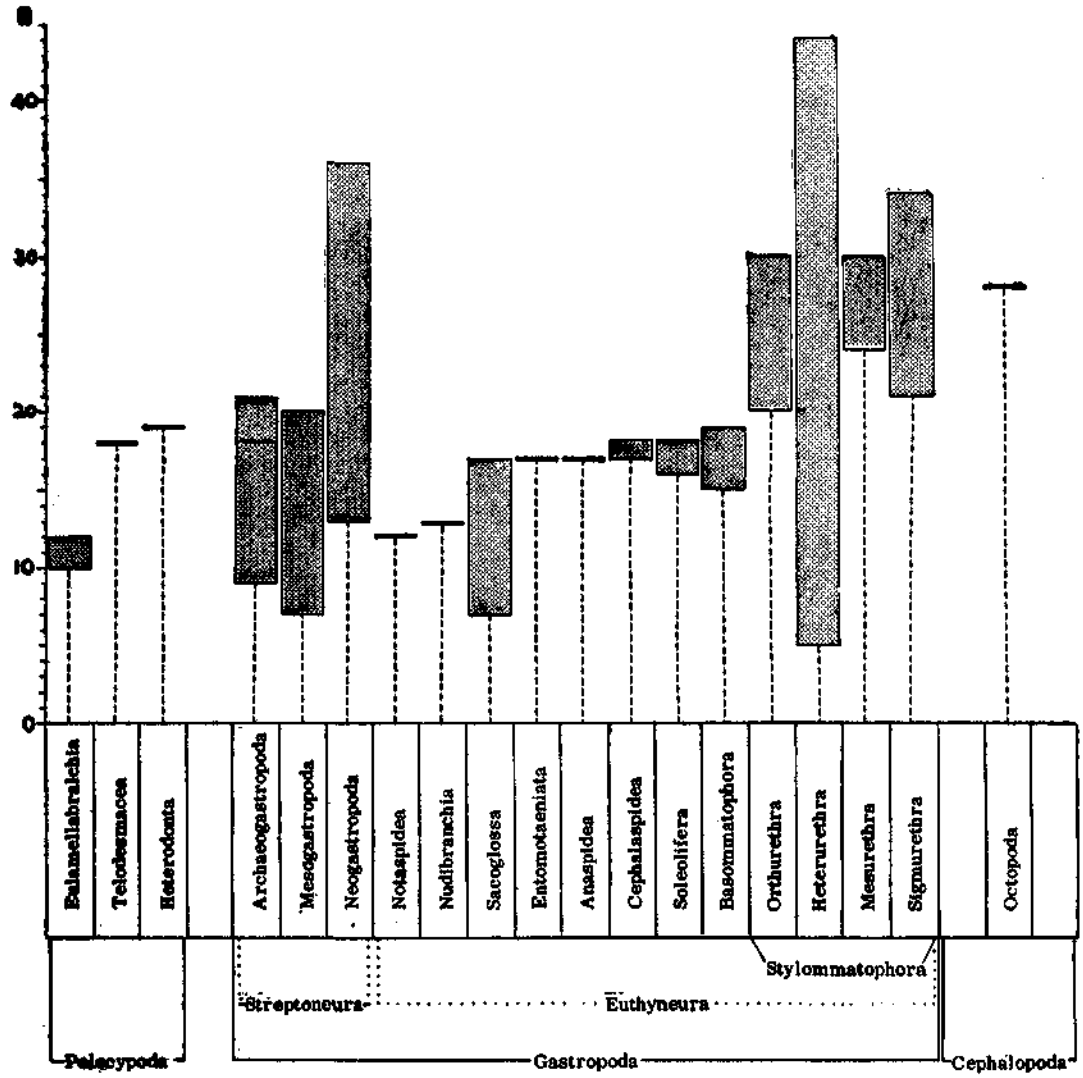

FIG. 1. Haploid chromosome numbers (n) of the Mollusca (excluding polyploidy). The stippled areas indicate the range between the lowest and highest numbers.
[*See* Table I for revisions and additions concerning the Pelecypoda].

Macroschisma sinensis, $n = 16$; *M. dilatata*, $n = 16$; and *Clypina picta*, $n = 17$. In the Patellacea chromosome information is available for 2 of the 3 families, *i.e.*, the Acmaeidae and Patellidae; all 9 species studied have the haploid number $n = 9$. Chromosome data are available for 2 of the 6 families in the Trochacea. The species of Trochidae and Turbinidae have the haploid chromosome number $n = 18$, except for *Stomatella lyrata*, which has $n = 21$. In the superfamily Neritacea, 7 species belonging to 2 of the 6 recent families have been studied cytologically. Haploid chromosome numbers in the family Neritidae vary with the species; *Puperita japonica* ($n = 11$), *Clithon retropictus* ($n = 12$), *Septaria* sp. ($n = 12$) and *Dostia violacea* ($n = 14$). The 3 species of the Helicinidae studied all have a higher number ($n = 18$).

A total of 86 species and subspecies of the streptoneuran order Mesogastropoda have been investigated (Tables III–V). They have chromosome numbers that range from $n = 7$ to $n = 20$

(excluding species that may be polyploids). The superfamily Cyclophoracea (Table III) contains 5 recent families with chromosome information available for 3 species belonging to 2 families. They have the haploid chromosome numbers $n = 13$ and $n = 14$.

TABLE II

Haploid chromosome numbers of the Archaeogastropoda

Species	Haploid No.	Source	Reference
Order *ARCHAEOGASTROPODA*			
Superfamily **PLEUROTOMARIACEA**			
Family HALIOTIDAE			
Haliotis japonic..	17	Japan	Nishikawa, 1962
Superfamily **FISSURELLACEA**			
Family FISSURELLIDAE			
Macroschisma sinensis	16	Japan	Nishikaw., 1962
M. dilatata	16	Japan	Nishikawa, 1962
Clypina picta	17	Japan	Nishikawa, 1962
Superfamily **PATELLACEA**			
Family ACMAEIDAE			
Patelloida saccharina lanx	9	Japan	Nishikawa, 1962
P. pygmaea	9	Japan	Nishikawa, 1962
P. lampanicola	9	Japan	Nishikawa, 1962
Notacmea schrenckii	9	Japan	Nishikawa, 1962
N. concinna	9	Japan	Nishikawa, 1962
N. fuscoviridis	9	Japan	Nishikawa, 1962
Family PATELLIDAE			
Cellana toreuma	9	Japan	Nishikawa, 1962
C. eucosmia	9	Japan	Nishikawa, 1962
C. nigroilneata	9	Japan	Nishikawa, 1962
Superfamily **TROCHACEA**			
Family TROCHIDAE			
Cantharidus callichroa	18	Japan	Nishikawa, 1962
Thalotia japonica	18	Japan	Nishikawa, 1962
Monodonta labio	18	Japan	Nishikawa, 1962
M. neritoides	18	Japan	Nishikawa, 1962
Tegula lischke	18	Japan	Nishikawa, 1962
T. nigerrima	18	Japan	Nishikawa, 1962
T. rustica	18	Japan	Nishikawa, 1962
T. pfeifferi carpenteri	18	Japan	Nishikawa, 1962
Stomatella lyrata	21	Japan	Nishikawa, 1962
Family TURBINIDAE			
Turbo cornutus	18	Japan	Nishikawa, 1962
Lunella coronata coreensis	18	Japan	Nishikawa, 1962
Astralium haematragum	18	Japan	Nishikawa, 1962
Superfamily **NERITACEA**			
Family NERITIDAE			
Puperita japonica	11	Japan	Nishikawa, 1962
Clithon retropictus	12	Japan	Patterson, 1967 *a*
Septaria sp.	12	India	Natarajan (pers. comm.)
Dostia violacea	14	Japan	Patterson, 1967 *a*
Family HELICINIDAE			
Palaeohelicina sp.	18	Solomon Is.	Burch, 1967 *a*
Pleuropoma sp.	18	Solomon Is.	Burch, 1967 *a*
Pleuropoma sp.	18	Solomon Is.	Burch, 1967 *a*

The Viviparacea contains two families, the Viviparidae and Pilidae (Table IV). In the most primitive viviparid subfamily, Bellamyinae, the haploid chromosome numbers of the 4 species studied range from 8 to 11. In the subfamily Viviparinae, the haploid numbers of 8 species range from $n = 7$ to $n = 13$, while in the most advanced subfamily, the Lioplacinae, the range for 5 species is $n = 13$–14. Four species of the closely related, but somewhat more advanced family Pilidae have the chromosome number $n = 14$.

In the superfamily Valvatacea, *Valvata tricarinata* has 10 pairs of chromosomes (Table III). The Littorinacea (Table III) contains 4 recent families with chromosome numbers ranging from 13 to 18 haploid among 6 species of the two families studied cytologically, the Pomatiasidae and Littorinidae. The superfamily Rissoacea (Table III) is large and diverse with 18 recent families; chromosome numbers known for species of only 3, the Hydrobiidae, Bithyniidae and Assimineidae. In the Hydrobiidae, the chomosome numbers for the 11 species and subspecies investigated range from $n = 16$ to $n = 18$. Rhein (1935) reported the diploid chromosome number of *Potamopyrgus jenkinsi* from Europe to be 20–22 and Sanderson (1940) found British specimens to have a diploid number of 36–44 suggesting that her specimens may be from a polyploid race. To settle this point, the exact chromosome number needs to be determined for this species. All 4 species of the Bithyniidae studied have the chromosome number $n = 17$. Seven species of the family Assimineidae have been studied cytologically. The haploid chromosome numbers of $n = 12$ or $n = 15$ occur in the genus *Assiminea*, while *Adelomorpha* sp. has 17 pairs of chromosomes.

Only 5 of the 17 recent families in the superfamily Cerithiacea have representatives with known chromosome numbers (Table V). *Turitella attenuata*, the only species of the Turitellidae studied cytologically, has 16 pairs of chromosomes. In the Thiaridae, the range in haploid chromosome numbers is from $n = 16$ to $n = 60$ in the 8 species studied. Jacob (1957, 1959 *a*) reported *Melanoides tuberculatus* to have a haploid number of $n = 16$, and the so-called polyploid race of this species to have $n = 46$–47. He further reported *M. lineatus* and *Thiara scabra* to have chromosome numbers of $n = 35$–36 and $n = 38$–39 respectively. These studies should be confirmed. *Balanocochlis* sp. ($n = 60$) may also be a polyploid species.

The greatest variation in chromosome numbers (excluding possible polyploidy mentioned above) of any mesogastropod family occurs in the Pleuroceridae (Table V). Haploid chromosome numbers in 11 species and 1 subspecies of *Semisulcospira* range from $n = 7$ to $n = 20$.[8] Two species of *Goniobasis*, *G. laqueata* and *G. livescens* have 18 and 20 pairs of chromosomes respectively.

All 7 members of the families Potamididae and Cerithiidae studied have a haploid chromosome number of $n = 18$.

There are 3 recent families in the superfamily Hipponicacea, with chromosome information reported for 1 species; *Amalthea conica* has 17 pairs of chromosomes (Table III). The superfamily Naticacea (Table III) contains only 1 family, the Naticidae, in which *Neverita didyma* has the chromosome number $n = 16$.

Twenty-three species have been studied in the Neogastropoda (Table VI), the most advanced order of the Streptoneura. The chromosome numbers of 22 of these species range from $n = 28$ to $n = 36$. In the Muricidae (Muricacea), 4 species have chromosome numbers of $n = 30$, 1 species has $n = 34$ and 2 species have $n = 35$. Uniquely, in *Purpura lapillus*[9] the haploid numbers 13 and 18 are found in different populations. These chromosome numbers are by far the lowest reliably reported in the Neogastropoda.

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[8] The number of chromosomes observed in *Semisulcospira habei* varied from individual to individual, the lowest number observed in mitotic cells being 16 (*see* Patterson, 1967 *b*). However, Burch (1967 *e*) deduced the basic haploid number to be $n = 7$.

[9] Three generic names have been applied to this species: *Purpura*, *Thais* and most recently *Nucella*.

TABLE III

Haploid chromosome numbers of the Mesogastropoda

Species	Haploid No.	Source	Reference
Order *MESOGASTROPODA*			
Superfamily CYCLOPHORACEA			
Family CYCLOPHORIDAE			
Cochlostoma septemspirale	13	Europe	Rainer, 1967
Cyclophorus aurantiacus	14	India	Natarajan (pers. comm.)
Family PUPINIDAE			
Pupina sp.	13	Solomon Is.	Burch, 1967 *a*
Superfamily VIVIPARACEA (Table IV)	7–14		
Superfamily VALVATACEA			
Family VALVATIDAE			
Valvata tricarinata	10	U.S.A.	Burch (pers. comm.)
Superfamily LITTORINACEA			
Family POMATIASIDAE			
Pomatias elegans	13	Europe	Rainer, 1967
P. costulatus hyrcanus	13	Europe	Rainer, 1967
Family LITTORINIDAE			
Nodilittorina picta	15	Japan	Nishikawa, 1962
N. granularis	18	Japan	Nishikawa, 1962
Littorina brevicula	17	Japan	Nishikawa, 1962
Littoraria strigata	17	Japan	Nishikawa, 1962
Superfamily RISSOACEA			
Family HYDROBIIDAE			
Pomatiopsis cincinnatiensis	16	U.S.A.	Burch, 1960 *c*; Patterson, 1963
P. lapidaria	17	U.S.A.	Burch, 1960 *c*; Patterson, 1963
P. californica	17	U.S.A.	Burch, 1967 *a*
P. binneyi	17	U.S.A.	Burch, 1967 *a*
Oncomelania hupensis formosana	17	Taiwan	Burch, 1960 *c*; Patterson, 1963
O. hupensis nosophora	17	Japan	Burch, 1960 *c*
O. hupensis hupensis	17	China	Burch, 1960 *c*
O. hupensis quadrasi	17	Philippines	Burch, 1960 *c*
Hydrobia neglecta	18	Europe	Butot and Kiauta, 1966
H. ulvae	18	Europe	Butot and Kiauta, 1966
H. stagnorum	18	Europe	Butot and Kiauta, 1966
Family BITHYNIIDAE			
Bithynia tentaculata	17	Europe	Butot and Kiauta, 1966
B. leachi	17	Europe	Butot and Kiauta, 1966
B. ussertensis	17	Japan	Patterson, 1967 *a*
Mysorella costigera	17	India	Patterson (unpublished)
Family ASSIMINEIDAE			
Assiminea grayana	12	Europe	Butot and Kiauta, 1966
A. japonica	12	Japan	Patterson, 1967 *a*
A. parasitologica	12	Japan	Patterson, 1967 *a*
A. castanea	15	Japan	Patterson, 1967 *a*
A. yoshidayukioi	15	Japan	Patterson, 1967 *a*
Assiminea sp.	15	Solomon Is.	Burch, 1967 *a*
Adelomorpha sp.	17	Solomon Is.	Burch, 1967 *a*
Superfamily CERITHIACEA (Table V)	7–60		
Superfamily HIPPONICACEA			
Family HIPPONICIDAE			
Amalthea conica	17	Japan	Inaba, 1958 *a*
Superfamily NATICACEA			
Family NATICIDAE			
Neverita didyma	16	Japan	Nishikawa, 1962

TABLE IV

Haploid chromosome numbers of the superfamily Viviparacea

Species	Haploid No.	Source	Reference
Superfamily VIVIPARACEA			
Family VIVIPARIDAE			
Subfamily BELLAMYINAE			
Sinotaia histrica	8	Japan	Inaba, 1965
Cipangopaludina malleata	9	U.S.A.	Pollister and Pollister, 1940, 1943
C. malleata	9	Japan	Inaba and Tanaka, 1953
Bellamya dissimilis	11	India	Ramamoorthy, 1958
B. bengalensis	11	India	Ramamoorthy, 1958
Subfamily VIVIPARINAE			
Viviparus contectus	7	Europe	Franz, 1932; Rainer, 1963
V. ater	9	Europe	Franz, 1932; Rainer, 1963
V. viviparus	10	Europe	Franz, 1932; Rainer, 1963
V. georgianus	12	U.S.A.	Pollister and Pollister, 1940, 1943
V. contectoides	13	U.S.A.	Pollister and Pollister, 1940, 1943; Patterson (unpublished)
V. intertextus	13	U.S.A.	Pollister aud Pollister, 1940
Tulotoma magnifica	12	U.S.A.	Pollister and Pollister, 1940, 1943
T. angulata	13	U.S.A.	Patterson, 1965
Subfamily LIOPLACINAE			
Lioplax subcarinata	13	U.S.A.	Pollister and Pollister, 1940, 1943
Campeloma decisum	13 or 14	U.S.A.	Pollister and Pollister, 1940
C. ponderosum	14	U.S.A.	Pollister and Pollister, 1940, 1943
C. p. coarctatum	14	U.S.A.	Patterson (unpublished)
C. subsolidum	13 or 14	U.S.A.	Pollister and Pollister, 1940
Family PILIDAE			
Pila ovata	14	Egypt	Lutfy and Demian, 1964
P. virens	14	India	Ramamoorthy, 1967
Lanistes bolteni	14	Egypt	Lutfy and Demian, 1964
Marisa cornuarietis	14	Egypt	Lutfy and Demian, 1964

The superfamily Buccinacea has 7 recent families with representatives of 4 having reported chromosome numbers. Five species of the family Pyrenidae have haploid chromosome numbers ranging from $n=28$ to $n=35$. The family Buccinidae contains 5 species with the chromosome number $n = 35$, while *Babylonica japonica* has $n = 36$, the highest number known in the Neogastropoda. In the family Nassariidae, *Nassarius livescens* and *Tritia festiva* both have the chromosome number $n = 34$ and in the Fasciclariidae, *Fasciolaria lignaria* is reported to have the haploid number 35.

In the superfamily Mitracea, *Pusia hizenensis* (Mitridae) has a chromosome number of $n = 30$.

Chromosome numbers of the euthyneuran "opisthobranch" orders appear in Tables VII–IX. These chromosome numbers range from $n = 7$ to $n = 18$. In the order Notaspidea, 2 species of the Pleurobranchidae have the haploid chromosome number $n = 12$ (Table VII). In the order Nudibranchia, 44 species and subspecies in 4 suborders and 16 families have been studied cytologically (Table VIII). The chromosome number $n = 13$ has been reported for all of these species, making the nudibranchs the most cytologically conservative euthyneuran order yet studied.

Eleven species representing 5 of the 14 recent families in the order Cephalaspidea have the haploid chromosome number $n = 17$ or $n = 18$ (Table VII).

Four of the 8 recent families of the order Sacoglossa have information on chromosome numbers (Table VII). Of these, 11 species have the chromosome number $n = 17$ and one

TABLE V

Haploid chromosome numbers of the superfamily Cerithiacea

Species	Haploid No.	Source	Reference
Superfamily CERITHIACEA			
Family TURITELLIDAE			
Turitella attenuata	16	India	Natarajan (pers. comm.)
Family THIARIDAE			
Melanoides tuberculatus	16	India	Jacob, 1959 *a*
M. tuberculatus (polyploid)	45–47	India	Jacob, 1959 *a*
Melanoides sp.	18	Solomon Is.	Burch, 1967 *a, f*
M. (Tarebia) lineatus	35–36	India	Jacob, 1959 *a*
Thiara scabra	38–39	India	Jacob, 1959 *a*
Melania (Radina) crenulata	18	India	Jacob, 1959 *a*
Paludomus transchaurica	19	India	Jacob, 1959 *a*
Balanocochlis sp.	60	Solomon Is.	Burch, 1967 *a*
Family PLEUROCERIDAE			
Semisulcospira habei	7	Japan	Burch, 1967 *e*
S. h. yamaguchi	7	Japan	Burch, 1967 *e*
S. decipiens	12	Japan	Burch, 1967 *e*
S. niponica	12	Japan	Burch, 1967 *e*
S. reticulata	12	Japan	Burch, 1967 *e*
S. nakasekoae	13	Japan	Burch, 1967 *e*
S. multigranosa	14	Japan	Burch, 1967 *e*
S. kurodai	18	Japan	Burch, 1967 *e*
S. libertina	18	Japan	Patterson, 1967 *a*; Burch, 1967 *e*
S. ornata	18	Japan	Patterson, 1967 *a*
S. trachea	18	Japan	Patterson, 1967 *a*
S. reiniana	20	Japan	Burch, 1967 *e*
Goniobasis laqueata	18	U.S.A.	Burch, 1967 *e, f*
G. livescens	20	U.S.A.	Burch, 1967 *e, f*
Family POTAMIDIDAE			
Cerithidea rhizophorarum	18	Japan	Nishikawa, 1962
C. cingulata	18	Japan	Nishikawa, 1962
C. djadjariensis	18	Japan	Nishikawa, 1962
Batillaria zonalis	18	Japan	Nishikawa, 1962
B. multiformis	18	Japan	Nishikawa, 1962
Family CERITHIIDAE			
Proclava kochi	18	Japan	Nishikawa, 1962
Contumax kobelti	18	Japan	Nishikawa, 1962

species, *Bosellia mimetica* (family Polybranchiidae), has $n = 7$, the lowest haploid chromosome number known among the opisthobranchs. The only other species of the Polybranchiidae studied has the more typical chromosome number of $n = 17$. *Tiberia fasciatus*, the only member of the order Entomotaeniata studied cytologically, has the chromosome number $n = 17$ (Table VII). In the order Anaspidea (Table IX) the haploid chromosome number $n = 17$ is known for 6 species of the family Aplysiidae.

Five species representing 2 of the 3 recent families contained in the order Soleolifera have reported chromosome numbers (Table IX). In the family Veronicellidae, *Veronicella floridana* has the haploid number $n = 16$ while *Laevicaulis alte* has $n = 17$. Variation in chromosome number is also evident in the Onchidiidae; *Onchidella kurodai* has $n = 17$, *O. evelinae*, $n = 18$ and *Onchidium verraculatum*, $n = 18$.

The order Basommatophora has 12 families arranged in 2 suborders, the Archaeopulmonata and the Branchiopulmonata (Tables X–XIV). Chromosome numbers are available for 129 species

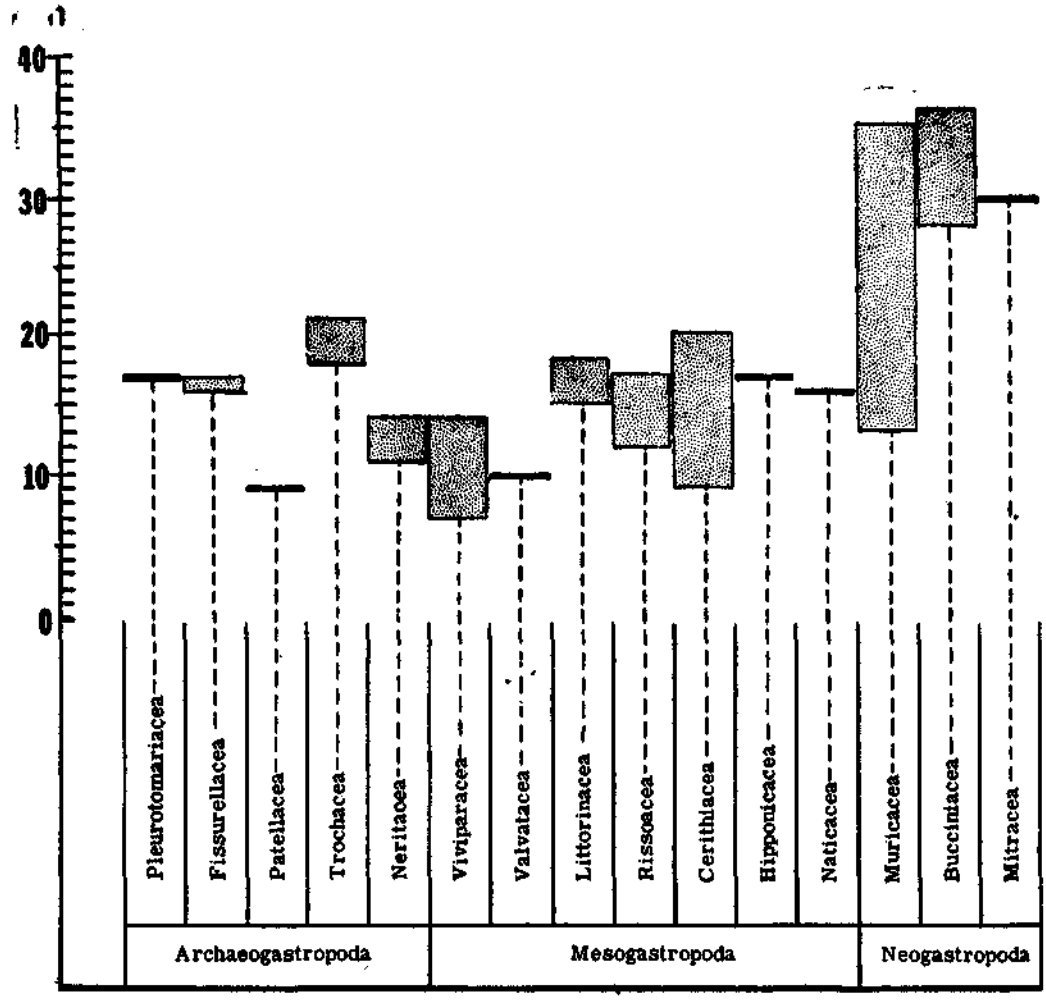

FIG. 2. Haploid chromosome numbers (n) in the streptoneuran superfamilies. The stippled areas indicate the range between the lowest and highest numbers. The polyploid chromosome numbers (in the Cerithiacea) are not shown.

and subspecies in 10 of the families. In the primitive Archaeopulmonata, 21 species and subspecies of 6 families have information on chromosome numbers (Table X). All 7 species of the family Siphonariidae studied have 16 pairs of chromosomes. Haploid chromosome numbers range from $n = 17$ to $n = 19$ in the 10 species and subspecies of the Ellobiidae studied. It is interesting to note that the variation in haploid chromosome numbers in the Ellobiidae occurs among congeneric species as well as genera. Interestingly enough, 1 individual of *Melampus coffeus* in an otherwise diploid population was found by Natarajan and Burch (1966) to be tetraploid ($n = 38$). The only representatives of each of the other 4 archaeopulmonate families studied has the chromosome number $n = 18$, the number most frequently found in the suborder Branchiopulmonata (the "higher limnic Basommatophora").

In the Branchiopulmonata, a number of species of all 4 families have been studied. Eleven species of the Ancylidae have haploid chromosome numbers ranging from $n = 15$ to $n = 60$, with

TABLE VI

Haploid chromosome numbers of the Neogastropoda

Species	Haploid No.	Source	Reference
Order *NEOGASTROPODA*			
Superfamily MURICACEA			
Family MURICIDAE			
Bedevina birileffi	30	Japan	Nishikawa, 1962
Purpura lapillus	13, 18	Europe	Staiger, 1950, 1954
P. bronni	30	Japan	Nishikawa, 1962
P. clavigera	30	Japan	Nishikawa, 1962
P. luteostoma	30	Japan	Nishikawa, 1962
Chicoreus asianus	34	Japan	Nishikawa, 1962
Hexaplex trunculus	35	Europe	Staiger, 1950
Tritonalia erinaceus	35	Europe	Staiger, 1950
Superfamily BUCCINACEA			
Family PYRENIDAE			
Columbella versicolor	28	Japan	Nishikawa, 1962
C. rustica	34	Europe	Staiger, 1950
Anachis misera	32	Japan	Nishikawa, 1962
Pyrene bicincta	34	Japan	Nishikawa, 1962
P. testudinaria tylerae	35	Japan	Nishikawa, 1962
Family BUCCINIDAE			
Pisania ferrea	35	Japan	Nishikawa, 1962
P. maculosa	35	Europe	Staiger, 1950
Euthria cornea	35	Europe	Staiger, 1950
Buccinum undatum	35	Europe	Staiger, 1950
Cantharus subrubiginosus	35	Japan	Nishikawa, 1962
Babylonia japonica	36	Japan	Nishikawa, 1962
Family NASSARIIDAE			
Nassarius livescens	34	Japan	Nishikawa, 1962
Tritia festiva	34	Japan	Nishikawa, 1962
Family FASCIOLARIIDAE			
Fasciolaria lignaria	35	Europe	Staiger, 1950
Superfamily MITRACEA			
Family MITRIDAE			
Pusia hizenensis	30	Japan	Nishikawa, 1962

polyploidy undoubtedly involved (Table XI). Both laevapecine limpets studied have 17 pairs of chromosomes. In the subfamily Ancylinae the chromosome numbers $n = 15$, 30 and 60 were reported, and $n = 17$, 18 and 30 occur in the Ferrissinae.

Haploid chromosome numbers range from $n = 18$ to $n = 72$ in the 52 species and subspecies of the family Planorbidae studied (Tables XII–XIII). Their basic chromosone number is $n = 18$. Twenty-four species and subspecies of the subfamily Bulininae have chromosome numbers ranging from $n = 18$ to $n = 72$ (Table XIII). Polyploid species have $n = 36$, $n = 54$, or $n = 72$. Also, chromosomes additional to the basic diploid complement occur in 2 species, *Bulinus forskalii* and *B. natalensis*.

The haploid chromosome number $n = 18$ occurs in 5 species of the subfamily Helisomatinae while a sixth species, *Planorbula crassilabris*, has 19 pairs of chromosomes (Table XII). All 8 representatives of the subfamily Biomphalariinae investigated have the basic planorbid number

$n = 18$. Three species of the subfamily Segmentininae and 1 species of the Camptocerinae also have the chromosome number $n = 18$. In the subfamily Planorbinae, polyploidy has also been found. *Gyraulus parvus* has the haploid chromosome number $n = 36$ while the other 9 *Gyraulus* species studied have the chromosome number $n = 18$. Additional chromosomes have also been reported for individuals of a population of *G. deflectus*.

TABLE VII

Haploid chromosome numbers of 'Opisthobranchs'

Species	Haploid No.	Source	Reference
Order *NOTASPIDEA*			
Family PLEUROBRANCHIDAE			
Pleurobranchaea novaezealandiae	12	Japan	Inaba, 1959 *c*
P. meckeli	12	Italy	Mancino and Sordi, 1965 *b*
Order *NUDIBRANCHIA* (Table VIII)	13		
Order *CEPHALASPIDEA*			
Family ACTEONIDAE			
Cylichnatys angusta	17	Japan	Inaba (pers. comm.)
Family PHILINIDAE			
Philine japonica	17	Japan	Inaba, 1959 *c*
P. quadripartita	17	Italy	Mancino and Sordi, 1965 *b*
Family AGLAJIDAE			
Aglaja gigliolii	17	Japan	Inaba, 1959 *c*
A. depicta	17	Italy	Mancino and Sordi, 1965 *b*
A. tricolorata	17	Italy	Mancino and Sordi, 1965 *b*
Family ATYIDAE			
Haminoea linda	17	Eniwetok	Burch and Natarajan, 1967
H. musetta	17	Eniwetok	Burch and Natarajan, 1967
H. crocata	17	India	Natarajan (pers. comm.)
Family SMARAGDINELLIDAE			
Lathophthalmus smaragdinus	18	Eniwetok	Burch and Natarajan, 1967
Smaragdinella calyculata	18	Eniwetok	Burch and Natarajan, 1967
Order *SACOGLOSSA*			
Family ELYSIIDAE			
Elysia amakusana	17	Japan	Inaba, 1959 *c*
E. viridis	17	Italy	Mancino and Sordi, 1964 *c*
Family POLYBRANCHIIDAE			
Bosellia mimetica	7	Italy	Mancino and Sordi, 1964 *b*, 1965 *a*
Calliphylla mediterranea	17	Italy	Mancino and Sordi, 1965 *b*
Family STILIGERIDAE			
Stiliger vesiculosus	17	Italy	Mancino and Sordi, 1964 *b*
Placida viridis	17	Italy	Mancino and Sordi, 1964 *b*
P. dendritica	17	Italy	Mancino and Sordi, 1964 *b*
Erocolania trinchesii	17	Italy	Mancino and Sordi, 1964 *c*
Hermaeopsis variopicta	17	Italy	Mancino and Sordi, 1965 *b*
Hermaea bifida	17	Italy	Mancino and Sordi, 1965 *b*
Alderia nigra	17	Japan	Inaba, 1961 *a*
Family JULIIDAE			
Berthelinia limax	17	Japan	Inaba, 1961 *a*
Order *ENTOMOTAENIATA*			
Family PYRAMIDELLLIDAE			
Tiberia fasciata	17	Japan	Inaba (pers. comm.)
Order *ANASPIDEA* (Table IX)	17		
Order *SOLEOLIFERA* (Table IX)	16–18		

Chromosome numbers reported for the family Lymnaeidae range from 16 to 19 haploid among the 40 species and subspecies that have been studied (Table XIV). Three species have the chromosome number $n = 16$. Nearly all *Radix* have 17 pairs of chromosomes (*R.* "*limosa*" has been reported to have 18 pairs of chromosomes, but this report needs to be verified). All of the species but 1 studied in the genera *Lymnaea, Stagnicola, Acella, Pseudosuccinea, Bulimnea, Fossaria, Lanx* and *Fisherola* have 18 pairs of chromosomes. *Fossaria rustica* has 19 pairs of chromosomes.

All 7 species of the family Physidae studied cytologically have 18 pairs of chromosomes.

The great majority of land snails belong to the large order Stylommatophora, which contains 63 recent families. Chromosome numbers have been reported for 32 of these families (Tables XV–XX). Haploid chromosome numbers range from $n = 20$ to $n = 30$ among the 18 species studied in 7 families of the suborder Orthurethra (Table XV). The lowest numbers ($n = 20$–23) occur in 5 species of the Achatinellidae while the highest haploid chromosome number ($n = 30$) is found among members of the family Chondrinidae. The intermediate numbers are $n = 24$ in the Enidae, $n = 26$ in the Cionellidae, $n = 28$ in the Valloniidae and $n = 29$ in the Partulidae. Conservatism in chromosome number at the family level is evident in the Orthurethra.

There is cytological information for 2 of the 6 families in the suborder Mesurethra, with chromosome numbers ranging from 24 to about 30 haploid (Table XV). *Cerion incanum* (Ceriidae) has 27 pairs of chromosomes. Fifteen species and subspecies of Clausiliidae have the chromosome number of $n = 24$, 3 species have $n = 28$ and 3 species have a haploid number of about 30.

The greatest range in haploid chromosome numbers ($n = 5$–44) of any stylommatophoran group occurs among members of the Heterurethra (Table XVI),[4] an aberrent suborder containing 3 families, the Succineidae, Aillyidae and Athoracophoridae. In 33 species and 1 subspecies of the family Succineidae, the haploid chromosome numbers range from $n = 5$ to $n = 25$. Species of the most primitive succineid genus studied, *Catinella*, have the lowest number of chromosomes yet found in the Mollusca, $n = 5$ and $n = 6$ in the 5 species so far investigated. *Succinella oblonga* has been reported to have 12 pairs of chromosomes, but at least one population has only 11 pairs. Haploid numbers range from $n = 15$ to $n = 19$ among the 10 *Oxyloma* species studied, and in *Succinea* the range is $n = 17$–25, except for 1 species which has only 11 pairs of chromosomes. One species of the aberrant slug-like succineid genus, *Omalonyx*, has 21 pairs of chromosomes. *Aneitea* of the Athoracophoridae has the surprisingly high chromosome number of $n = 44$, the highest yet found in the Stylommatophora.

The largest suborder of the Stylommatophora, the Sigmurethra, contains 3 infraorders (Holopodopes, Aulacopoda and Holopoda), from which cytological information is available for 21 families (Tables XV, XVII-XX). The chromosome numbers of 9 species from 5 of the 14 recent families of the Holopodopes range from $n = 25$ to $n = 31$ (Table XV). It is interesting that the highest and lowest numbers occur in the family Subulinidae. All other species have either 29 or 30 pairs of chromosomes.

The Aulacopoda contains 18 families with chromosome numbers of $n = 20$ to $n = 34$ reported for 38 species in 11 families. One species of the family Endodontidae has a haploid chromosome number of $n = 29$, 2 other species have 31 pairs of chromosomes.

The sigmurethran slugs, comprising the families, Arionidae, Philomycidae, Milacidae, Limacidae and Testacellidae, have chromosome numbers ranging from 24 to 34 haploid (Table XVII). Two species of *Incilaria* (Philomycidae) have 24 pairs of chromosomes; 6 species of *Arion* (Arionidae)

[4] The name *C.* cf. *oregonensis* mentioned by Burch and Patterson (1965) was later changed to *C.* cf. *gabbi* by Burch, Patterson and Natarajan (1966) and is so listed in Table XVI.

TABLE VIII

Haploid chromosome numbers of Nudibranchia

Species	Haploid No.	Source	Reference
Order *NUDIBRANCHIA*			
Family DORIDIDAE			
Doris verrucosa	13	Italy	Mancino and Sordi, 1964 *a*
Anisodoris stellifera	13	Italy	Mancino and Sordi, 1965 *b*
Family AEGIRETIDAE			
Aegires leuckarti	13	Italy	Mancino and Sordi, 1965 *b*
Homiodoris japonica	13	Japan	Inaba and Saiki, 1967
Family GLOSSODORIDIDAE			
Glossodoris festiva	13	Japan	Inaba and Hirota, 1958
G. pallescans	13	Japan	Inaba and Hirota, 1958
G. gracilis	13	Italy	Mancino and Sordi, 1965 *b*
Family ALDISIDAE			
Aldisa sanguinea	13	Japan	Inaba and Saiki, 1967
Family DISCODORIDIDAE			
Discodoris pardalis	13	Japan	Inaba, 1959 *c*
D. concinna	13	Japan	Inaba and Saiki, 1967
Family ROSTANGIDAE			
Rostanga arbutus	13	Japan	Inaba, 1959 *c*
Family GONIODORIDIDAE			
Okenia barnardi	13	Japan	Inaba and Hirota, 1958
Goniodoris castanea	13	Italy	Mancino and Sordi, 1965 *b*
G. sugashimae	13	Japan	Inaba and Saiki, 1967
Trapania sp.	13	Italy	Mancino and Sordi, 1964 *c*
Trapania fusca	13	Italy	Mancino and Sordi, 1965 *b*
Family POLYCERIDAE			
Polycera quadrilineata	13	Italy	Mancino and Sordi, 1965 *b*
Caloplocamus ramosus	13	Italy	Mancino and Sordi, 1965 *b*
Family DENDRODORIDIDAE			
Dendrodoris miniata	13	Japan	Inaba and Hirota, 1958
D. nigra	13	Japan	Inaba and Hirota, 1958; Inaba, 1959 *a*
		Eniwetok	Burch and Natarajan, 1967
D. rubra nigromaculata	13	Japan	Inaba and Saiki, 1967
Family ARMINIDAE			
Dermatobranchus striatus	13	Japan	Inaba and Hirota, 1958
Armina tigrina	13	Italy	Mancino and Sordi, 1965 *b*
Family FIMBRIIDAE			
Melibe papillosa	13	Japan	Inaba, 1959 *c*
Family CUTHONIDAE			
Cuthona futairo	13	Japan	Inaba and Saiki, 1967
Catriona purpureoanulata	13	Japan	Inaba and Saiki, 1967
C. signifera	13	Japan	Inaba and Saiki, 1967
C. pupillae	13	Japan	Inaba, 1961 *a* ; Inaba and Saiki, 1967
Trinchesia coerulea	13	Italy	Mancino and Sordi, 1965 *b*
T. aurantia	13	Italy	Mancino and Sordi, 1965 *b*
Family FACELINIDAE			
Sukuraeolis (Hervia) enosimensis	13	Japan	Inaba and Hirota, 1958
S. japonica	13	Japan	Inaba and Hirota, 1958
Facelina rubrovittata	13	Italy	Mancino and Sordi, 1965 *b*
Hervia peregrina	13	Italy	Mancino and Sordi, 1964 *c*
Caloria maculata	13	Italy	Mancino and Sordi, 1964 *c*
Herviella mietta	13	Eniwetok	Burch and Natarajan, 1967
H. affinis	13	Japan	Inaba and Saiki, 1967
Rizzolia lineata	13	Japan	Inaba and Saiki, 1967
Family DOTOIDAE			
Doto bella	13	Japan	Inaba, 1961 *a*; Inaba and Saiki, 1967
Family EUBRANCHIDAE			
Eubranchus misakiensis	13	Japan	Inaba and Saiki, 1967
E. inabai	13	Japan	Inaba and Saiki, 1967
Eubranchopsis virginalis	13	Japan	Inaba and Saiki, 1967
Family AEOLIDIIDAE			
Spurilla neapolitana	13	Italy	Mancino and Sordi, 1965 *b*
Limenandra fusiformis	13	Japan	Inaba and Saiki, 1967

have haploid chromosome numbers that range from $n=25$ to $n=29$. In the family Limacidae, 4 species of *Deroceras* have the haploid number $n = 30$, 3 species of *Limax* have $n = 31$ and *Lehmannia marginata* has only 24 pairs of chromosomes. Chromosome numbers of $n = 33$ or 34 are reported for 3 species of *Milax* (Milacidae). Only 1 species, *Testacella haliotidea*, of the family Testacellidae has been studied and it has the chromosome number $n = 32$.

In the family Vitrinidae, *Vitrinopsis* sp. has 28 pairs of chromosomes and in the Zonitidae, 4 species have haploid numbers ranging from $n=28$ to $n=31$ and 1 species, *Vitrea diaphana* has the lowest chromosome number reported in the Aulacopoda, $n = 20$ (Table XV). There are only a few chromosome studies in the Trochomorphidae and Helicarionidae (Table XV). The species studied have the chromosome numbers $n = 28$ and $n= 30$. Six species of the family Ariophantidae have chromosome numbers that range from $n = 25$ to $n = 32$.

Five of the 10 families in the Holopoda have information on chromosome numbers. Haploid chromosome numbers range from $n = 26$ to $n = 31$ in 21 species and subspecies of the family Polygyridae (Table XVIII); 17 of them have the chromosome number $n = 29$. The family Bradybaenidae is also conservative in regard to chromosome numbers — 18 species have either 28 or 29 pairs of chromosomes (Table XIX). The 9 species studied that belong to the family Camaenidae have haploid chromosome numbers ranging from $n=27$ to $n=29$ (Table XV). One species of the Helminthoglyptidae (*Monodenia fidelis*) has the haploid chromosome number $n = 29$ (Table XV).

The greatest number (45) of stylommatophoran species and subspecies studied cytologically belong to the family Helicidae (Table XX). Haploid chromosome numbers range from a low of 21 to a high of 30. The Helicellinae, which is sometimes given its own familial rank (Helicellidae), has chromosome numbers ranging from $n=23$ to $n=27$ among the 11 species studied. As shown in Table XX, one particular haploid chromosome number does not seem to consistently characterize the particular subfamilies. For example, the haploid number $n = 27$ occurs in 3 different subfamilies and $n = 30$, $n = 26$ and $n = 23$ each occur in 2 different subfamilies.

Chromosome Numbers and Systematics

Changes in chromosome numbers in animals may occur by (1) chromosomal fusion; (2) fragmentation; (3) mitotic or meiotic non-disjunction; (4) translocations involving a supernumerary fragment serving as a donor of a centromere and 2 telomeres and (5) duplication of entire chromosomal complements through polyploidy. Chromosomal fusion produces a decrease in chromosome number while the other conditions result in an increase in chromosome number. Changes in the chromosomal complement that involve the loss or gain of incomplete chromosomal sets occur by aneuploidy in contrast to polyploidy. Because most variation in molluscan chromosome numbers within a taxon is seldom greater than $\pm$ 1 or 2 bivalents, mechanisms for addition or deletion of chromosomes must operate at a low or inefficient level. Nevertheless, nearly all changes in chromosome numbers that did occur and are now evident undoubtedly represent aneuploid conditions which became stabilized. Furthermore, basic diploid species can tolerate duplications or additions of chromosomes more easily than loss of chromosomes (*see* Husted and Burch, 1946); therefore, variation in chromosome number within a taxon probably more usually reflects addition of rather than loss of chromosomes. When the variation in chromosome number between lower taxonomic categories exceeds 1 or 2 bivalents, *i.e.*, 3 or 4 or more (but less than an additional complement of chromosomes), this probably represents successive aneuploid conditions, each change followed by a period of stability in the new chromosome number. Accordingly, we may expect to find more primitive molluscs to have lower chromosome numbers and species derived from them too often have a greater number of chromosomes. When an unexpectedly low chromosome number is found in an advanced group, this number either represents an unchanged ancestral condition or it has resulted from successive reductions in the chromosome number. Subsequent chromosome number increases may be expected in either case, which would be primary

increases in the former case and secondary increases in the latter. However, in referring to chromosome number change as an increase or decrease, care must be taken in assessing which groups are morphologically primitive or advanced. It often appears that the determination of whether or not a taxon is "primitive" or "specialized" ("advanced") is quite subjective and is not determined by careful analysis of many detailed characters. The lack of sufficiently critical morphological studies and careful analyses relating to phylogeny pose serious problems for the molluscan cytotaxonomist. Until more species' chromosome numbers are determined which can be related to reliable information on molluscan comparative morphology, systematics and phylogeny, we can only present suggestions concerning the cytotaxonomic relationships in the Mollusca.

A general conservativeness with regard to chromosomal change is evident in most mollusc groups. Many higher taxa have characteristic chromosome numbers, with only a few species having chromosome numbers that deviate widely from the "basic" haploid number for their particular groups. In the Archaeogastropoda, for example, all members of the Patellacea have the haploid chromosome number $n = 9$; in the Mesogastropoda all members of the rissoacean families Hydrobiidae and Bithyniidae have 16 to 18 pairs of chromosomes; the opisthobranch order Nudibranchia is characterized by the single haploid number $n = 13$ (44 species), and the other opisthobranch orders are also conservative with all species studied (except 1) having $n = 16$–18; the basommatophoran Lymnaeidae all have $n = 16$–19 ($n = 18$ is the most prevalent), nearly all planorbids have $n = 18$ or a polyploid multiple of $n = 18$; and an example of conservatism in the Stylommatophora is the 17 (out of 21) polygyrid species that have 29 pairs of chromosomes.

Figure 1 shows the range of haploid chromosome numbers in the Gastropoda, excluding polyploid numbers. It has not been possible in many cases to correlate low chromosome numbers with species' groups considered to be "primitive" by taxonomists and morphologists, but in other cases such a correlation deserves mention. In the most conservative groups, chromosome numbers *per se*, may have no bearing on phylogenetic schemes because chromosomal changes may have not occurred.

The range of haploid chromosome numbers excluding polyploidy, in the Archaeogastropoda is $n = 9$–21; in the Mesogastropoda it is $n = 7$–20; and in the Neogastropoda $n = 13$–36 (with

TABLE IX

Haploid chromosome numbers of Anaspidea and Soleolifera

Species	Haploid No.	Source	Reference
Order *ANASPIDEA*			
Family APLYSIIDAE			
Petalifera punctulata	17	Japan	Inaba, 1959 *c*
Notarchus leachii freeri	17	Japan	Inaba, 1959 *c*
Dolabrifera dolabrifera	17	Eniwetok	Burch and Natarajan, 1967
Stylocheilus longicauda	17	Eniwetok	Burch and Natarajan, 1967
Aplysia benedicti	17	India	Natarajan (pers. comm.)
Bursatella leachii	17	India	Natarajan (pers. comm.)
Order *SOLEOLIFERA*			
Family VERONICELLIDAE			
Veronicella floridana	16	U.S.A.	Burch, 1965 *b*
Laevicaulis alte	17	India	Natarajan, 1960
Family ONCHIDIIDAE			
Onchidella kurodai	17	Japan	Inaba, 1961 *a*
O. evelinae	18	Eniwetok	Burch and Natarajan, 1967
Onchidium verraculatum	18	India	Natarajan, 1959

the lower limit due to one species, *Purpura lapillus* with all other species have 28 or usually 30 or more pairs of chromosomes). The higher chromosome numbers of the Neogastropoda, considered to be the most advanced streptoneuran order, correlate well with the hypothesis that higher chromosome numbers occur in advanced taxonomic groups. Nishikawa (1962) found difficulty in explaining the great difference in the chromosome numbers reported for *P. lapillus* ($n = 13$; $n = 18$) and the other 3 species of that genus he studied which had the haploid number 30. He suggested that the basic chromosome number of the order is one of the higher numbers.

Because of their wide range of chromosome numbers (Table IV, Fig. 3), it is interesting to compare the taxa within the Viviparacea. Bellamyinae, the more primitive subfamily, has general lower chromosome numbers ($n = 8$–11). In the Viviparinae, the range of haploid chromosome numbers is $n = 7$–13, with the greater number of species having haploid chromosome numbers above 11, the highest number of species having reported in the Bellamyinae. Furthermore, the most advanced subfamily in the Viviparidae, the Lioplacinae, is characterized by haploid chromosome numbers of 13 and 14. Those members studied of the most advanced family of the Viviparacea the Pilidae, have only the haploid number 14, the highest number found in the Viviparidae. The higher chromosome numbers seem to characterize the more "advanced" taxa within the Viviparacea based on the information now available.

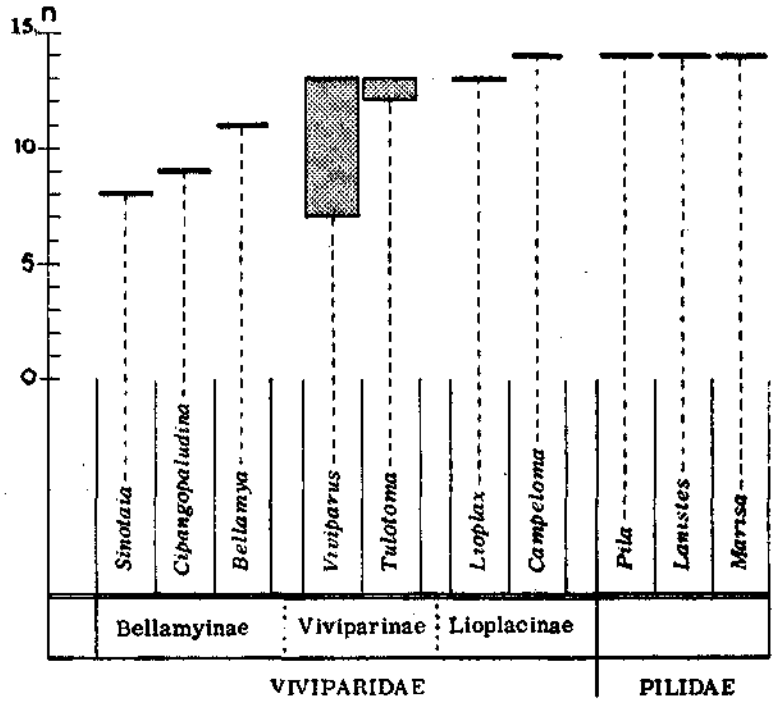

FIG. 3. Haploid chromosome numbers (n) in the Viviparacea. The stippled areas indicate the range within the genera.

Four species of *Assiminea* from Japan have been studied cytologically. This genus is currently divided into three subgenera (*Assiminea* s.s., *Pseudomphala*, *Angustassinminea*) containing nine species and subspecies. Of the Japanese species studied, *A. japonica* ($n = 12$) is placed in the subgenus *Assiminea* s.s. *A. parasitologica* ($n = 12$), *A. castanea* ($n = 15$) and *A. yoshidayukioi* ($n = 15$) are placed together in the subgenus *Angustassiminea*. Cytological data tends to show that *A. parasitologica* is perhaps more closely related to *A. japonica* (each with 12 pairs of chromosomes) than to either *A. castanea* ($n = 15$) or *A. yoshidayukioi* ($n = 15$). Furthermore, the taxonomic status of

the species comprising these subgeneric groups does not correlate with their susceptibility to *Paragonimus* infection (Miyazaki *et al.*, 1960). A detailed karyotype analysis combined with a critical morphological study of the assimineid species in Japan is desirable to help clarify the relationships among members of this medically important genus.

At this time, the information on chromosome numbers of streptoneuran gastropods is much too fragmentary to suggest a general, clear-cut correlation between low chromosome numbers and "primitiveness" or higher numbers and greater "advancement" among the various groups.

Information on chromosome numbers is more complete for the Euthyneura (Opisthobranchia + Pulmonata). The more advanced groups, or those usually considered to be more specialized by morphologists, often have the greater number of chromosomes (Burch, 1965*b*, 1967*d*). However, certain deviations from this general pattern are evident, which necessitates caution in assessing systematic position based on chromosome numbers alone.

Haploid chromosome numbers range from $n = 7$ to $n = 18$ in the 7 opisthobranchiate orders (Tables VII–IX; Fig. 1). The Pleurobranchidae is the only family of the order Notaspidea for which there is any cytological information. *Pleurobranchaea novazealandica* and *P. meckeli* both have 12 pairs of chromosomes (Tables VII). According to Pruvot-Fol (1954), the systematic status of the families placed in the order Notaspidea is still controversial because in some aspects they resemble tectibranchs (the mantle covers an internal shell), while in other ways they resemble nudibranchs (a highly concentrated nervous system). Many of the families placed in the Tectibranchia by Pelseneer (1897) now belong to groups characterized by 17 or 18 pairs of chromosome while all members of the Nudibranchia studied have only 13 pairs of chromosomes. Pelseneer (1894) considers that the nudibranchs have arisen from a pleurobranchid ancestor. Morton (1958) states that "the Notaspidea are rather generalized opisthobranchs, foreshadowing the true nudibranchs, to which they have almost certainly given rise". The genus *Pleurobranchaea* is, however, somewhat different from all other members of the Pleurobranchidae in that the nervous system is less concentrated than in other members and the nerve connectives are distinct (Pruvot-Fol, 1954). Therefore, the Pleurobranchid species for which we have cytological information ($n = 12$) may be aberrent and not indicative of the basic chromosome number for the Notaspidea. However, if the chromosome number $n = 12$ is found in other species of the Notaspidea, then the contention of Pelseneer (1894) and Morton (1958) that the Pleurobranchidae or Notaspidea ($n = 12$) are ancestral to the Nudibranchia ($n = 13$) is perhaps strengthened.

The Nudibranchia is the most thoroughly studied opisthobranch order with respect to chromosome numbers (Table VIII). The only chromosome number reported for 44 species of this group is $n = 13$, making it the most cytologically conservative euthyneuran order. The Notaspidea, with $n = 12$, as previously mentioned, may be ancestral to the Nudibranchia. The nudibranchs are an extremely diverse group and most malacologists consider them to be the most "advanced" group of opisthobranchs, whereas the Cephalaspidea, characterized by 17 or 18 pairs of chromosomes is considered the most "primitive" opisthobranchiate group. Nevertheless, the chromosome number of the Nudibranchia, $n = 13$, may suggest that perhaps they are more primitive than the Cephalaspidea ($n = 17$–18).

The order Cephalaspidea has classically been thought of as the most "primitive" opisthobranch group which forms the transition between the Streptoneura and the Opisthobranchia (Pruvot-Fol, 1954). Since all eleven species studied have the haploid number $n = 17$ or $n = 18$ chromosome numbers; *per se*, do not corroborate the classical phylogenetic position of the Cephalaspidea. In fact, chromosome numbers of this group may indicate that they are somewhat more "advanced" than the Pleurobranchidae and the Nudibranchia. Natarajan (1964) and Burch and Natarajan (1967) pointed out that the haploid number 18 occurring in the Smaragdinellidae may be significant in regard to the origin of the Basommatophora from the Cephalaspidea, since $n = 18$ appears to be the basic haploid chromosome number for the Basommatophora.

TABLE X

Haploid chromosome numbers of Basommatophora

Species	Haploid No.	Source	Reference
Suborder *ARCHAEOPULMONATA*			
Family SIPHONARIIDAE			
Siphonaria japonica	16	Japan	Inaba, 1953
S. guamensis	16	Marshall Is.	Natarajan and Burch, 1966
S. laciniosa	16	New Caledonia	Natarajan and Burch, 1966
S. alternata	16	Bermuda	Natarajan and Burch, 1966
S. pectinata	16	U.S.A.	Natarajan and Burch, 1966
S. acmaeoides	16	Japan	Nishikawa, 1967
S. sirius	16	Japan	Burch, 1967 *a*; Nishikawa, 1967
Family ELLOBIIDAE			
Cassidula mustelina	17	India	Natarajan, 1958
C. verpertillonis	17	New Caledonia	Natarajan and Burch, 1966
Pythia plicata	17	India	Natarajan, 1958
P. scarabaens	18	Solomon Is.	Burch, 1967 *a*
Phytia myosotis	18	Europe	Meyer, 1955
P. m. marylandica	18	U.S.A.	Burch, 1960 *b*
Detracia floridana	18	U.S.A.	Burch, 1960 *b*
Melampus bidentatus lineatus	18	U.S.A.	Burch, 1960 *a, b*; Natarajan and Burch, 1966
M. ceilonicus	18	India	Natarajan, 1958
M. coffeus	19	U.S.A.	Natarajan and Burch, 1966
M.? coffeus	19 (38)	U.S.A.	Natarajan and Burch, 1966
Family AMPHIBOLIDAE			
Salinator takii	18	Japan	Inaba, 1950, 1953
Family CHILINIDAE			
Chilina fluviatilis	18	Uruguay	Natarajan and Burch, 1966
Family LATIIDAE			
Latia neritoides	18	New Zealand	Burch and Patterson, 1963
Family ACROLOXIDAE			
Acroloxus lacustris	18	England	Burch, 1961 *a*, 1962
Suborder *BRANCHIOPULMONATA*			
Family ANCYLIDAE (Table XI)	15–60		
Family PLANORBIDAE (Tables XII, XIII)	18–72		
Family LYMNAEIDAE (Table XIV)	16–19		
Family PHYSIDAE			
Physa acuta	18	Europe	LeCalvez and Certain, 1950
P. anatina	18	U.S.A.	Burch, 1960 *a*
P. fontinalis	18	Europe	LeCalvez and Certain, 1950
P. gyrina	18	U.S.A.	Burch and Bush, 1960
P. heterostropha	18	U.S.A.	Burch and Bush, 1960
P. sayii crassa	18	U.S.A.	Burch and Bush, 1960
Aplexa hypnorum	18	U.S.A.	Burch and Bush, 1960

The order Sacoglossa contains members of diverse morphology, some being comparable to cephaspideids while others are nudibranch-like (Morton, 1958). Eleven species have 17 pairs of chromosomes, but *Bosellia mimetica* has only 7 pairs (Table VII), the lowest haploid chromosome number reported in the Euthyneura, with the exception of the land snail genus *Catinella*. The taxonomic position of *Bosellia* is uncertain, but it is most probable that it belongs with ascoglossans possessing papillae (Pruvot-Fol, 1954). In any case, *Bosellia* stands alone with its low chromosome number among the cytologically conservative opisthobranchs, and

TABLE XI

Haploid chromosome numbers of Ancylidae

Species	Haploid No.	Source	Reference
Family ANCLIDAE			
Subfamily ANCYLINAE			
Rhodacmea cahawbensis	15	U.S.A.	Burch, Basch and Bush, 1960
Ancylus sp.	30	Ethiopia	Burch, 1967 *a*
A. fluviatilis	60	England	Burch, Basch and Bush, 1960
Subfamily FERRISSINAE			
Pettancylus nipponica	17	Japan	Inaba, 1965
P. japonica	18	Japan	Burch, 1965 *a*
P. sp.	18	Tanzania	Burch, 1967 *a*
Gundlachia japonica	18	Japan	Burch, 1965 *a*
Ferrissia tarda	30	U.S.A.	Burch, Basch and Bush, 1960
F. parallela	30	U.S.A.	Burch, 1960 *a*
Subfamily LAEVAPECINAE			
Laevapex fuscus	17	U.S.A.	Burch, 1960 *a*
Burnupia sp.	17	S. Africa	Burch, 1962 *a*

it may well deserve its own taxonomic group separate from other Sacoglossa. Burch and Natarajan (1967) regarded *B. mimetica* as either an extremely aberrent species, or one in which its cytological evolution has been much more rapid than has been the evolution of its morphology in respect to other opisthobranchs so far studied. The order Sacoglossa with a basic haploid chromosome number of $n = 17$ does not differ cytologically from the opisthobranch orders Cephalaspidea, Entomotaeniata and Anaspidea.

Only 1 member of the Entomotaeniata, *Tiberia fasciata*, has been studied cytologically; it has 17 pairs of chromosomes (Table VII). This pyramidellid is a rather specialized ectoparasite.

The Anaspidea, an opisthobranch order containing relatively few species, is characterized by the haploid number $n = 17$ (Table IX). Some members of the Anaspidea have certain affinities with the Cephalaspidea and the anaspideid chromosome number of $n = 17$ would seem to indicate relationships to cephalaspideids rather than pleurobranchids ($n = 12$) or nudibranchs ($n = 13$).

TABLE XII

Haploid chromosome numbers of Planorbidae

Species	Haploid No.	Source	Reference
Family PLANORBIDAE			
Subfamily BULININAE (Table XIII)	18–72		
Subfamily HELISOMATINAE			
Helisoma anceps	18	U.S.A.	Burch, 1960 *a*
H. subcrenatum	18	U.S.A.	Bonham, 1955
H. trivolvis	18	U.S.A.	Burch, 1960 *a*
Planorbarius corneus	18	Netherlands	Burch, 1961 *b*
Promenetus exacuous	18	U.S.A.	Burch, 1960 *a*
Planorbula crassilabris	19	U.S.A.	Burch, 1960 *a*
Subfamily BIOMPHALARIINAE			
Biomphalaria a. alexandria	18	Egypt	Natarajan *et al.*, 1965

TABLE XII—(*Contd.*)

Species	Haploid No.	Source	Reference
B. c. choanomphala	18	Tanzania	Burch, 1965 *b*
B. glabrata	18	Puerto Rico	Burch, 1960 *c*
B. s. sudanica	18	Sudan	Burch, 1960 *c*
B. s. tanganyicensis	18	Tanzania	Natarajan *et al.*, 1965
B. p. pfeifferi	18	Tanzania, Rhodesia, S. Africa	Natarajan *et al.*, 1965
B. p. gaudi	18	Liberia, Ghana	Natarajan *et al.*, 1965
B. p. madagascariensis	18	Madagascar	Natarajan *et al.*, 1965
Subfamily PLANORBINAE			
Anisus vortex	18	Europe	LeCalvez and Certain, 1950
A. crassilabrum	18	Madagascar	Natarajan *et al.*, 1965
Armiger crista	18	U.S.A.	Burch, 1960 *a*
Gyraulus spirillus	18	Japan, Taiwan	Burch *et al.*, 1964; Burch and Natarajan, 1965
G. perstriatulus	18	Japan	Burch *et al.*, 1964
G. tokyoensis	18, 18+	Japan	Burch *et al.*, 1964
G. deflectus	18	U.S.A.	Burch, 1960 *a*
G. circumstriatus	18	U.S.A.	Burch, 1965 *b*
G. parvus	36	U.S.A.	Burch, 1965 *b*
G. sp.	18	Taiwan	Burch, 1965 *a*
Subfamily SEGMENTININAE			
Segmentina hemisphaerula	18	Japan, Taiwan	Burch *et al.*, 1964; Burch and Natarajan, 1965
Helicorbis umbilicalis	18	Taiwan	Burch and Natarajan, 1965
Hippeutis cantori	18	Taiwan	Burch and Natarajan, 1965
Subfamily CAMPTOCERINAE			
Camptoceras hirasei	18	Japan	Burch *et al.*, 1964

The systematic position of the Soleolifera is uncertain. Their haploid chromosome numbers range from $n = 16$ to $n = 18$ (Table IX). Some contend that the Soleolifera should be placed with the opisthobranch orders (*e.g.*, Fretter, 1943; Plate, 1893; Boettger, 1955; Zilch, 1959–60; Taylor and Sohl, 1962), while others place them with the Pulmonata (*e.g.*, Pilsbry, 1948; Hoffmann, 1925, 1929; and Van Mol, 1967). Hyman (1967, p. 624), following Fretter (1943), seems to favour removing the Onchidiacea from the systellamatophoran pulmonates (=Soleolifera + Onchidiacea) to the Opisthobranchia. Van Mol (1967), on the basis of cerebral nervous system structure, recognized the pulmonate order Soleolifera (+ Systellamatophora) to contain a natural grouping of the families Oncidiidae, Rathouisiidae and Veronicellidae. For reasons of convenience only, I have followed in the tables the classification of Taylor and Sohl (1962). Chromosome numbers, *per se*, have no bearing on whether or not the Soleolifera should be classified with the Pulmonata or Opisthobranchia, although Van Mol (1967) has erroneously stated that "Burch (1962)[6] considére quil faut ranger les Veronicellidae among les Opisthobranches parce que le nombre haploide de leurs chromosomes est de 17, nombre inferior à 18 qui semble etre celui de tous les Pulmonés primitifs". Van Mol (1967) considers the family Oncidiidae to be the most primitive of the Soleolifera and the families Veronicellidae and Rathouisiidae to be derived from the Oncidiidae.

[6] *In*: Cytotaxonomic studies of freshwater limpets, (Gastropoda, Basommatophora). I. The European lake limpet, *Acroloxus lacustris*. *Malacologia*, 1 (1): 55–72. (In that paper, Burch did not mention the family Veronicellidae but did show a graph of chromosome numbers where the Soleolifera were included with the opisthobranch orders following the classification of Boettger (1955). Additionally, the haploid chromosome number shown on the graph was 16 and not 17 as Van Mol (1967) stated. In reviewing chromosome numbers and systematics of the Euthyneura, Burch (1965 *b*) states, "Only two species of the Soleolifera have been investigated, *Veronicella floridana* with the haploid chromosome number 16 and *Onchidium verruculatum* with the haploid number 18. The systematic relations of the Soleolifera are still uncertain and some authors maintain that they should more properly be placed with the 'pulmonates'. If they should be included among the opisthobranch orders as suggested by Fretter (1943) then *Onchidium* has the highest chromosome number known for these groups [opisthobranchs]".

TABLE XIII

Haploid chromosome numbers of the planorbid subfamily Bulininae

Species	Haploid No.	Source	Reference
Subfamily BULININAE			
Indoplanorbis exustus	18	India	Natarajan, 1960
Bulinus africanus group			
B. africanus ovoideus	18	Tanzania	Burch, 1967 *b*
B. globosus	18	Liberia, Ghana, Zambia, Rhodesia, S. Africa	Natarajan *et al.*, 1965
B. n. nasutus	18	Tanzania	Burch, 1967 *b*
B. n. productus	18	Tanzania	Burch, 1967 *b*
B. jousseaumei	18	Senegal	Natarajan *et al.*, 1965
B. ugandae	18	Tanzania	Burch, 1967 *b*
Bulinus forskalii group			
B. beccarii	18	W. Aden	Natarajan *et al.*, 1965
B. forskalii	18	W. Aden, Ghana, Tanzania, S. Africa	Natarajan *et al.*, 1965
	19	Angola	Natarajan *et al.*, 1965
B. reticulatus	18	W. Aden	Natarajan *et al.*, 1965
B. senegalensis	18	Senegal	Natarajan *et al.*, 1965
Bulinus tropicus group			
B. guernei	18	Senegal	Natarajan *et al.*, 1965
B. natalensis	18–21	Rhodesia, S. Africa	Burch, 1963, 1964 *a*
B. t. tropicus	18	Kenya, S. Africa, Rhodesia	Burch, 1963, 1964 *a*
B. t. angolensis	18	Zambia	Burch, 1963, 1964 *a*
B. t. zanzebaricus	18	Tanzania	Burch, 1963, 1964 *a*
B. sp.	18	Ethiopia	Burch, 1964 *a*
Bulinus truncatus group			
B. coulboisi	36	Tanzania	Burch, 1963, 1964 *a*
B. schackoi	36	Ethiopia	Burch, 1967 *b*
B. t. truncatus	36	Sardinia, Iran, Iraq, Egypt, Sudan	Burch, 1960 *c*, 1963, 1964 *a*
B. t. rohlfsi	36	Ghana	Burch, 1964 *a*
B. sp.	36	Ethiopia, W. Aden	Burch, 1964 *a*, 1967 *b*
B. sp.	54	Ethiopia	Burch, 1967 *b*
B. sp.	72	Ethiopia, W. Aden	Burch, 1964 *a*, 1967 *b*

The range in haploid chromosome numbers among non-polyploid members of the order Basommatophora is $n = 15$–19; polyploid species extend this range to $n = 72$ (Tables X–XIV). Classification of the "lower" Basommatophora is, at this time, still uncertain. It does seem justified to follow the suggestion of Morton (1955) in separating the order Basommatophora into the more primitive ("lower") basommatophoran species (suborder Archaeopulmonata) and the more specialized ("higher") limnic Basommatophora (suborder Branchiopulmonata). The question arises however as to which groups belong to these respective suborders. Originally, Morton (1955) included the following families in the Archaeopulmonata: Ellobiidae, Otinidae, Chilinidae, Latiidae, Amphibolidae, Gadiniidae and Siphonariidae. The Lymnaeidae, Physidae, Planorbidae and Ancylidae belonged to the Branchiopulmonata.[6] However, Van Mol (1967) includes only the Ellobiidae and Otinidae in the Archaeopulmonata, which he raised to an order. Similarly, disagreement are prevalent as to which familial group if the most primitive pulmonate with the major argument centered about the families Siphonariidae and Ellobiidae. Hyman (1967) feels that *Siphonaria* is probably the most primitive pulmonate. Natarajan and Burch (1966) state that "the lower

[6] Morton considered *Acroloxus* to be a member of the Ancylidae. Bondeson (1950), Burch (1961 *a*, 1962) and Hubendick (1962) have convincingly argued for familial status of *Acroloxus*.

chromosome numbers of the Siphonariidae may indicate that they are the most primitive family of the suborder [Archaeopulmonata], if the suggestion (Burch, 1961 *b*) that changes in chromosome numbers in the Euthyneura usually involve an increase rather than a decrease in numbers and that higher numbers are nearly always found in the phylogenetically more advanced taxa". The Siphonariidae have the lowest chromosome number characterizing any single basommatophoran group. Seven widely distributed species of *Siphonaria* have only 16 pairs of chromosomes (Table X).

In the Ellobiidae (Table X), the genera *Pythia*, *Ovatella* (= *Phytia*), *Ophicardelus* and *Cassidula* are placed in the primitive subfamily, Pythiinae, by Morton (1955) with *Pythia* considered the most primitive. *Melampus*, and presumably *Detracia*, are considred by Morton to belong to one of the most advanced groups of the family. Haploid chromosome numbers in the Ellobiidae

TABLE XIV

Haploid chromosome numbers of Lymnaeidae

Species	Haploid No.	Source	Reference
Subfamily LYMNAEINAE			
Lymnaea stagnalis	18	England	Burch, 1965 *b*
L. s. lacustris	18	Europe	Perrot, J. L., 1930
L. s. rhodani	18	Europe	Perrot, J. L., 1930, 1934
L. s. jugularis	18	U.S.A.	Burch, 1960 *a*
Acella haldemani	18	U.S.A.	Burch, 1960 *a*
Pseudosuccinea columella	18	U.S.A.	Burch, 1960 *a*
Bulimnea megasoma	18	U.S.A., Canada	Burch, 1960 *a*
Fossaria modicella	18	U.S.A.	Burch, 1960 *a*
F. parva	18	U.S.A.	Burch, 1960 *a*
*F. truncatula**	18	Japan	Burch *et al.*, 1964
F. rustica	19	U.S.A.	Burch, 1960 *a*
Stagnicola caperata	18	U.S.A.	Burch, 1960 *a*
S. catascopium	18	U.S.A.	Burch, 1960 *a*
S. desidiosa	18	U.S.A.	Burch, 1960 *a*
S. elodes	18	U.S.A.	Burch, 1960 *a*
S. emarginata	18	U.S.A.	Burch, 1960 *a*
S. exilis	18	U.S.A.	Burch, 1960 *a*
S. lanceata	18	U.S.A.	Burch, 1960
S. montanensis	18	U.S.A.	Burch, 1963 *a*
S. palustris	18	Europe	Perrot and Perrot, 1938; Burch, 1960 *a*
S. reflexa	18	U.S.A.	Burch, 1960 *a*
S. umbrosa	18	U.S.A.	Burch, 1960 *a*
Radix "limosa" (?)	18	Europe	LeCalvez and Certain, 1950
R. ollula	16	Japan, Taiwan	Burch *et al.*, 1964; Burch and Natarajan, 1965; Inaba, 1965
Austropeplea tomentosa (= *Radix* ?)	16	Australia	Inaba, 1969
Peplimnea lessoni (= *Radix* ?)**	16	Papua	Inaba, 1969
R. auricularia	17	Europe	Perrot and Perrot, 1938
R. a. swinhoei	17	Taiwan	Burch and Natarajan, 1965
R. hovarum	17	Madagascar	Burch, 1965
R. ovata	17	Europe	Perrot and Perrot, 1938
R. pereger	17	Europe	Perrot and Perrot, 1938; Burch, 1960 *a*
R. sp.	17	Italy	Burch, 1965 *b*
R. japonica	17	Japan	Burch *et al.*, 1964
R. onychia	17	Japan	Burch *et al.*, 1964
R. luteola	17	India	Natarajan, 1960
Subfamily LANCINAE			
Lanx spp. (3 species)	18	U.S.A.	Burch, 1965 *b*, 1967 *a*
Fisherola spp. (2 species)	18	U.S.A.	Burch, 1967 *a*

* *F. truncatula* = *F.* sp. in Burch, 1965 *b*.

** See addendum.

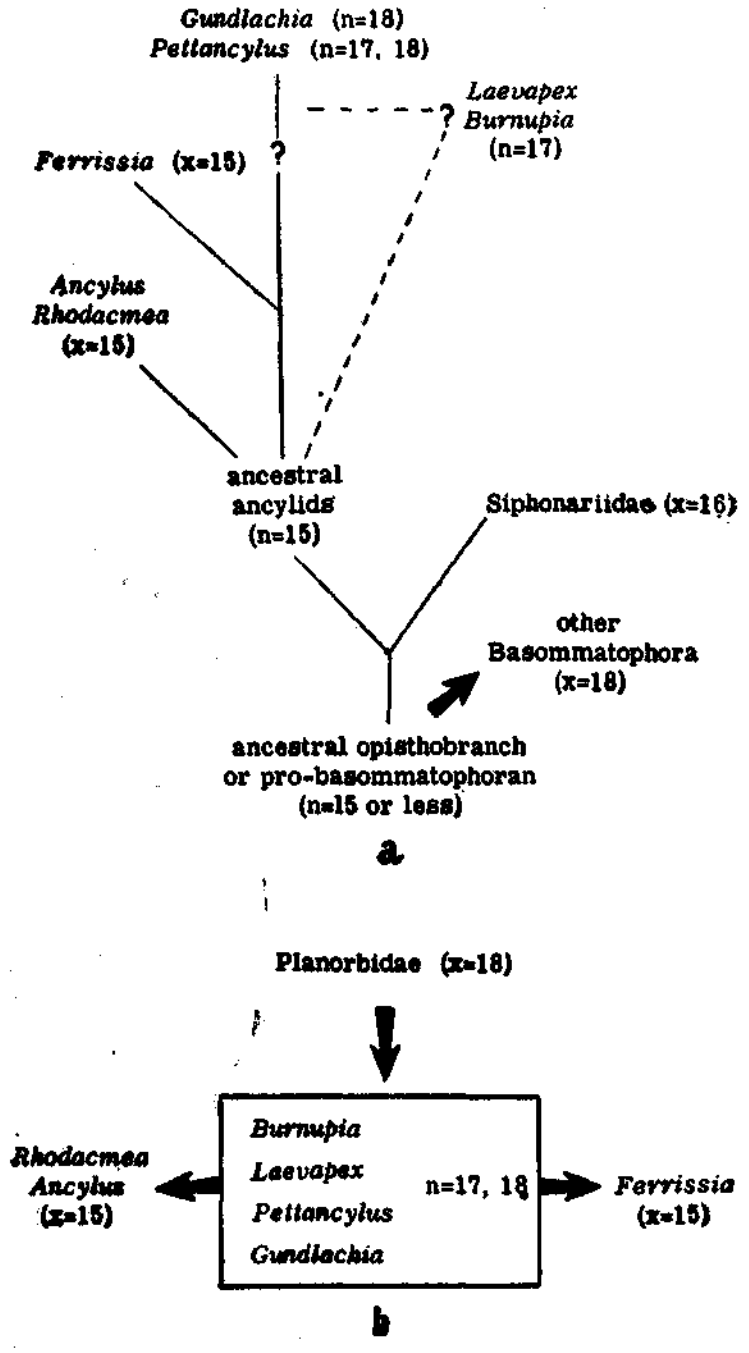

FIG. 4. Two possible evolutionary trends in the derivation of the Ancylidae. (*a*) increases in chromosome number through aneuploidy and polyploidy; (*b*) decreases in chromosome number through aneuploidy followed by subsequent increases through ploypoloidy.

TABLE XV

Haploid chromosome numbers of Stylommatophora

Species	Haploid No.	Source	Reference
Suborder *ORTHURETHRA*			
Family ACHATINELLIDAE			
Achatinella mustelina	20	Hawaii	Burch, 1965 *b*
A. bellula	20	Hawaii	Burch and Natarajan (pers. comm.)
A. producta stewartii	21	Hawaii	Burch and Natarajan (pers. comm.)
Auriculella auricula	23	Hawaii	Burch and Natarajan (pers. comm.)
Family CIONELLIDAE			
Cionella lubrica	26	Europe	Rainer, 1967
Family PYRAMIDULIDAE			
Pyramidula sp.	26	Europe	Natarajan, 1965
Family PARTULIDAE			
Partula turneri	29	New Hebrides	Burch, 1968 *c*
Family CHONDRINIDAE			
Abida secale	30	Europe	Rainer, 1967
Chondrina avenacea	30	Europe	Rainer, 1967
C. similis	30	Europe	Rainer, 1967
Family VALLONIIDAE			
Vallonia costata	28	U.S.A.	Burch and Heard, 1962
V. pulchella	28	U.S.A.	Burch and Heard, 1962
Family ENIDAE			
Ena andersoniana	24	Japan	Inaba, 1959 *a*
E. japonica	24	Japan	Inaba, 1959 *a*
E. montana	24	Europe	Rainer, 1967
E. obscura	24	Europe	Rainer, 1967
Zebrina detrita	24	Europe	Rainer, 1967
Rhachis punctata	24	India	Natarajan, 1960
Suborder *MESURETHRA*			
Family CERIIDAE			
Cerion incanum	27	U.S.A.	Burch and Kim, 1962
Family CLAUSILIIDAE			
Clausilia bidentata	24	Europe	Natarajan, 1965
C. parvula	24	Europe	Thaler, 1963; Rainer, 1967
C. dubia tettelbachiana	24	Europe	Thaler, 1963
C. d. obsoleta	24	Europe	Thaler, 1963
C. d. huttneri	24	Europe	Thaler, 1963
C. d. vindobonensis	24	Europe	Thaler, 1963
Megalophaedusa martensii	24	Japan	Inaba, 1959;
Phaedusa subaculus	24	Japan	Inaba, 1959;
Iphigena ventricosa	24	Europe	Thaler, 1963; Rainer, 1967
I. plicatula	24	Europe	Rainer, 1967
I. p. grossa	24	Europe	Thaler, 1963
Cochlodina laminata	24	Europe	Thaler, 1963; Rainer, 1967
Herilla bosniensis	24	Europe	Thaler, 1963
Laciniaria plicata	24	Europe	Rainer, 1967
L. biplicata	24	Europe	Thaler, 1963; Burch, 1965 *b*
Graciliaria strobeli	24	Europe	Rainer, 1967
Euphaedusa pseudosheridani	28	Taiwan	Burch, 1965 *b*
E. tau	28	Japan	Natarajan, 1965
Hemiphaedusa similaris	28	Taiwan	Burch, 1965 *b*
Stereophaedusa japonica	30	Japan	Natarajan, 1965
Delima itala	*ca.* 30	Europe	Rainer, 1967
Papillifera papillaris	*ca.* 30	Europe	Rainer, 1967
Suborder *HETERURETHRA* (Table XVI)	5–44		
Suborder *SIGMURETHRA*			
Infraorder HOLOPODOPES			
Family FERRUSSACIIDAE			
Glessula sp.	30	India	Natarajan (pers. comm.)

TABLE XV—(*Contd.*)

Species	Haploid No.	Source	Reference
Family SUBULINIDAE			
Lamellaxis mauritianus	25	Solomon Is.	Burch, 1967 *a*
Subulina octona	31	Australia, Solomon Is.	Laws, 1965; Burch, 1967 *a*
Family ACHATINIDAE			
Achatina fulica	30	India	Natarajan, 1960
Family HAPLOTREMATIDAE			
Haplotrema sportella	29	U.S.A.	Ford, 1962
H. vancouverense	30	U.S.A.	Burch, 1965 *b*
Family BULIMULIDAE			
Bulimulus nux	29	Galapagos Is.	Burch and Natarajan (pers. comm.)
Placostylus miltocheilus	30	Solomon Is.	Burch, 1967 *a*
Diplomorpha sp.	30	New Hebrides	Burch, 1968 *c*
Infraorder AULACOPODA			
Family ENDODONTIDAE			
Discus rotundatus	29	Europe	Rainer, 1967
D. patula	31	U.S.A.	Husted and Burch, 1946
Anguispira alternata	31	U.S.A.	Husted and Burch, 1946
Family ARIONIDAE (Table XVII)	25–29		
Family PHILOMYCIADAE (Table XVII)	24		
Family VITRINIDAE			
Vitrinopsis sp.	28	Solomon Is.	Burch, 1967 *a*
Family ZONITIDAE			
Vitrea diaphana	20	Europe	Rainer, 1967
Dyakia striata	28	Fiji	Burch, 1968 *c*
Mesomphix oxycoccus	29–30	U.S.A.	Husted and Burch, 1946
M. cupreus	31	U.S.A.	Husted and Burch, 1946
Oxychilus alliarius	30	Australia	Laws, 1966
Family MILACIDAE (Table XVII)	33–34		
Family LIMACIDAE (Table XVII)	24–31		
Family TROCHOMORPHIDAE			
Trochomorpha sp.	28	Solomon Is.	Burch, 1967 *a*
T. sp.	30	Solomon Is.	Burch, 1967 *a*
Family HELICARIONIDAE			
Helicarion sp.	28	Solomon Is.	Burch, 1967 *a*
Family ARIOPHANTIDAE			
Mariella dussumieri	25	India	Natarajan, 1960
Euplecta travancorica	27	India	Natarajan, 1960
Cryptozona bistrialis	27	India	Natarajan, 1958 *b*
C. semirugata	28	India	Natarajan, 1958 *b*
C. ligulata	32	India	Natarajan, 1958 *b*
Macrochlamys vilipensa	30	India	Natarajan, 1960
Family TESTACELLIDAE (Table XVII)	32		
Infraorder HOLOPODA			
Family POLYGYRIDAE (Table XVIII)	26–31		
Family CAMAENIDAE			
? *Draparnaudia singularis*	27	New Hebrides	Burch, 1968 *c*
Papuina sp.	28	Papua	Burch, 1968 *c*
Satsuma myomphala	29	Japan	Inaba, 1959 *a*
Planispira fallaciosa	29	India	Natarajan (pers. comm.)
Meracomelon sp.	29	Australia	Laws, 1965
Sinumelon sp.	29	Australia	Laws, 1965
Hadra sp.	29	Australia	Laws, 1965
Xanthomelon sp.	29	Australia	Laws, 1965
Pleuroxia sp.	29	Australia	Laws, 1965
Chlorritobadistes sp.	29	Australia	Laws, 1965
Family BRADYBAENIDAE (Table XIX)	28–29		
Family HELMINTHOGLYPTIDAE			
Monadenia fidelis	29	U.S.A.	Burch, 1965 *b*
Family HELICIDAE (Table XX)	21–30		

range from $n = 17$-19. Three members of the Pythiinae have the lowest chromosome numbers in the Ellobiidae, $n = 17$ and the highest number, $n = 19$, occurs in *Melampus* which belongs to one of the most advanced ellobiid groups. Morton (1955) considers *Pythia* to be structurally the most primitive ellobiid even though some of its species may occupy terrestrial habitats characteristic of more advanced or specialized pulmonates. It is of interest to note that *Pythia plicata* collected from an estuarine environment has 17 pairs of chromosome (Natarajan, 1958 *a*), whereas *Pythia scarabeans* which lives in terrestrial habitats often far removed from the seashore has 18 pairs of chromosomes (Burch, 1967 *a*).

The haploid chromosome number $n = 18$ is reported for the 1 species studied in each of the archaeopulmonate families Amphibolidae, Chilinidae, Latiidae, and Acroloxidae (Table X). Chromosome numbers, *per se*, are of little help in establishing the phylogenetic positions of the above families within the Archaeopulmonata since all have 18 pairs of chromosomes, the basic haploid chromosome number characterizing the Branchiopulmonata. Cytological characteristics and information on egg morphology convinced Burch (1962) and Bondensen (1950) that *Acroloxus lacustris* should be removed from the Ancylidae (Branchiopulmonata) and placed in its own family, the Acroloxidae. Natarajan and Burch (1966) included the Acroloxidae in the Archaeopulmonata. Burch and Patterson (1963) pointed out that *Acroloxus* is quite distinct from *Latia* in contrast to the view of Hubendick (1962).

The freshwater limpet family Ancylidae has the chromosome numbers $n = 15$, 30, 60 and $n = 17$ or 18 (Table XI). Cytological information for the 3 related species of Ancylinae indicates the establishment of a polyploid series with $x = 15$ (found in *Rhodacmea cahawbensis*) considered to be the basic chromosome number. *Ancylus* sp. ($n = 30$) from Ethiopia is probably a tetraploid species and *A. fluviatilis* an octoploid.

Two species of *Ferrissia* also have 30 pairs of chromosomes and may be tetraploids derived from an ancestral species with 15 pairs of chromosomes. *Laevapex fuscus* from North America and its widely separated relative *Burnupia* sp. from Africa both have 17 pairs of chromosomes. Only 3 of the 11 species of Ancylidae studied have the haploid number $n = 18$, characteristic of most other Branchiopulmonata. Ancylidae has been less conservative in regard to changes in chromosome number, since there is evidence of evolutionary change accompanied by both polyploidy and aneuploidy.

Pelseneer (1901) contended that the Ancylidae were derived from the Planorbidae, but Burch (1962) followed by Hubendick (1964) felt that the ancestral ancylid was perhaps more primitive than any recent or "higher limnic Basommatophora". Two possible evolutionary trends in the derivation of the Ancylidae are shown in Fig. 4 *a, b*. If the primitive ancylids were derived from a pro-basommatophoran or an opisthobranch ancestor with less than 15 pairs of chromosomes, there would have been an increase in haploid chromosome numbers by aneuploidy and polyploidy. However, if a planorbid with 18 pairs of chromosomes served as the ancestor, there would have been successive loss of bivalents (as many as three) resulting in the $n = 17$ and $n = 15$ representatives and a subsequent increase through polyploidy to derive the $n = 30$ and $n = 60$ individuals from the $n = 15$ stock. The former is perhaps the "easiest" derivation since gain in chromosome numbers are easier to account for than reductions of chromosome numbers.

The Planorbidae are characterized by the basic haploid chromosome number $x = 18$; all of the genera have 18 pairs of chromosomes (or a multiple of 18), except *Planorbula* ($n = 19$), [*Gyraulus tokyoensis* ($n = 19$)], one specimen of a population of *Bulinus forskalii* ($n = 19$), and individuals of a population of *B. natalensis* which had one to three extra bivalents (Tables XII, XIII). Tetraploid, hexaploid and octoploid species occur among the Planorbidae and will be discussed in greater detail later in this paper.

Most of the 40 species and subspecies of Lymnaeidae studied have 18 pairs of chromosomes (Table XIV), but those of the widely distributed genus *Radix* have only 17 pairs of chromosomes.

Burch (1965 *b*) speculated that the original lymnaeid ancestors had less than 18 pairs of chromosomes, and that other lymnaeid groups were derived from *Radix* or a pro-*Radix* stock and gained an extra bivalent during or after separation from this ancestral group. Dr. Yoshio Kondo (*see* Burch, 1967*a*), referring to the low chromosome number (n=16) reported by Burch *et al.* (1964) for "*Radix*" *ollula* Gould, suggested that this species is perhaps an archaic form that entered the Pacific with the Orthurethra during the late Paleozoic to Early Mesozoic, and gave rise to various of the current Pacific species. This hypothesis is strengthened by finding *Austropeplea tomentosa* (= *Radix*?) of Australia and *Peplimnea* (= *Radix*?) *lessoni* of Papua to have 16 pairs of chromosomes. But, Dr. H. J. Walter (pers. comm. to J. B. Burch) considers *P. lessoni* to have the anatomy of the *Radix auricularia*-group and "*R.*" *ollula* to be definitely radicine, but in some respects, similar to the *R. peregra*-group. Therefore, "*R.*" *ollula* and the species *A. tomentosa* and *P. lessoni* may well be primitive lymnaeids, belonging to a group which gave rise to *Radix*, which in turn gave rise to the other lymnaeids, just as Burch suggested.

Dr. Walter (pers. comm. to J. B. Burch) has suggested that the "*Radix*" *limosa* of LeCalvez and Certain (1950) may have been a stagnicoline form. If this is the case, then their report of the anomalous chromosome number ($n = 18$) for a *Radix* species would be explained, since $n = 18$ characterizes *Stagnicola* species.

Burch *et al.* (1964) referred to "*Lymnaea*" *ollula* Gould as "*Radix*" partly because of cytological considerations and partly on anatomical information from Hubendick (1951). Burch (1967*a*) later referred this species to *Bakerilymnaea*, following the recommendation of Dr. D. W. Taylor. However, there is no critical evidence that this Oriental species has any close relationship with the group of Western Hemisphere stagnicoline snails which Baker (1928) called *Nasonia* (= Bakerilimnaea) (Dr. J. B. Burch, personal communication). Anatomically, "*L.*" *ollula* appears to be related to the Eurasian *Radix* (Dr. H. J. Walter, pers. to comm. J. B. Burch), and perhaps should be retained in that genus for the time being.

All other species of the Lymnaeidae not mentioned above have 18 pairs of chromosomes except *Fossaria rustica* which has 19 pairs. The fact that *F. rustica* ($n = 19$), a species at present difficult to distinguish from nominal species of *Fossaria* (e.g., *F. obrussa*, *F. exigua*), has been found to have 19 pairs of chromosomes in Michigan, Ohio and Idaho, shows that this cytological form has a wide geographical range and is not just an isolated, cytologically aberrent population. Its cytological distinction from the common *F. obrussa* would argue against the opinion of Hubendick (1951) that there is only one fossarid-type species (*F. humilis*) in most of the U.S.A.

Little can be said about cytological relationships in the Physidae except all 7 members of this conservative group have 18 pairs of chromosomes, and in this way do not differ from species of most other higher limnic basommatophoran families.

Pulmonate land snails are almost exclusively members of the order Stylommatophora. A commonly held view is that the ancestors of the stylommatophoran land snails were the basommatophoran Ellobiidae. The Stylommatophora has been divided into four suborders according to the structural arrangement of the kidney and ureter. The Orthurethra is considered by Pilsbry (1900*a*) to be the most primitive suborder because the kidney and ureter resemble those of the Basommatophora. He apparently considered the Heterurethra to be somewhat more advanced than the Orthurethra, and he considered the Sigmurethra to be the most specialized suborder. Pilsbry (1900*b*) implies that both the Heterurethra and Sigmurethra were evolved separately from the Orthurethra. Boettger (1955) follows this same scheme. In contrast, Baker (1955) feels that the ancestral group of the Sigmurethra had heterurethrous pallial organs and an aulacopod foot. Baker (1955, 1961) proposed the establishment of the suborder Mesurethra to distinguish the cerionoids and clausiliids from other stylommatophoran snails. The Mesurethra is believed to be phylogenetically close to the Orthurethra (Baker, 1961; Pilsbry, 1946).

TABLE XVI

Haploid chromosome numbers of the Heterurethra

Species	Haploid No.	Source	Reference
Superfamily SUCCINACEA			
Family SUCCINEIDAE			
Subfamily CATINELLEINAE			
Catinella rotundata	5	Hawaii	Burch, 1964 *b*
C. cf. gabbi	6	U.S.A.	Burch, Patterson and Natarajan, 1966
C. texana	6	U.S.A.	Natarajan, Hubricht and Burch, 1966
C. vermeta	6	U.S.A.	Burch, 1964 *c*
C. arenaria	6	England	Butot and Kiauta, 1967
Subfamily SUCCINEINAE			
Succinella oblonga	11, 12	Europe	Rainer, 1967
	12	Europe	Butot and Kiauta, 1964.
Oxyloma japonica	15	Japan	Burch, 1965 *b*
O. hirasei	17	Japan	Koyama, 1955
O. kwansae	17	Japan	Koyama, 1955
O. elegans	17	Europe	Butot and Kiauta, 1964
O. sarsi	17	Europe	Butot and Kiauta, 1964
O. cleopatraea	18	Egypt	Patterson (unpublished)
O. sanibelensis	18	U.S.A.	Patterson, (unpublished)
O. haydeni	19	U.S.A.	Franzen, 1966
O. salleana	19	U.S.A.	Natarajan, Hubricht and Burch, 1966
O. retusa	19	U.S.A.	Burch, Patterson and Natarajan, 1966
Omalonyx felina	21	Antigua	Patterson (unpublished)
Succinea sp.	11	Tanzania	Patterson (unpublished)
S. horticola	17	Japan	Inaba, 1945
S. girata	17	Curacoa	Butot and Kiauta (pers. comm.)
S. concordialis	18	U.S.A.	Natarajan, Hubricht and Burch, 1966
S. greeri	18	U.S.A.	Natarajan, Hubricht and Burch, 1966
S. urbana	18	U.S.A.	Natarajan, Hubricht and Burch, 1966
S. campestris	18	U.S.A.	Burch, Patterson and Natarajan, 1966
S. luteola	18	U.S.A.	Natarajan, Hubricht and Burch, 1966
S. sp.	18	U.S.A.	Patterson (unpublished)
S. cf. strubelli	18	Papua	Patterson (unpublished)
S. kuntziana	18	New Hebrides	Patterson (unpublished)
S. grosvenori	19	U.S.A.	Natarajan, Hubricht and Burch, 1966
S. ovalis	21	U.S.A.	Husted and Burch, 1946; Burch, Patterson and Natarajan, 1966
S. putris	22	Europe	Perrot, 1938 *a*; Butot and Kiauta, 1964
S. lauta	22	Japan	Burch, 1965 *b*
S. l. sphaerica	22	Japan	Burch, 1965 *b*
S. gravelyi	25	India	Patterson (unpublished)
Superfamily ATHORACOPHORACEA			
Family ATHORACOPHORIDAE			
Aneitea sp.	44	New Hebrides	Patterson, (unpublished)

Mörch (1865) united the trachaeopulmonate slugs *Janella* and *Aneitea* with the succineids *Omalonyx* and *Succinea* in the group Elasmognatha on the basis of jaw structure. However, Pilsbry (1948) felt that the tracheopulmonates (Athoracophoridae) were so different from the Succineidae that they should be retained in the separate suborder, Trachaeopulmonata, leaving the Succineidae alone in the Elasmognatha (= Heterurethra). Baker (1955) placed the trachaeopulmonate slugs in the same group with succineids, the Heterurethra. Likewise, Van Mol (1967) unites these 2 groups retaining Elasmognatha, the name designated by Mörch (1865). The important difference in Van Mol's classification from those of other authors is that Van Mol considers the Elasmognatha (= Heterurethra) to be the most primitive stylommatophoran suborder; the Orthurethra to be

derived from an heterurethran ancestor and the Sigmurethra from an orthurethran predecessor. For convenience, I have followed the classification presented by Taylor and Sohl (1962) for the Stylommatophora.

The range in haploid chromosome numbers of the stylommatophoran land snail suborders is shown in Fig. 1. The greatest aneuploid range of haploid chromosome numbers occurs in the aberrant suborder Heterurethra ($n = 5$–44, Table XVI). Its most prominent family, the Succineidae, is of particular interest cytologically because the lowest chromosome numbers found in molluscs ($n = 5$ or 6) occur in several of its species. Haploid chromosome numbers range from $n = 5$–25 in the Succineidae. This family contains 2 subfamilies, the Catinellinae which lacks a penial sheath and the Succineinae which has a penial sheath. The chromosome numbers of the succineids fall into 2 groups corresponding to these morphological divisions. Members of the Catinellinae have the chromosome number $n = 5$ or $n = 6$ in all 5 species studied while members of the Succineinae have haploid chromosome numbers ranging from $n = 11$ to $n = 25$. There appears to be a consistent correlation between low haploid chromosome numbers in the most primitive group, Catinellinae, and higher haploid numbers in the more advanced group, Succineinae. It will be interesting to observe whether further cytological data bears out this relationship.

TABLE XVII

Haploid chromosome numbers of sigmurethran slugs

Species	Haploid No.	Source	Reference
Family PHILOMYCIDAE			
Incilaria confusum	24	Japan	Inaba, 1959
I. fruhstorferi	24	Japan	Inaba, 1959
Family ARIONIDAE			
Arion subfuscus	25	Britain	Beeson, 1960
A. ater	26	Britain	Beeson, 1960
A. rufus	26	France	Beeson, 1960
A. hortensis	28	Britain	Beeson, 1960
A. intermedius	28	Britain	Beeson, 1960
A. circumscriptus	29	Britain	Beeson, 1960
Family LIMACIDAE			
Lehmannia marginata	24	Britain	Beeson, 1960
Deroceras agreste	30	Britain	Beeson, 1960
D. caruanae	30	Britain	Beeson, 1960
D. laeve	30	Britain	Beeson, 1960
D. reticulatum	30	Britain	Beeson, 1960
Limax cinereoniger	31	Britain	Beeson, 1960
L. flavus	31	Britain	Beeson, 1960
L. maximus	31	Britain	Beeson, 1960
Family MILACIDAE			
Milax gracilis	33	Britain	Beeson, 1960
M. gagates	33–34	Britain	Beeson, 1960
M. sowerbii	34	Britain	Beeson, 1960
Family TESTACELLIDAE			
Testacella haliotidea	32	Britain	Beeson, 1960

Butot and Kiauta (1964) reported 8 specimens from populations of *Succinella oblonga* from North Holland and Belgium to have 12 pairs of chromosomes. Rainer (1967) examined 2 specimens of 1 population from Baden (Switzerland) and found the haploid chromosome number to be predominantly $n = 11$ in 1 specimen, and a variation in the other of $n = 10$–12, with $n = 12$ predominant. He concluded that $n = 11$ is probably the normal haploid chromosome number of *Succinella oblonga* and that a connection to *Succinea putris* ($n = 22$) through polyploidy is concei-

vable. However, it seems that the population Rainer sampled was perhaps aberrant and that the normal haploid chromosome number of *Succinella oblonga* is $n = 12$ as Butot and Kiauta (1964) reported. Furthermore, the data do not suggest a connection to *Succinea putris* through polyploidy since there are species with intermediate numbers of $n = 17$, 18, 19 and 21 in the genus *Succinea*. Such a progressive increase in chromosome number could more likely be explained through aneuploidy. Also, anatomically the 2 genera are different.

TABLE XVIII

Haploid chromosome numbers of the Polygyridae

Species	Haploid No.	Source	Reference
Allogona profunda	26	U.S.A.	Husted and Burch, 1946
A. townsendiana	26	U.S.A.	Burch, 1965 *b*
Polygyra cereolus	29	U.S.A.	Burch and Patterson (unpublished)
Stenotrema hirsutum	29	U.S.A.	Husted and Burch, 1946
S. leai aliciae	29	U.S.A.	Husted and Burch, 1946
S. spinosum	29	U.S.A.	Husted and Burch, 1946
S. stenotrema	29	U.S.A.	Husted and Burch, 1946
Mesodon andrewsae normalis	29	U.S.A.	Husted and Burch, 1946
M. appressus	29	U.S.A.	Husted and Burch, 1946
M. inflectus	29	U.S.A.	Husted and Burch, 1946
M. mitchelleanus	29	U.S.A.	Husted and Burch, 1946
M. rugeli	29	U.S.A.	Husted and Burch, 1946
M. thyroides	29	U.S.A.	Husted and Burch, 1946
M. zaletus	29	U.S.A.	Husted and Burch, 1946
Triodopsis albolabris	29	U.S.A.	Husted and Burch, 1946
T. dentifera	29	U.S.A.	Husted and Burch, 1946
T. tridentata	29	U.S.A.	Husted and Burch, 1946
T. fraudulenta vulgata	29	U.S.A.	Husted and Burch, 1946
T. f. fraudulenta	29–31	U.S.A.	Husted and Burch, 1946
T. germana	31	U.S.A.	Ford, 1962
Vespericola columbiana	30	U.S.A.	Ford, 1962

The trachaeopulmonate slugs are not only unique morphologically (e.g., *see* Burch, 1968 *a*) but also cytologically. The single species studied has 44 pairs of chromosomes during the first meiotic division (late diakinesis), the highest haploid chromosome number reported in the Stylommatophora.

If, as suggested by Burch (1965 *b*), evolutionary change in the Euthyneura has usually been accompanied by an increase in chromosome number, then the contention of Van Mol (1967) that a heterurethran snail is ancestral to the other stylommatophoran groups is strengthened by the low chromosome numbers of some succinieids.

Haploid chromosome numbers range from $n = 20$ to $n = 30$ in the suborder Orthurethra (Table XV). Pilsbry (1900 *a*) considers the Achatinellidae to be one of the most primitive Orthurethran

groups but Solem (1959) placed this family in a more advanced phylogenetic position. It may be of significance that members of this family have the lowest haploid chromosome numbers ($n = 20$–23) in the Orthurethra, which may strengthen Pilsbry's contention.

Zilch (1959–60) does not recognize the suborder Mesurethra (Table XV) but includes the members of this group in various superfamilies in the suborder Sigmurethra. The current classification of the Clausiliidae is very complicated; there are 10 subfamilies with various tribes. There is cytological data for species that have been placed in 5 different subfamilies. Haploid chromosome numbers of clausiliids do not correspond to their current classification into subfamily groups. A critical morphological-cytological study of this family would probably lead to certain revisions in the current classification. The only other mesurethran studied, *Cerion incanum* (Ceriidae), has 27 pairs of chromosomes and therefore is within the range of the Clausiliidae.

TABLE XIX

Haploid chromosome numbers of Bradybaenidae

Species	Haploid No.	Source	Reference
Bradybaena similaris	28	Japan	Inaba, 1959 *a*
B. gainesi	29	Japan	Kawabe, 1947
Euhadra callizona	28	Japan	Inaba, 1959 *a*
E. congenita	28	Japan	Inaba, 1959 *a*
E. eoa	28	Japan	Inaba, 1959 *a*
E. grata	28	Japan	Inaba, 1959 *a*
E. peliomphala	28	Japan	Inaba, 1959 *a*
E. sadoensis	28	Japan	Inaba, 1959 *a*
E. sandai	28	Japan	Inaba, 1959 *a*
E. senckenbergiana	28	Japan	Inaba, 1959 *a*
E. awaensis	29	Japan	Inaba, 1959 *a*
E. idzumonis	29	Japan	Inaba, 1959 *a*
E. quaesita	29	Japan	Inaba, 1959 *a*
E. scaevola	29	Japan	Inaba, 1959 *a*
Eulota fruticum	29	Europe	Perrot, 1938 *a*
Fruticicola despecta	29	Japan	Inaba, 1959 *a*
Aegista vatheleti	29	Japan	Inaba, 1959 *a*
Acuta despecta	29	Japan	Burch (pers. comm.)

The largest stylommatophoran suborder, the Sigmurethra, is divided into 3 infraorders, the Holopodopes, Aulacopoda and Holopoda. Baker (1955, 1962) proposed the infraorder Holopodopes and included in it several primitive "Achatinidae like" genera and families that had a less distinct aulacopod foot and had similar kidneys. The chromosome numbers characterizing members of the Holopodopes so far investigated are $n = 25$, 29, 30 and 31 (Table XV). However, cytological information for this group is too fragmentary to draw any conclusions concerning cytotaxonomic relationships.

The lowest ($n = 20$) and the highest ($n = 34$) chromosome numbers in the Sigmurethra occur in members of the infraorder Aulacopoda, a morphologically diverse group considered to be somewhat more advanced than the Holopodopes. The family Endodontidae seems to be the most primitive aulacopod group (Solem, 1959). Chromosome numbers in this family ($n = 29$–30) are higher than those reported for members of other aulacopod families (Table XV). The lowest chromosome number in the Aulacopoda occurs in *Vitrea diaphana* (Zonitidae), but the other 4 species studied in this family have 28–31 pairs of chromosomes. There is also a considerable amount of variation in chromosome numbers in the families Arionidae, Limacidae (Table XVII) and Ariophantidae (Table XV).

Chromosome numbers of the sigmurethran slugs are shown in Table XVII. In the family Limacidae each genus is characterized by a different chromosome number. But, in the Arionidae the chromosome number varies from $n = 25$ to $n = 29$ in the genus *Arion*. Nevertheless, Burch (1965 *b*) pointed out that the different chromosome numbers correspond to subgeneric groupings based on gross morphology.

The Philomycidae exhibit several morphological characters that differentiate them from other slugs. Pilsbry (1948) felt that this family was perhaps an early branch of the endodontid stock from which the Arionidae also arose. It is of interest that both philomycid species examined have 24 pairs of chromosomes, the lowest haploid chromosome number reported for the 2 endodontid slug families. Pilsbry considers the limacid slugs (including Limacidae and Milacidae) to have arisen independently from different ancestors than those of the endodontid slugs. The chromosome numbers in the Limacidae and Milacidae are higher than those of the endodontid slugs (except for *Lehmannia*). *Lehmannia* is a form intermediate to *Limax* and *Deroceras*, which is given a subgeneric ranking in *Limax* by Pilsbry (1940). However, its specialized penial structure and chromosome number suggest that perhaps *Lehmannia* deserves full generic ranking.

The infraorder Holopoda includes some rather morphologically specialized groups of land snails. Their chromosome numbers range from $n = 21$ in 1 of the Helicidae to $n = 31$ in a mosaic polygyrid specimen (Table XV). Most members of the Polygyridae studied have 29 pairs of chromosomes (Table XVIII). But, both species of *Allogona* have the haploid number $n = 26$. Pilsbry (1940) felt that polygyrid genera which retain an epiphallus and flagellum are primitive and that these structures are lost in the more specialized forms. *Allogona* has retained both an epiphallus and flagellum and is therefore considered more primitive; it also has a lower chromosome number than the other genera.

Chromosome numbers in the Helicidae range from $n = 21$ to $n = 30$ (Table XX), a rather wide variation compared to most other stylommatophoran families. Several of the chromosome numbers of this apparently specialized land snail family are relatively low. Recently, Rainer (1967) has determined the chromosome numbers of 19 species and subspecies of helicid snails and discussed some of their cytotaxonomic relationships. He studied representatives of 5 helicid subfamilies and 2 tribes of the subfamily Helicellinae. The tribe Helicelleae includes the genera *Candidula* ($n = 27$), *Helicella* ($n = 26$), *Trochoidea* ($n = 26$) and *Cochlicella* ($n = 23$). He feels that *Cochlicella* should be assigned to the tribe Monacheae of which 2 species of *Monacha* have also been found to have 23 pairs of chromosomes. *If* the member of *Cochlicella* and *Monacha* are similar morphologically, Rainer's contention may be justified. Rainer has also suggested that a division of the genus *Cepaea* into 2 subgenera based on cytology is appropriate. One subgenus would contain the species with $n = 22$ and the other subgenus the species with $n = 25$. Such a suggestion is not justified unless there are correlative morphological characters. It is interesting to note that Rainer found the European *Euparypha* (= *Theba*) *pisana* to have 28 pairs of chromosomes while Laws (1965) reported that species introduced into Australia to have chromosome number $n = 30$.

Rainer (1967) states that "in general, the cytological findings correlate well with the modern classification of the Stylommatophora". However, there are a sufficient number of exceptions to such a correlation with current classification of the Stylommatophora that such a broad statement

is not justified at present. Also, within and among a great many taxa, chromosome numbers, *per se*, neither support nor detract from present systematic schemes.

TABLE XX

Haploid chromosome numbers of Helicidae

Species	Haploid No.	Source	Reference
Subfamily HELICELLINAE			
Monacha cartusiana	23	Europe	Perrot, 1938 *a*; Rainer, 1967
M. cantiana	23	Europe	Rainer, 1967
Cochlicella ventrosa	23	Australia	Laws, 1965
C. acuta	23	Europe	Perrot, 1938 *a*; Laws, 1965
Trochoidea cretica	26	Europe	Rainer, 1967
Helicella virgata	26	Australia	Laws, 1965
H. stolismena	26	Australia	Laws, 1965
H. neglecta	26	Australia	Laws, 1965
H. itala	26	Europe	Rainer, 1967
H. obvia	26	Europe	Perrot, 1938 *a*
Candidula unifasciata	27	Europe	Rainer, 1967
Subfamily HYGROMIINAE			
Hygromia cinctella	21	Europe	Perrot, 1938 *a*
Euomphalia arpatschiana sewanica	23	Iran	Rainer, 1967
Trichia sericea	23	Europe	Rainer, 1967
T. villosa	23	Europe	Rainer, 1967
T. unidentata	23	Europe	Rainer, 1967
Perforatella incarnata	24	Europe	Perrot, 1938 *a*
Subfamily HELICODONTINAE			
Helicodonta obvoluta	27	Europe	Rainer, 1967
Subfamily HELICIGONINAE			
Helicogona lapicida	29	Europe	Perrot, 1938 *a*; Rainer, 1967
H. setosa	29	Europe	Rainer, 1967
H. pouzolzi	29	Europe	Rainer, 1967
H. arbustorum	30	Europe	Perrot, 1938 *a*; Rainer, 1967
H. achates rhaetica	30	Europe	Rainer, 1967
Isognomostoma isognomostoma	30	Europe	Rainer, 1967
I. holosericum	30	Europe	Rainer, 1967
Subfamily HELICINAE			
Pseudotachea litturata	22	Europe	Perrot, 1938 *a*
Cepaea nemoralis	22	Europe	Perrot, 1938 *a*; Rainer, 1967
C. hortensis	22, 30	Europe	Perrot, 1938 *a*; Rainer, 1967
C. silvatica	25	Europe	Perrot, 1938 *a*; Rainer, 1967
C. vindobonensis	25	Europe	Baltzer, 1931; Rainer, 1967
Iberus soluta	25	Europe	Perrot, 1938 *b*
Archelix dupotetiana	26	Europe	Perrot, 1938 *a*
A. hieroglyphicula	26	Europe	Perrot, 1938 *a*
A. punctata	26	Europe	Perrot, 1938 *a*
A. xanthodon	26	Europe	Perrot, 1938 *a*
Eobania vermiculata	27	Europe	Rainer, 1967
Helix pomatia	27	Europe	Perrot, 1938 *a*
H. dormitoris bosnica	27	Europe	Rainer, 1967
H. lucorum trapezuntis	27	Europe	Rainer, 1967
H. aspersa	27	Europe	Perrot, 1938 *a*; Laws, 1965; Rainer, 1967
H. aperta	27	Europe	Perrot, 1938 *a*
H. cincta	27	Europe	Perrot and Perrot, 1938
H. melanostoma	27	Europe	Perrot and Perrot, 1938
Euparypha (= *Theba*) *pisana*	28	Europe	Rainer, 1967
	30	Australia	Laws, 1965
Caucasotachea lenkoranea	30	Iran	Rainer, 1967

A final point I wish to discuss in this section is the utility of cytological information in taxonomic discrimination at the generic and specific levels. Cytological information has been helpful in 2 cases in the Lymnaeidae. The lymnaeid species *ollula* of the Orient was long considered to be a member of the genus *Fossaria*. However, Burch *et al.* (1965) found this species to have the chromosome number $n = 16$, quite distinct from other fossarids ($n = 18, 19$). It was provisionally assigned to the radicine group, and later Dr. H. J. Walter (personal communication) found this species to have radicine-like anatomy. Hubendick (1951) considered all North American fossarids, excluding those of the southernmost region of the Gulf coast states, to be only one species, *Lymnaea* (= *Fossaria*) *humilis*. But the chromosome number of *F. rustica* ($n = 19$) with a wide geographic range demonstrates its distinction from other fossarids ($n = 18$) and supports those who contend that there are several species of the genus in North America.

Cytological information has been helpful in discerning various planorbid species. Burch (1965 *b*) reported *Gyraulus circumstriatus* to have 18 pairs of chromosomes, and the closely related and often indistinguishable form, *G. parvus*, to have 36 pairs (Table XII). These 2 species have sometimes been considered synonymous by malacologists, but their difference in chromosome number demonstrates their distinctness (*i.e.*, one is a normal diploid, the other tetraploid).

Burch (1967 *b*, *c*) found Ethiopian bulinine snails to have chromosome numbers of $n = 18$, $n = 36$, $n = 54$ and $n = 72$ (Table XIII). Each of these various cytologically distinct populations was also found in different ecological habitats. Mandahl Barth (1965) considered only 1 species of *Bulinus* s.s. to occur in Ethiopia, but these polyploid populations represent distinct biological species. There are, then, at least 4 species of the subgenus *Bulinus* s.s. in Ethiopia.

Chromosome cytology has been helpful in the systematics of 3 streptoneuran genera, *Tulotoma* (Table IV), *Oncomelania* (Table III) and *Semisulcospira* (Table V). The genus *Tulotoma* has been considered by some authors (*e.g.*, Haldeman, 1843; Clench, 1962) to be monotypic, containing only the species *T. magnifica* ($n = 12$, Pollister and Pollister, 1940, 1943) with several variants. Other authors (*e.g.*, Wetherby, 1876; Walker, 1918; Goodrich, 1944) consider the genus to include as many as 3 distinct species. The fact that specimens identified as *T. angulata* have not 12, but 13 pairs of chromosomes (Patterson, 1965) strengthens the contention that *Tulotoma* is a polytypic genus.

Burch (1964 *d*) studied F_1 hybrids of 4 nominal species of *Oncomelania* and concluded, on the basis of chromosomal features coupled with ease of hybridization, the non-reduced viability of the hybrids and morphological and biochemical similarities of the parents, that these representatives comprised only races of 1 nominal species, *O. hupensis*.

Although more than 30 Japanese species and subspecies of *Semisulcospira* have been described, Habe (1964) recognized only 4 of them as valid; *S. libertina*, *S. decipiens*, *S. kurodai* and apparently *S. niponica*. But Burch (1967 *e*) found that there were at least 8 species of *Semisulcospira* within the Lake Biwa basin alone, based on chromosome numbers and karyotype analyses with *S. habei* and *S. habei yamaguchi* being discovered for the first time. The karyotype analyses of these species are discussed in greater detail in the cytogenetics section of this paper.

Cytogenetic Studies

Hybrid Studies

Hybrid studies of molluscs combined with a cytological investigation of the parents and offspring have been reported in one paper on pelecypods and in two papers on gastropods. Menzel and Menzel (1965) crossed females of *Mercenaria campechiensis* with males of *M. mercenaria* and reported the chromosomal condition of the F_1 hybrids as well as that of the parents. They found oocyte nuclei to be at Metaphase I within 15 seconds to 5 minutes after spawning. After fertilization, the first polar body appeared in 10 minutes, Metaphase II in 15 minutes and the first

cleavage within 20–30 minutes. Some F_2 embryos from reciprocal backcrosses to the F_1 parents were examined with one lot showing various degrees of polyploidy while the other lots did not exhibit such a condition. The chromosomes of the F_1 hybrids were regularly paired to form 19 bivalents with a chiasma frequency similar to that of the parents. Due to the ease of hybridization, the authors suggest that a certain amount of gene flow may occur between the 2 species. The regularity of the chromosomes of F_1 hybrids "demonstrate that there is no gross chromosomal barrier to such gene flow". The authors point out that genic exchange under experimental conditions does not imply that such an event occurs in nature.

Various populations of the muricid snail, *Purpura lapillus*, were studied by Staiger (1954). In hybrid populations between $n = 13$ and $n = 18$ forms individuals were found in which, during meiosis, 5 metacentric chromosomes were paired with 10 acrocentrics. Presumably the 10 acrocentrics arose by splitting of the centromeres of 5 metacentric chromosomes. The arms of the 10 acrocentric chromosomes remained homologous to the 10 arms of the 5 metacentric chromosomes of the 13-chromosome race.

Burch (1960 *c*) reported the chromosomal features of the 4 nominal species of *Oncomelania* and later (1964 *d*) studied the cytology of hybrids of the 4 so-called species. All specimens studied of each of the hybrid populations had 17 pairs of chromosomes, and a comparative examination of late prophase of diakinesis chromosomes of the various F_1 hybrids revealed no apparent anomalies that did not also occur as prevalently in the parental stocks. Only normal bivalents with 1, 2 or 3 chiasmata were observed, and no univalents, trivalents or quadrivalents were found. Furthermore, all segments of each chromosome seemed to pair completely. Considering this information in conjunction with the great ease of hybridization of the various nominal species of *Oncomelania*, the non-reduced viability of those hybrids, and the morphological and biochemical similarities of the parental populations, it was concluded that these 4 nominal species, although isolated and widely separated geographically, were in reality only races of 1 species. Recent attempts (Davis, 1967) to cross species of *Oncomelania* with species of the closely related North American *Pomatiopsis* failed, indicating that these 2 species are perhaps not as closely related as previously thought.

Polyploidy:

Polyploidy in molluscs has been previously discussed by Burch and Huber (1966). A polyploid condition is evident where there is an exact multiplication of the normal chromosomal complement. The degree of polyploidy is indicated by the number of multiples of the basic haploid number. For example, if the haploid number of a snail is 18, a closely related species with a diploid number of $2n = 144$ is an octoploid (8×18). Polyploidy is the only clearly established mechanism of instantaneous speciation.

There are 2 types of polyploidy, autopolyploidy and allopolyploidy. An autopolyploid condition arises when more than 2 haploid chromosome sets of a single species participate in zygote formation. Thus, autopolyploidy is the multiplication of 1 basic genome. For example, an autotriploid can be produced by the union of an unreduced ($2n$) gamete with a normal (n) gamete, or a tetraploid can result from an aberrent mitotic division where the chromosomes duplicate and divide but the nucleus fails to divide, thereby producing a $4n$ condition. Polyploids produced artificially by such drugs as colchicine are of this type. Allopolyploids, on the other hand, have a chromosomal complement composed of 2 or more genomes, each from a different species. The fact that the 2 species can cross may imply that at least partial homology (homeology) might be expected between chromosomes of the parents. The pattern of association of chromosomes should differ with the species involved in the crosses. Two species having no cytologically detectable evidence of homology between genomes would show only bivalent formation while 2 species with completely homologous genomes would show multivalents or bivalents that are formed at random from the members of the group of 4 homologues. Between these 2 extremes are cases of constituent genomes with intermediate degrees of homology where the homologous segments are probably

interspersed with others along the length of the chromosomes. All molluscan polyploids appear to be allopolyploids.

Polyploidy is rare among sexually-reproducing animals presumably because the chromosomal balance for sex determination would be upset. Most polyploids so far discovered are permanently hermaphroditic species capable of self-fertilization or asexual reproduction, or are parthenogenetic. Disturbance of the sex-determining mechanism, improbability of a newly arisen polyploid finding a similar mate, or developmental aberrancies are some of the reasons given for the rarity of polyploidy among bisexual animals.

Euthyneuran snails are hermaphroditic and have no sex chromosomes. The streptoneuran snails for which polyploidy has been reported are parthenogenetic, except for *Balanocochlis*, for which there is no information available as to its mode of reproduction. Thus, it would seem that the only barriers to polyploidy would be the rarity of accidentally producing gametes with double sets of chromosomes, the infrequency of self-fertilization (in normally cross-fertilizing hermaphrodites) after the production of such a cell, or the reduced viability of interspecies hybrids and possible developmental difficulties. Polyploidy has been found in less than 3 per cent of the euthyneuran species (Burch, 1965 *b*) indicating that these factors may be important in reducing the appearance of polyploid species. Self-fertilization is known to exist in both the Planorbidae and Lymnaeidae; polyploidy in the former is known to occur in 15 per cent of the species studied, and not at all in the Lymnaeidae. This indicates that factors other than lack of self-fertilization do operate to reduce polyploidy, especially in the Lymnaeidae.

Polyploidy has been reported to occur in the streptoneuran families Hydrobiidae (Table III) and Thiaridae (Table V). Sanderson (1940) studied the chromosomes of *Potamopyrgus jenkinsi* (Hydrobiidae) collected from Scotland. She found that there was only 1 non-reductional maturation division in the egg and that the diploid number of chromosomes appeared to be $n = 36$ in some cells and $n = 44$ in others. Rhein (1935) reported the Continental race of *P. jenkinsi* to have 20–22 pairs of chromosomes. Sanderson concluded that the British snails appeared to be a tetraploid race of the Continental diploid parthenogenetic members of the species.

Melanoides tuberculatus is a parthenogenetic member of the Thiaridae. Jacob (1959 *a*) studied the chromosomes of 2 "races" that differed in shell characters but occurred together in the same habitat. He found one "race" to be diploid ($2n = 32$) and devoid of male individuals, while the other "race" was considered to be polyploid ($2n = 90$–94) and had 3 per cent males, presumably sterile. Burch (personal communication) feels that 2 different sympatric species were involved, each with its distinct morphological differences accompanying the cytological differences. Jacob considered the polyploid to be an autoallopolyploid arising from a cross between an autotetraploid parthenogenetic female of one species and a diploid male of another species. *M. lineatus* ($2n = 71$–73) and *M. scabra* ($2n = 76$–78) were also studied and considered by Jacob to be polyploid because of their high chromosome numbers.

Among the Euthyneura, polyploidy has been reported in the families Ellobiidae, Ancylidae and Planorbidae. Natarajan and Burch (1966) reported *Melampus coffeus* to have a haploid number of 19 and one member of a nearby normally diploid but somewhat morphologically different population to have 38 pairs of chromosomes (Table X). This specimen was obviously a tetraploid.

Species of the freshwater limpet family Ancylidae, have chromosome numbers of $n = 15$, $n = 30$, $n = 60$, and $n = 17$ and 18 (Burch *et al.*, 1960; Burch, 1965 *a*, *b*) (Table XI). *Rhodacmea cahawbensis* has the chromosome number $n = 15$, while 2 species each of *Ancylus* and *Ferrissia* have chromosomes that are multiples of 15 (*i.e.*, $n = 30$ and $n = 60$). That there is a distinct polyploid series, with no intermediate haploid numbers known in the subfamily Ancylinae, strengthens the contention that $n = 30$ for *Ferrissia* (Ferrissinae) is also a polyploid (tetraploid) condition.

Polyploidy in the family Planorbidae has been reported to occur in representatives of 2 different subfamilies. In the subfamily Planorbinae, *Gyraulus circumstriatus* has 18 pairs of chromosomes and the closely related *G. parvus* has 36 pairs of chromosomes (Burch, 1960 *b*, 1965 *b*).

The greatest number of polyploid species yet studied are members of the subgenus *Bulinus* S.S. (Bulininae) (Table XIII). Seven of the 13 species and subspecies of the *Bulinus* s.s. group studied are polyploids (*ca.* 54%). However, considering the subfamily Bulininae as a whole, about 29% are polyploid species. An extensive cytological study of the bulinine species occurring in Ethiopia has been reported by Burch (1967 *b*, *c*) (also *see* Brown and Burch, 1967). Burch found the Ethiopian species belonging to the subgenus *Bulinus* s.s. to have haploid numbers of $n = 18$, $n = 36$, $n = 54$ and $n = 72$. Thus, the degrees of ploidy within this relatively limited geographic range include tetraploidy, hexaploidy and octoploidy. These polyploid species are, no doubt, reproductively isolated from individuals of other populations with different chromosome number. Cytologically, these polyploids resembled normal diploids in that all the chromosomes of the first meiotic divisions are bivalents. Therefore, it was concluded that these polyploids were most likely allopolyploids rather than autopolyploids.

There has been some discussion concerning the possibility of polyploidy within the land snail family Succineidae (*e.g.*, Rainer, 1967). However, there is no correlation between chromosome numbers and morphological characters of the species having multiples of basic haploid numbers. Therefore, polyploidy is not indicated in the Succineidae.

Chromosome Morphology, Chromosome Behaviour and Karyotype Analyses

Chromosome morphology of snails has been discussed by Burch (1960 *a*, *e*) in considerable detail. He pointed out that the shapes of chromosomes in aquatic and land pulmonate snails was similar in that the mitotic chromosomes are elongate and monocentric, with median, submedian or terminal centromeres forming the primary constriction. At anaphase, the larger of such chromosomes exhibit the appearance of V's or J's, depending on the position of the centromere (the point of attachment to the spindle). The commonly observed lighter or non-staining nature of the primary constriction and the occasional presence of secondary constrictions was also described by Burch.

The morphological and behavioral aspects of chromosomes during the mitotic and meiotic cycles has been described in detail for the land snail, *Catinella vermeta*, which has only 6 pairs of relatively large chromosomes (Patterson and Burch, 1966). The chromosome cycle in *Catinella* does not differ from that typically found in other animals and especially resembles that observed in other gastropods.

The orientation of chromosomes on the spindle and as seen in the mitotic metaphase plate is probably random. Sometimes homologues may be observed to be close to each other, but just as often they appear to be some distance apart or on opposite sides of the cell. Jacob (1959 *a*) designates homologues by their close proximity on the assumption of a regular or determined arrangement of chromosomes within the nucleus. Such an arrangement has not been observed in our laboratories.

Heterochromatic chromosomes have been incidentally reported in several cytological studies of molluscs. Jacob (1959 *b*) reported the sex chromosomes (X and Y) of *Paludomus tanschaurica* to change from a positive heterochromatic (deeply stained) state in the early stages of Meiosis I to a slight negative heteropyknotic appearance in Metaphase I. Three supernumerary chromosomes of one individual of *Gyraulus deflectus* were more deeply stained (positively heteropyknotic) than the normal bivalents of meiotic Metaphase I (Burch, 1960 *a*). In spermatogonial cells of *Pomatiopsis lapidaria*, there are 16 pairs of chromosomes and a large, subterminally constricted sex chromosome which appeared to be negatively heterochromatic (Burch, 1960 *c*; personal communication).

One bivalent in meiotic Metaphase I plates of 2 Japanese neritids, *Dostia violacea* ($n = 14$) and *Clithon retropictus* ($n = 12$) appeared to be almost entirely non-staining or negatively heteropyknotic. Both individuals examined were males but it was not ascertarned if these heterochromatic bivalents were associated with sex determination (Patterson, 1967*a*).

Pairing of chromosomes during meiosis in snails has been discussed most often with regard to chiasma frequency. Homologous chromosomes become associated in pairs during zygonema after which genetic interchange presumably takes place through the process of crossing over. The points of such cross-overs can be identified and are called chiasmata. As terminalization of chiasmata begins and chromosomal contraction continues through diplonema, the chromosomes form ring-rod, cross or multiple loop-shaped figures characteristic of diakinesis. The shapes of diakenetic bivalents depends on the number and position of chiasmata remaining (*i.e.*, those which have not terminalized) (Patterson and Burch, 1966). The average frequency of chiasmata per cell has been used for some time as a species specific character in many animal groups.

When more than 2 homologous or homeologous chromosomes are present in the complement, multivalent associations may occur. Trivalents and quadrivalents have been most recently reported by Burch (1967*e*) for several species of *Semisulcospira*. Non-homologous chromosomes do not have pairing partners and hence are usually observed as univalents in meiotic Metaphase I cells (Burch, 1960*a*, 1967*e*). Univalents can also be derived by non-disjunction, failure of meiotic pairing or as a consequence of aberrant meiotic pairing as reported in one cell of *Catinella vermeta* which had a quadrivalent, 3 bivalents and 2 univalents (Patterson and Burch, 1966). The occurrence of heteromorphic bivalents has been described in *Triodopsis* (Husted and Burch, 1946).

A cross-over within a paracentric inversion will result in a bridge and acentric fragment. Bridges and fragments have been reported in *Allogona profunda* (Husted and Burch, 1953) and *Gyraulus deflectus* (Burch, 1959). Sometimes mitotic chromosomes have difficulty in separating at anaphase and tend to "stick" together after the other chromosomes have reached their respective poles. Such "sticky chromosomes" (*see* Patterson and Burch, 1966) form a bridge in mitotic cells that resembles the bridge during anaphase following a cross-over within a paracentric inversion except for the lack of the fragment. Therefore, these 2 aberrencies are distinguishable cytologically. Also, sometimes bivalents have difficulty in terminalization of the remaining chiasmata and the dyads may lag behind the others in Anaphase I movement (*see* Patterson and Burch, 1966).

With a clearer knowledge of chromosome morphology and behaviour, combined with the use of modern techniques of slide preparation and optical equipment, it has been possible to ascertain karyotypes of several molluscs. A karyotype is usually represented by a drawing or photograph in which the homologous pairs of mitotic chromosomes are arranged together according to size and position of the centromere. Karyotypes have been presented for *Laevapex fusca* (Burch, 1962); *Acroloxus lacustris* (Burch, 1962); *Tulotoma angulata* (Patterson, 1965); *Catinella vermeta* (Patterson and Burch, 1966; Burch, 1968*b*); *C. arenaria* (Butot and Kiauta, 1967); *Catinella texana*, *Succinea greeri*, *S. urbana*, *S. grosvenori* (Natarajan *et al.*, 1966); *Melampus bidentatus lineatus* (Natarajan and Burch, 1966); *Semisulcospira decipiens*, *S. niponica*, *S. nakasekoae*, *S. reticulata* (Burch, 1967*e*); *Assiminea grayana* (Kiauta and Butot, 1967); *Helix aspersa* (Rainer, 1967) and *H. pomatia* (Burch, 1968*b*). The karyotype is usually a species specific character and hence is potentially useful in species discrimination, especially in taxonomically poorly understood groups.

The most thorough comparative karyotype analysis of a snail group is that of Burch (1967*e*) for species of the Japanese melaniid genus *Semisulcospira*. Prior to this study, the systematics of that group of morphologically similar snails in the Lake Biwa basin was so poorly understood that it was not known how many nominal species really existed. In addition to validating species that had been previously named, an additional cytologically distinct and previously unrecognized species and its subspecies were discovered with the use of karyotype analyses. Chromosome numbers of the *Semisulcospira* species studied are shown in Table V. *S. libertina* ($n = 18$) has mostly metacentrically constricted mitotic chromosomes while *S. kurodai*, which also has a haploid number of

$n = 18$, has 7 chromosome pairs that are medianly constricted or nearly so, 7 pairs are subterminally constricted or nearly subterminal and 4 pairs are acrocentric. The karyotypes of these 2 species are clearly different even though a cytological difference is not evident in examining meiotic cells. *S. nakasekoae* ($n = 13$), and some specimens of *S. niponica* ($n = 12$), *S. decipiens* ($n = 12$) and *S. reticulata* ($n = 12$) with extra chromosomes have 26 chromosomes in mitotic metaphase plates. However, the karyotype of each is distinct. *S. nakasekoae* has 2 pairs of metacentrics, 4 pairs of submetacentrics and 7 pairs of acrocentrics. The karyotype of *S. niponica* includes 9 pairs of median or submedianly constricted chromosomes, 2 pairs that are subterminally constricted and 2 pairs of acrocentrics. In *S. decipiens* the mitotic chromosomes appear as 9 metacentric or submetacentric chromosomes, 2 subterminally constricted chromosomes and 2 acrocentrics. The karyotypes of these latter 2 species are similar, except that they are distinguished by the larger size of the acrocentrics in *S. decipiens* as compared to *S. niponica*. *S. reticulata* can be clearly distinguished from all other species having the chromosome number $2n = 26$ in that its mitotic chromosomes are all acrocentric. The importance of these karyotype analyses in species discrimination is clearly evident from this comparative study, the only such study so far reported in the Mollusca.

Sex Chromosomes and Sex Determination in Bisexual Snail Species

There has been some doubt concerning the presence of sex chromosomes in molluscs. However, sex chromosomes have been reported in several species of marine gastropods, 3 freshwater species, *Melania crenulata*, *Paludomus tanschaurica* and *Tulotoma angulata* and 2 amphibious species, *Pomatiopsis cincinnatiensis* and *P. lapidaria*. The investigations on marine gastropods are mostly older reports based on the paraffin section technique and therefore need confirmation. Concerning these reports, Nishikawa (1962) states: "According to the author's view, the X-element designated by some authors is no other than the chromosome which is mechanically displaced unusually by the influence of technical procedures." In his study of 53 species of marine gastropods, Nishikawa found no evidence of sex chromosomes. Jacob (1959 *a*, *b*) reported *Melania crenulata* to have a chromosome number of $2n = 35$ with an XO sex-determining mechanism. The data of these 2 studies, as they concern sex chromosomes, should be verified since the material was prepared by paraffin section technique and the morphology of their chromosomes was somewhat obscure, making accurate karyotype analyses difficult. Jacob also reports *Paludomus tanschaurica* to have a chromosome number of $2n = 38$ with X and Y sex chromosomes present in males. These sex chromosomes apparently are slightly heterochromatic during meiosis and do not pair to form a sex bivalent, thereby being rather atypical in this regard (Jacob, 1959 *b*). Very often the Y is present merely to act as a pairing partner for the X in animals. Jacob designates the largest sex chromosome an X and the smallest a Y. However, he examined only male individuals and until females are studied one cannot necessarily designate either of these chromosomes as an X or Y.

Burch (1960 *c*) and Patterson (1963) found heteromorphic chromosomes, presumably related to sex determination, in 2 species of *Pomatiopsis*. In *P. cincinnatiensis*, the male chromosome number is $2n = 32$ ($n = 16$) apparently with a male XY sex-determining mechanism. In males of *P. cincinnatiensis* what are considered to be the X and Y elements are the 2 largest chromosomes of the complement. These 2 chromosomes are clearly dimorphic, one being medianly constricted and the other subterminally constricted. Neither has a homologue. In light of this evidence, it was suggested that these 2 elements are dimorphic sex chromosomes. Female members of the species must be investigated cytologically before the X and Y chromosomes can be specifically designated. In *P. lapidaria*, the male chromosome number is $2n = 33$ ($n = 17$), apparently with a male XO sex-determining mechanism. The X chromosome is the largest of the complement; it is subterminally constricted and usually negatively heteropyknotic.

Sex chromosomes in both male and female individuals of the viviparid species, *Tulotoma angulata*, have been determined (Patterson, 1965). The somatic karyotype of *T. angulata* consists of 3 pairs of metacentric chromosomes, 7 pairs of submetacentrics and 2 pairs of almost acrocentric chromosomes. Two relatively large chromosomes in spermatogonial metaphase cells cannot be

matched with morphologically similar homologues, as was the case in *P. cincinnatiensis*. One is medianly constricted, the other is distinctly submedianly constricted. These are presumably the sex chromosomes, one being an "X", the other a "Y". In oögonial metaphase cells, the corresponding submedianly constricted chromosome is duplicated and the metacentric chromosome is lacking. This indicates that the submetacentric chromosomes are the X's and that the metacentric is a Y chromosome. Therefore, the sex-determining mechanism is XX in the female and XY in the male in *T. angulata*. It is concluded from the above observation that morphologically distinguishable sex chromosomes do exist in at least some bisexual streptoneuran species, and probably they will be demonstrated in additional species with the use of modern cytological techniques.

Many discussions have been devoted to the evolution of sex-determining mechanisms in both plants and animals (White, 1954; Swanson, 1957; Bacci, 1965; Mittwoch, 1967). Most authors agree that the XX-XY mechanism is probably the most primitive (all other forms can be derived from it) and most likely arose from a state of hermaphroditism with the establishment of differentiated sex chromosomes from essentially undifferentiated autosomes. Separation of the sexes could have first evolved from genetic recombination between cross-fertilizing (self-sterile) hermaphrodites with this separation being maintained and strengthened by selection. Sex chromosomes must then have attained distinction with the accumulation in the X chromosome of factors controlling the development of the homogametic sex and similar accumulation in the Y (or in the autosomes) of factors which control development of the heterogametic sex. This was followed by isolation of the X from the Y, perhaps by reduction in crossing over enabling the preservation of favorable gene combinations. If a region became isolated from crossing over, ordinary genes in other autosomes could conceivably be changed (mutate) to assume the functions that would be "lost". In male animal individuals the Y chromosome is often genetically inert and serves only as a pairing partner for the X. This inertness presumably resulted from the isolation process. Thus, a loss of the Y can be tolerated as evident in XO individuals. However, dosage compensation may then have evolved. Such a loss of the Y leads to "isolation" of the X which has become heterochromatized in some species where the only function of the X is sex determination as in the XO males of grasshoppers. It is interesting that the "X" in *Pomatiopsis lapidaria* (XO) is usually visibly negatively heterochromatic.

In regard to the apparent difference in sex-determining mechanisms (XY *vs.* XO) in the pomatiopsid snails, Butot and Kiauta (1966) state: "The difference in this mechanism shown by Patterson (1963) to exist in the two *Pomatiopsis* species in the subfamily Truncatellinae might be only very slight as this phenomenon could easily be explained by the fusion of the original X-element thus forming a neo-XY system." However, such fusions have commonly been found only in insects, an invertebrate group exhibiting many unconventional cytological features, most of which have not been demonstrated in other invertebrate groups and especially not in molluscs. In light of the evidence of cytological conservativeness in molluscs and that concerning sex chromosomes and sex determination (e.g., *Tulotoma*), I would like to offer a simpler and perhaps more plausible explanation for the difference in sex-determining mechanisms in the 2 *Pomatiopsis* species. In the first place, I feel that *P. cincinnatiensis* (and *Tulotoma angulata*) retains the more primitive sex-determining mechanism, XX-XY. The XO system in *P. lapidaria* could easily have been derived from the loss of the Y in an ancestral XX-XY individual. That this is likely the case is strengthened by the heterochromatization of the X in *P. lapidaria*. This sequence has been found in other animal groups, *i.e.*, progressive inertness of the Y in an XY individual, followed by loss of the Y, and subsequent heterochromatization of the remaining X chromosome. The difference in chromosome number of *P. lapidaria* and *P. cincinnatiensis* could have been derived through aneuploidy involving the loss of a pair of autosomes in the latter species. Since most members of the Hydrobiidae have 17 pairs of chromosomes, it is likely that the aneuploid change took place in *P. cincinnatiensis*, with a decrease of 1 bivalent. An analysis of the precise karyotypes of these 2 species might shed some light on the cytological changes involved in these related species.

Supernumerary or Additional Chromosomes

Supernumerary chromosomes have usually been defined as those that are additional to the karyotype and that are non-homologous or homeologous to members of the normal complement

(White, 1954). Non-homology of the extra chromosomes reported to occur in molluscs has not been proven except perhaps in *Helix*. Thus, I am provisionally including all extra chromosomes reported for molluscs in the category of supernumeraries. These additional chromosomes, when present, appear in variable frequency among individuals of a wild population and may not necessarily characterize all populations of a species. In fact, supernumerary chromosomes have not been observed commonly in those molluscs that have been studied cytologically. This may be due to the fact that supernumerary chromosomes often have deleterious effects on fertility and viability. Supernumerary chromosomes have been reported to occur in 1 streptoneuran genus and in 4 euthyneuran genera.

Burch (1967 *e*) found seven species and subspecies of *Semisulcospira* (*S. niponica, S. kurodai, S. multigranosa, S. reticulata, S. decipiens, S. habei* and *S. h. yamaguchi*) to have extra chromosomes, which are most likely supernumerary chromosomes. But, the possibility that they are associated with sex determination cannot definitely be excluded. These extra chromosomes exhibit variable associations in the first meiotic division in both female and male individuals. For example, female individuals of *S. habei yamaguchi* had diploid chromosome numbers ranging from $2n = 17$ to $2n = 20$ and meiotic cells showed the extra chromosomes to be associated as trivalents and univalents in different individuals. In male individuals there were similar associations but with a quadrivalent evident in one of them.

Individuals of one population of *Gyraulus deflectus* (normally $n = 18$) were reported by Burch (1960 *a*) to have three extra elements which were associated variously as 3 univalents, a univalent and a bivalent or 1 trivalent at diakinesis of the first meiotic division. These supernumeraries stained more deeply than the bivalent chromosomes of the complement.

Supernumerary chromosomes were found in 2 populations of *Bulinus natalensis* (Burch, 1964 *a*). The number of additional chromosomes varied from 1 to 3 ($n = 19$–21) among the individuals of one population and 1 extra chromosome ($n = 19$) was present in some cells of individuals of the other population. Burch suggested that these varying chromosome numbers may be the result of hybrid origins of the 2 populations.

Husted and Burch (1946) found individuals of *Triodopsis fraudulenta* (normally $2n = 58$) to have chromosome numbers of $2n = 58$, 59, 60, 61 (?) or 62 and the bivalents to number 29 or 30 or $n = 29$ with an additional univalent. The authors suggest that the extra chromosomes are perhaps duplications of other chromosomes of the complement. If this should be the case, the chromosomes would be at least partially homologous (homeologous), thereby not qualifying as supernumeraries in the strict sense. Nearly 40% of the specimens of *T. fraudulenta* examined were found to have extra chromosomes. One individual was also found to be mosaic, having cells with 62, 64 and 66 chromosomes ($2n$). In a footnote to the same paper, Husted and Burch mentioned that some individuals of *Mesomphix oxycoccus* had 29 pairs of chromosomes, while others had 30 pairs.

The most thorough study of supernumerary chromosomes of a mollusc is that of Evans (1960) in which he investigated their occurrence in wild populations of *Helix pomatia*. From 1 to 6 extra chromosomes were found in cells containing the basic diploid complement. All of the extra chromosomes were the same in the 2 populations studied in that they were submedianly constricted and smaller than chromosomes of the basic complement. If only 1 extra chromosome was present it usually did not pair or come to lie on the metaphase plate of the first meiotic division, but divided mitotically at the second meiotic division and was always included in daughter nuclei. If 2 additional chromosomes were present, they were usually associated as bivalents in Meiosis I, and more than 2 extra chromosomes appear as univalents or bivalents in the first meiotic division. Multivalent associations as have been reported in other gastropods were not observed. Supernumerary bivalents seemed to behave normally during meiosis. Evans felt that the supernumerary chromosomes may have arisen from chromosome change occurring after a period of genotype instability due to inbreeding of these normally cross-fertilizing hermaphrodites. The author also presented

a statistical analysis of the frequencies of supernumerary chromosomes between individuals and between primary spermatocytes within individuals. However, this analysis was performed on very small samples of individuals (11 from population F and 14 from K), and will not be discussed further here.

Two other cases of chromosome number variation in the Helicidae are mentioned by Rainer (1967). He found a population of *Cepea hortensis* which had a chromosome number of $n = 30$ as compared to $n = 22$ reported by Perrot (1930) for this species. He also found the haploid chromosome number of *C. nemoralis* to vary from $n = 28$ to $n = 29$ in spermatocytes. Haploid chromosome number variation in *Succinella oblonga* was previously discussed.

Cytology and Medical Zoology of Molluscs

Members of several gastropod genera have been the subject of cytological studies in recent years due to their importance as intermediate hosts of human schistosomiasis and paragonimiasis (*see* Burch, 1963, 1964 *a, d*, 1966, 1967 *b, c*; Natarajan, Burch and Gismann, 1966; Brown and Burch, 1967; Brown *et al.*, 1967).

The gastropod genus *Bulinus* is of medical importance because several of its members transmit urinary schistosomiasis (*Schistosoma haematobium*) in Africa and the near East. Species of the subgenus *Bulinus* s.s. occur in most of the African continent and comprise two species groups, the "*tropicus*" and "*truncatus*" groups.[7] Members of the "*truncatus*" group occur in the northern portion of the continent, are polyploid ($n = 36$, 54 or 72), and appear to be actual or potential intermediate hosts of *S. haematobium*. Members of the more southerly "*tropicus*" ($n = 18$) group are not implicated in the transmission of schistosomiasis haematobium. In his various studies, Burch has referred to the correlation between polyploidy and the ability of tetraploid species of the "*truncatus*" group to transmit schistosomiasis haematobium (Burch, 1964 *a*, 1966, 1967 *c*). Therefore, because of the great difficulty in distinguishing the species morphologically, chromosome numbers are of potential value in discerning which field populations are capable of transmitting schistosomiasis. The study of the Ethiopian species of *Bulinus* s.s. was previously discussed in the section on polyploidy. That study showed the current inadequate taxonomy and pointed out that before the actual and potential vectors of *S. haematobium* can be determined and their distributions recorded, it is necessary to know how many species occur in Ethiopia and how they can be distinguished. Cytological studies have already proven to be a useful tool in studying this problem in Ethiopian bulinids (*see* Burch, 1967 *c*).

Species of the genus *Biomphalaria* serve as intermediate hosts of *Schistosoma mansoni* in Africa and South America. Chromosome numbers have been determined for 7 species and subspecies of *Biomphalaria* (Table XII) which are known to be vectors. Since all species have the haploid number $n = 18$, chromosome numbers have not been helpful in determining systematic relationships in this genus as in the case of *Bulinus*.

Oncomelania serves as the intermediate host of oriental *Schistosoma japonica*. The cytotaxonomic studies of this genus and its hybrid forms have been previously discussed in the section on hybrid studies. The haploid chromosome number of *Oncomelania hupensis* and its various subspecies is $n = 17$ (Table III).

At least 3 genera, *Semisulcospira*, *Assiminea* and *Pomatiopsis* are intermediate hosts of *Paragonimus*. *Semisulcospira libertina* (= *bensoni*) is thought to be the most important intermediate

[7] According to Brown *et al.* (1967) there is a third species group, the "*B. natalensis* group". However, Burch and Lindsay (personal communication) could not find any immunological evidence for such a group, and contend that *B. natalensis* belongs to the "*tropicus* group".

host of *P. westermani*, which causes oriental human paragonimiasis (Yokogawa *et al.*, 1960). Several nominal subspecies of *S. libertina* have also been implicated as intermediate hosts of paragonimiasis in humans and other mammals in the orient. However, little is known concerning the biological relationships of the nominal species of *Semisulcospira* over its wide geographic range (Japan, Korea, Okinawa and Formosa), and the relationship of *S. libertina* to other melaniid snails. The haploid chromosome number of *S. libertina* is $n = 18$, and in this way it does not differ greatly from many other cerithieids.

In Japan, *Assiminea parasitologica* ($n = 12$) and *A. yoshidayukioi* ($n = 15$) serve as natural intermediate hosts of *Paragonimus ohirai*, a non-human mammalian trematode (Kawashima *et al.*, 1961). In addition, *A. parasitologica* has been proven to be the intermediate host of *P. iloktsuenensis* (non-human) in Japan (Miyazaki *et al.*, 1960). So far, there seems to be no correlation between chromosome numbers and current taxonomy of the species of *Assiminea* which transmit *Paragonimus*.

Pomatiopsis lapidaria ($n = 17$) is the species implicated as an intermediate host for *Paragonimus kellicotti*, a trematode infecting wild animals in North America. Due to the morphological similarities of *P. lapidaria* and *Oncomelania hupensis nosophora*, *P. lapidaria* has been considered a potential intermediate host for human oriental schistosomiasis and experimental infections with *Schistosoma japonica* have been effected (Berry and Rue, 1948). Regardless of their similarities, Davis (1967) has shown that *P. lapidaria* is distinct from *O. h. nosophora* and the 2 are not congeneric. Cytological studies also show that these two genera differ noticeably in regard to chromosome size (Burch, personal communication) and the presence of sex chromosomes in *Pomatiopsis*, which have not been discerned in *Oncomelania*.

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ADDENDUM

Since the presentation of this paper at the Symposium on Mollusca, continued cytological studies have yielded new information which is included in this brief addendum. The haploid chromosome numbers of most of the 34 species shown in Table XXI correspond well with those already reported for the respective groups. However, the haploid chromosome number of $n = 25$ for the genus *Quickia* is somewhat higher than the modal number reported for all other succineid genera and strikingly higher than that for other members of the Catinellinae. Karyotypes of several species are given by Inaba (1969) and of one species by Natarajan (1969). Natarajan (1969) reports the presence of an X sex chromosome in the male individuals of all the neritid species he studied and an XX sex chromosome condition in the females of *Septaria tessellata*.

TABLE XXI

Chromosome numbers of Mollusca

Species	Haploid No.	Source	Reference
Class GASTROPODA			
Subclass STREPTONEURA			
Order *ARCHAEOGASTROPODA*			
Superfamily NERITACEA			
Family NERITIDAE			
Nerita chameleon	12	Andaman Is.	Natarajan, 1969
N. plicata	12	Andaman Is.	Natarajan, 1969
N. dombeyi	12	Andaman Is.	Natarajan, 1969
N. rumphii	12	Andaman Is.	Natarajan, 1969
Neritina ovalaniensis	12	Andaman Is. India	Natarajan, 1969
N. retifera	12	India	Natarajan, 1969
N. laydri	12	India	Natarajan, 1969
N. (Dostia) crepidularia	12	India	Natarajan, 1969
* *Septaria tessellata*	12	India	Natarajan, 1969
S. compressa	12	India	Natarajan, 1969
Subclass EUTHYNEURA			
Order *BASOMMATOPHORA*			
Suborder *ARCHAEOPULMONATA*			
Family AMPHIBOLIDAE			
Salinator solida	18	Australia	Laws, 1967
Suborder *BRANCHIOPULMONATA*			
Family PLANORBIDAE			
Physastra sp.	18	New Zealand	Burch (pers. comm.)
Family LYMNAEIDAE			
Stagnicola wyomingensis	18	U.S.A.	Inaba, 1969
S. hinkleyi	18	U.S.A.	Inaba, 1969
S. idahoensis	18	U.S.A.	Inaba, 1969
S. cf. bonnevillensis	18	U.S.A.	Inaba, 1969
"*Erinna*" *aulacospira*	18	Kauai I.	Burch, (pers. comm.)
Radix natalensis	17	Liberia	Inaba, 1969
R. hamadai	17	Japan	Burch (pers. comm.)
† "*Lymnaea*" *lessoni*	16	Papua	Inaba, 1969

* Shown as *Septaria* sp. in Table II.

† Shown as *Peplimnea lessoni* (= *Radix*?) in Table XIV.

TABLE XXI—(*Contd.*)

Species	Haploid No.	Source	Reference
Order *STYLOMMATOPHORA*			
Suborder ***ORTHURETHRA***			
Family PARTULIDAE			
Partula taeniata	29	Moorea	Scvortzoff, 1966
P. suturalis strigosa	29	Moorea	Scvortzoff, 1966
P. s. vexillum	29	Moorea	Scvortzoff, 1966
P. mooreana	29	Moorea	Scvortzoff, 1966
P. aurantia	29	Moorea	Scvortzoff, 1966
P. mirabilis	29	Moorea	Scvortzoff, 1966
P. olympia	29	Moorea	Scvortzoff, 1966
Suborder ***MESURETHRA***			
Family CLAUSILLIDAE			
Alopia canescens	32	Romania	Burch (pers. comm.)
Suborder ***HETERURETHRA***			
Family SUCCINEIDAE			
Subfamily CATINELLINAE			
Catinella putamen	5	W. Samoa	Patterson (unpublished)
C. aprica	6	U.S.A.	Patterson (unpublished)
Quickia spurca	25	Liberia	Patterson, 1968 *b*
* *Q.* sp.	25	Aldabra	Patterson (unpublished)
† *Q. gravelyi*	25	India	Patterson (unpublished)
Subfamily SUCCINEINAE			
Succinea cf. *cepulla*	18	Hawaii I.	Patterson (unpublished)

* Shown as *Succinea* sp. in Table XVI. † Shown as *Succinea gravelyi* in Table XVI.

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* Title concerned with cytology of molluscs.

LYSOSOMES IN THE EARLY DEVELOPMENT OF THE SLUG, *ARION ATER RUFUS* L.

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ABSTRACT

Lysosomes and related bodies have been identified in ova and early embryonic cells of the slug *Arion*. They have been stained supravitally, and their cytochemical nature and fine structure have been investigated. They are chiefly concerned with intracellular digestion of albumen which is ingested from the very onset of development by a process akin to micropinocytosis. They also seem to play a significant role in the autolytic degeneration of polar bodies and may be involved in cell division and morphogenesis. Ontogenetic interrelationships between Golgi, lipochondria and lysosomes are discussed.

INTRODUCTION

RECENT studies have revealed that lysosomes are important constituents of most animal cells. Very little work, however, has been done on lysosomes in early embryonic cells. In some eggs, especially of molluscs, certain metachromatic granules have been identified, which are probably of lysosomal nature (Pasteels and Mulnard, 1957; Dalcq, 1960; Rebhun, 1960; Novikoff, 1961; Beams, 1964).

The main objectives of this investigation are to identify and trace these cell inclusions and their allies during the early development of the slug *Arion*, and to elucidate their structure, chemical nature, and function.

MATERIALS AND METHODS

Ova and early embryos up to the post-gastrula stage were studied by phase-contrast microscopy, vital staining, cytochemical methods, and electron microscopy. Centrifuged eggs were also studied by the first three methods.

The slugs were collected at Aberystwyth, Wales, and were bred outside the laboratory. The eggs were gently decapsulated in tap-water under a binocular microscope, rinsed in isotonic saline (0·28% NaCl), and then studied by various methods. Centrifuged embryos were obtained by subjecting the entire capsules to a centrifugal force of 950 g. for 5 mins. and then decapsulating them immediately afterwards.

For phase-contrast microscopy the embryos were squashed in isotonic saline and examined under oil-immersion with a Wild M 20 phase microscope.

Vital staining was carried out according to Baker (1958). Grubler's neutral red, nile blue, toluidine blue, and methylene blue were used. The embryos were stained for 5-40 mins. in a 0·01% solution of the stain in isotonic saline. These were then squashed in saline and were examined with an apochromatic oil-immersion objective.

A variety of cytochemical tests were carried out on both living and fixed embryos (Table I). Acid phosphatase and benzidine peroxidase activities were studied by incubating whole living embryos and examining them in much the same way as in vital staining—' vital enzymology '. Frozen sections were also used for the acid phosphatase test. Embryos were fixed in formaldehyde/saline (10% solution of neutral formalin in 0·28% NaCl) at 0–4° C. for 12–16 hours and then stored in Apathy's medium. They were then embedded in 15% gelatin (Pearse, 1961) and frozen sections 6 microns thick were cut in a cryostat. A few embryos were fixed in cold formaldehyde/saline or acetone/ethanol and incubated whole, squashed, and examined. The Gomori lead nitrate method and the standard coupling azo dye technique were used for acid phosphatase while Van Duijn's method was used for benzidine peroxidase (Pearse, 1961).

For other cytochemical tests various fixatives including Carnoy, Helly, Zenker, Ciaccio, Bouin, Gendre, and acetone/ethanol were used. The embryos were then processed and impregnated with celloidin and embedded in wax according to Peterfi's method (Pantin, 1960). A rapid method of embedding was employed to suit the embryos. Paraffin wax (56° C.) mixed with ½% ceresin wax was used in the final stages of embedding. Serial sections 4 microns thick were cut with a Cambridge rocking microtome. Various cytochemical tests were then carried out and colour reactions were visualised with a Zeiss apochromatic objective.

Illustrations were drawn with a Zeiss camera-lucida and photomicrographs were taken with a Zeiss photomicroscope using Ilford Pan-F film.

For electron microscopy the embryos were fixed in plain aqueous 1% osmium tetroxide for 1 hour at about 2° C., and then for ½ hour at room temperature (Baker, 1965). These were then embedded in araldite and sectioned with a Huxley ultramicrotome. Sections were mounted on formvar and carbon-coated copper grids, stained with uranyl acetate, and examined with an Akashi TRS-50 electron microscope.

Observations

Phase-contrast microscopy

When an ovum is squashed and examined under positive phase-contrast, there are dense rounded bodies ranging from 0·5 to 2 microns in maximum diameter, which are quite distinct from other granules like mitochondria, Golgi, and yolk. The smaller bodies are granular, extremely dense (denser than mitochondria) and appear dark brown, while the larger bodies (1–2 microns) are vesicular and distinctly heterogeneous in structure, there being darker blobs within each of them (Fig. 1). In centrifuged eggs these granules and small vesicles are seen in the hyaloplasm zone intermingled with the mitochondria, but are far less numerous than the latter. In centrifuged ova stained with toluidine blue these bodies are tinged purple and hence metachromatic.

Phase-contrast also shows a few larger but more homogeneous vesicles (2–5 microns), which appear brown or grey and which, unlike the above, stratify in the equator (*i.e.*, in the most centrifugal part of the hyaloplasm) forming a dividing line between the clear and yolky zones. These are tiny albumen vesicles and are also metachromatic with toluidine blue. Three or four of the bodies described above are sometimes found associated with these larger vesicles.

Similar granules and vesicles are found in later stages (2-cell, 4-cell, 8-cell and 16-cell embryos) and as the embryo develops their numbers increase and the albumen vesicles reach a diameter of about 6 microns or more. Lighter or darker crescentric areas are sometimes visible on the periphery of these vesicles (Fig. 1). These inclusions are more abundant round nuclei and asters and some are seen in the cortical region. In later stages (blastulae and gastrulae) some albumen vesicles become enormous, the largest being about 8 microns. Some show lighter

peripheral crescentric areas while others appear faintly foamy in consistency. The majority, however, are more or less homogeneous in appearance.

Vital staining

The granules and vesicles (small and large) are easily stainable with the vital dyes used and the results are superimposable. They stain in about 5–10 mins. with neutral red; 10–15 mins. with toluidine blue and nile blue. Optimum results, however, are obtained after 30–40 mins. staining. Methylene blue requires about 1–2 hours staining.

Metachromasy

Both granules and vesicles stain metachromatically with toluidine blue, methylene blue, and sometimes with nile blue. They stain in shades of purple blue, purple or pink purple and hence show varying degrees of metachromasy—α, β and γ (Pearse, 1961). The predominant shade is purple (β metachromasia).

The finest particles that stain metachromatically are the minute granules seen earlier under phase-contrast. Some are even smaller than mitochondria ($< 0{\cdot}5\mu$), and are best seen in polar bodies and in association with larger albumen vesicles in later embryos as 'satellites'. These stain red purple to purple with toluidine blue, purple to purple blue with methylene blue, and purple blue to blue with nile blue. In centrifuged eggs they stratify in the hyaloplasm zone with the mitochondria. This zone stains diffuse purple because RNA, which is also feebly metachromatic, stratifies here. Therefore, it is difficult to differentiate the finest granules in the hyaloplasm. On closer examination there are more intense purple granules of variable size ($0{\cdot}5$–1μ) and sometimes a few larger vesicles (1–2μ in diameter). The latter stain pink purple. A more intense metachromatic band is seen at the equator. This consists of larger purple vesicles ranging from about 2–5 μ in diameter. Some of these vesicles have finer purple granules associated with them.

In uncentrifuged eggs these metachromatic inclusions are randomly distributed in the cytoplasm. During cleavage myriads of these inclusions become associated with asters and nuclei forming halos around them. As development proceeds the vesicles increase in number and size. The cells of the gastrula and post-gastrula stages are choked with numerous albumen vesicles of varying sizes (2–8μ in diameter). Most of these vesicles are purple and show varying degrees of metachromasy. A gradual loss of metachromasy is seen as the vesicles increase in size. Some of the larger vesicles are almost colourless or are feebly orthochromatic. Colourless vesicles of all sizes are sometimes seen but these are rare. Some of the metachromatic vesicles are distinctly foamy or bubbly in consistency, apparently multivesicular in appearance. Others have crescentric darkly staining areas within their margins similar to those seen under phase-contrast. Still others have fine purple granules associated with them. These 'satellites' are either found dispersed evenly on the surface of the vesicle or conglomerate in mulberry-like groups (Fig. 1). Often smaller dark purple, sometimes heterogenous, vesicles are found superimposed on the larger ones. When the preparations begin to dry up the large vesicles gradually burst and disappear leaving these satellites behind almost as they were. The latter do not scatter and disappear showing that they were on the surface of the globule and not in its substance. In very advanced embryos, certain cells in the body and visceral regions have groups of intensely blue rounded bodies which are enclosed in vesicles or conglomerate in grape-like clusters (Fig. 1). Tiny granules may be seen sometimes amongst these bodies.

Neutral red

Parallel observations have been made after neutral red staining. The metachromatic granules and vesicles stain in shades of red to pink to colourless. The minute granules stain red and are

best seen in later stages of development as satellites and in polar bodies. In centrifuged eggs the hyaloplasm is uniformly diffuse pink with tiny darkly staining bodies. Most of the larger albumen vesicles and their satellites are stained bright red.

Nile blue

Similar results are obtained with nile blue. If the vesicles are overstained they stain orthochromatically. The finer granules are usually blue in colour. In centrifuged eggs, the hyaloplasm stains light blue, while the bodies are distinctly blue.

The smaller metachromatic bodies (or neutral red bodies) are randomly distributed in ova at the time of laying, that is, soon after the first maturation. In mature and late ova they begin to make their appearance around nuclei and asters which appear as clear areas. These tiny granules and vesicles increase in numbers during cleavage, and myriads of them become associated with asters and nuclei forming halos around the latter. These inclusions are more numerous in the micromeres than in macromeres, especially in 16–24 cell stages. This is because albumen first appears in the micromeres and then in the macromeres. If after neutral red staining the embryos were allowed to develop in saline for 2–3 hours, there is a gradual reduction of the dye which commences in the peripheral zone of the cytoplasm of each blastomere and gradually reaches the central nuclear region. At this stage, the neutral red halo is brightest and on prolonged standing these regions, too, become colourless, fading away in a centripetal manner. Thus neutral red has been very useful in the study of cell-lineage in *Arion*. With toluidine and methylene blue, a similar perinuclear purple zone is seen but development is retarded.

In later blastulae nearly all the blastomeres are full of albumen vesicles so that the metachromatic and neutral red zones are more dispersed in the cytoplasm, leaving only the nuclear and inter-blastomeric regions colourless. In gastrulae, the whole ectoderm becomes intensely metachromatic and neutral red positive while the inner cell mass is distinctly lighter in colour (blue or pink). In the late gastrula the dorsal ectoderm stains more intensely than the ventral. The differential staining exhibited by the post-gastrula (= early veliger stage) is very pronounced. The whole of the dorsal ectoderm now appears to form a cap on the cephalic vesicle and large purple or red blobs are seen in the ectoderm cells. The blobs represent groups of albumen vesicles. The inner cephalic mass and the visceral and body regions are far less intensely stained and appear dull in colour (blue or pink, sometimes grey). Each massive endoderm cell of the cephalic vesicle has an enormous albumen vacuole occupying almost the whole cell. These stain faint purple or faint blue, and pink with neutral red or are almost colourless. There are also three or four smaller vesicles at the inner nuclear end of the cell which are tinged or colourless. The body and visceral ectoderm, however, have minute purple or red spots distributed evenly on their surface. The region round the blastopore or the dorsal lip of the mouth in post-gastulae are sometimes stained more intensely.

The polar bodies show remarkable staining reactions. They are intensely metachromatic with toluidine blue and are also stainable with neutral red. The first polar body is usually more intense than the second. The staining is somewhat diffuse but closer examination reveals the presence of minute dark purple or bright red granules and vesicles. It appears that the polar bodies have more than their share of these granules, which may bear some functional significance, as we shall see later.

Acid phosphatase activity

The best results were obtained with living embryos by the azo dye method. Intracellular localization of the enzyme is good and the results are reproducible. The enzyme is, so to speak, 'fixed' by the substrate at its original site of activity. A slight diffusion of the enzyme into the cytoplasm was noted with frozen sections. Acetone/ethanol fixed material gave better localization of the enzyme by the lead method.

Apart from the Golgi, which are strongly acid phosphatase positive, the granules and small vesicles contain the enzyme.

Two salts, Fast red TR and Fast Garnet GBC, were used in acetate buffer for the azo dye method. The latter gave more intense staining reactions and better localisation of the enzyme (no precipitation was also observed). Ground cytoplasm is colourless or pale yellow brown. Embryos were incubated for about an hour for optimum results. They stain diffuse reddish-brown or crimson in more intense areas. The ovum prior to second maturation is uniformly stained reddish-brown. A later ovum prior to first cleavage shows a distinct intense crimson zone at the animal pole where the male and female pronuclei are just being transformed into asters. The two polar bodies are also intensely stained, the second being sometimes darker than the first; fine, more intense, granules are seen in them. In centrifuged eggs the hyaloplasm is more intensely stained than the yolky region. In later ova a darker crimson equatorial band and an even more intense zone in the most centripetal part of the hyaloplasm at the animal pole is distinctly seen (Fig. 5 *a*). On closer examination, the latter is found to consist of numerous minute crimson granules in groups or rows surrounding two unequal, clear, circular areas representing the copulating pronuclei. The band which is situated in the most centrifugal lower limit of the hyaloplasm consists mainly of less intensely stained reddish-brown vesicles associated with darkly stained granules. Crimson Golgi bodies are found in the yolky zone. In early cleavage stages, as in late ova, the granular and vesicular inclusions are found dispersed in the cytoplasm and of course associated with nuclei and asters, and are also more abundant in the subcortical zone. They are sometimes found in clefts between adjacent blastomeres within the vitelline membrane. The vesicles in particular are distinctly seen here. In blastula and gastrula stages the granules are more numerous and are found in rows or groups especially below the cortex. There are a few brown globules in this region but the larger albumen vesicles are absolutely colourless. In gastrulae a distinct red ring was seen around the blastopore.

Whole embryos incubated ½–1 hour in the lead medium turn brown or black in dilute ammonium sulphide. On squashing, there are myriads of fine black granules sometimes in clouds, rows or groups distributed in the faint yellowish-brown ground cytoplasm. In later stages they are seen to form mulberry-like aggregations in certain ectodermal cells similar to those seen staining metachromatically with vital dyes. There are also larger vesicular bodies (2–4 μ), staining less intensely (brownish-black to light brown) distributed randomly in the cells. In post-gastrulae the cephalic ectoderm, shell gland, and the dorsal region round the blastopore stain more intensely.

Peroxidase activity

A remarkable reaction was seen when centrifuged embryos were tested for benzidine peroxidase. When living stratified embryos were incubated a sharply defined, intense blue, equatorial band is formed in about 20 minutes (Fig. 5 *a*). Similar observations were made by Raven (1958) for *Limnaea*. The rest of the hyaloplasm is pale blue, while the perinuclear region is slightly more intense. This resembles, very much, the reaction seen in embryos incubated for acid phosphatase. Stratified 2-cell and 4-cell stages give similar results. When squashed and examined under apochromatic oil-immersion the blue band is seen to consist of fine irregular granules and larger rounded blobs. These are probably the granules and small vesicles seen above. Fewer inclusions are found in the hyaloplasm and more granules are found around the nuclei and asters. A few needle-like spicules are sometimes seen.

In unstratified embryos the reaction is weak and diffuse but becomes very intense around nuclei and asters on prolonged incubation (30–60 minutes). A blue staining was seen around pronuclei in late ova and around nuclei and asters in 2-, 4-, 8-, and 16-cell stages. These cleavage stages also show a more intense reaction in the vegetal zone. The blue inclusions are found on the outer ventro-lateral regions of the blastomeres (or megameres) instead of in the vegetal zone. In 8- and 16-cell stages the micromeres are more intense than the megameres.

The polar bodies also stain fairly intensely. In all cases the usual inclusions (granules, vesicles, and spicules) were seen as in stratified eggs. In blastulae a blue reaction was observed around nuclei, while in gastrulae no visible reaction was seen. In post-gastrulae the cephalic ectoderm and shell gland region stain intensely. Myriads of fine granules (' satellites ') are seen in ectoderm cells especially around nuclei. In these cases too, the usual inclusions are seen on squashing. The grape-like clusters that were seen after vital staining are also positive.

The distribution of these inclusions is very similar to those which were metachromatic and to those which contained acid phosphatase.

Carbohydrates

By far, the easiest way of demonstrating the granules and vesicles (small and large) in sections, is by the PAS test after McManus and Mowry (1964). Ritter and Oleson's combined Hale-PAS method (Pearse, 1961) was found to be particularly useful in distinguishing these inclusions from yolk. If either method is used after saliva digestion even the smaller bodies can be clearly differentiated from glycogen granules.

As in earlier observations, the granules are minute and very variable in size and shape. They are rounded or oval and range from 0·5 to 1 μ in maximum diameter (some are even smaller). On account of their diversity, they can be easily distinguished from other organelles. The small vesicles (1–2 μ in diameter) often appear heterogeneous—having darkly staining blobs within their substance. Sometimes minute granules are found associated with them; these seem to be on the surface of the vesicle giving it a warty appearance (Fig. 1). These vesicles gradually grow in size during development and become more and more homogeneous giving rise to larger albumen vesicles of assorted sizes (2–8 μ in diameter).

The granules, small vesicles, and large albumen vesicles are all intensely PAS-positive. They stain brilliant crimson red and are virtually resistant to saliva and even pectinase digestion. Most of the PAS reactivity of larger vesicles, however, is removed by pretreating acetone-ethanol fixed sections with pectinase (48 hours). This suggests the presence of galactogen in the albumen vesicles (Grainger and Shillitoe, 1952) together with mucoprotein or neutral mucopolysaccharide (NMP). It is difficult to say definitely whether the smaller inclusions are effected by pectinase because of their minuteness and the extreme intensity of the reaction. The presence of galactogen in the larger albumen vesicles has been confirmed by staining with Best's carmine, before and after pectinase digestion.

In the Hale/PAS sequence the granules and vesicles are more purplish crimson and they contrast beautifully with the ground cytoplasm and the yolk granules which are tinged pale purple and blue. If the glycogen is removed from the cytoplasm with saliva before this sequence, even the smaller granules are exceptionally distinct. With early embryos similar results could be obtained with the PAS reaction alone, if the sections are previously treated with saliva or pectinase.

By using these methods it is possible to study and trace the inclusions during early development. A few granules and small vesicles are already present below the cortex when the egg is laid. As the ovum matures and develops they increase in number and a few have already dispersed to other parts of the cytoplasm by the time the pronuclei begin to copulate. Soon afterwards the cleavage spindle appears and a few granules are seen associated with the asters while the rest are in the subcortical zone (Fig. 2). This clearly shows that in the ova the PAS-positive bodies are mainly in the peripheral zone of the cytoplasm, and that some of them are later attracted towards the cleavage aster. In centrifuged eggs the equator is most PAS-positive, and consists of numerous tiny vesicles associated with finer granules. The rest of the hyaloplasm is lighter but highly granular with larger darkly staining bodies.

When the 2-cell stage is reached, the bodies have further increased in number and are now distributed more randomly. They are, however, more numerous around asters in the animal half (Fig. 3). Some of the vesicles have grown in size (4–6 μ). As in the ovum, in centrifuged 2-cell stages the vesicles and associated granules form a distinct equatorial band (Fig. 5) while the granules are dispersed throughout the pink hyaloplasm. Some are still associated with the faint cleavage asters in the hyaloplasm. PAS-positive material can also be seen on the egg surface which is really capsule fluid that has not been washed off. All these observations were made on serial sections and may not be wholly substantiated by the microphotographs. Besides, in the sections photographed, glycogen has not been removed—hence the finer particles are indistinct.

The membranes surrounding the egg (and other stages) appear to be PAS-positive. Under very high magnifications, one finds myriads of extremely minute granules (about 0·2 μ or smaller in size) which are hardly visible. In fact, these are the bodies that are PAS-positive that are mainly responsible for the staining of the egg membranes as a whole. There are also larger bodies within the vitelline membrane and outside the plasma membrane, especially in clefts between cleavage furrows or in places where the vitelline membrane is elevated; for example, around the second polar body. The granules on the membranes can be best seen in sections of embryos fixed in Carnoy which have been either centrifuged and stained with PAS, or stained by the Hale/PAS sequence without prior centrifugation. The idea is to get the egg membrane to contrast with the inner cytoplasm. In centrifuged eggs the granules can be distinctly seen on the egg membranes delimiting the clear hyaline zone and oil-cap zone. In Hale/PAS preparations, the membranes are stained bright crimson and the tiny granules are seen on both vitelline and plasma membranes and in between them, and not on the interblastomeric parts of the plasma membrane. All these observations suggest that the capsule fluid (albumen) is being pinocytosed all around the egg surface right from the beginning of development. The tiny granules perhaps represent minute pinocytotic vesicles which later coalesce to form the larger vesicles seen below the cortex in ova. This has, indeed, been confirmed by electron microscopy as will be seen later.

As development proceeds the PAS-positive bodies steadily increase in number and they become more intimately associated with cleavage asters. In late 4-cell and 8-cell stages, large numbers are seen between the astral rays, the smaller granules being more numerous than vesicles. In the 8-cell stage the micromeres have relatively more bodies than the megameres, almost wholly located in the astral zone (Fig. 4). The vesicles in particular are more abundant and this agrees with the observations on vitally stained embryos where the micromeres were found to be more metachromatic. The 16-cell stage resembles an 8-cell stage, but there are more bodies in the peripheral regions of the blastomeres and round nuclei. Slightly larger albumen vesicles are just beginning to appear in the uppermost quartet of micromeres.

In the early blastula (24-cell stage) very large albumen vesicles (6 μ) are found in the animal regions of the micromeres (Fig. 6). There are numerous granules and smaller vesicles distributed everywhere and could best be seen in Hale/PAS preparations pretreated with saliva. Some of the large vesicles are associated with myriads of these fine bodies ('Satellites'), as in vital preparations (Fig. 6 *a*). In mid-blastula, the outer thirds or halves ('ectoplasm') of all the blastomeres are packed with large intensely PAS-positive albumen vesicles and their satellites. The inner parts of the cells ('endoplasm') and the mesoderm cell in the blastocoele are usually free of albumen (Fig. 7). The nuclei are situated between ectoplasmic and endoplasmic zones. Very exceptionally one or two albumen vesicles may be found in the 'endoplasm' and in the mesoderm cell. The primary mesoderm cell is formed by an oblique vertical division of the megamere 3D in such a way that most of the endoplasm of 3D is incorporated in it together with a thin slice of ectoplasm, which remains in contact with the vegetal surface for some time, and is later withdrawn. This explains why a little albumen is found in the primary mesoderm cell, which during later development has no connection with the outside world. In later blastulae the condition is similar but the albumen vesicles have increased in number and

size and are sometimes arranged in linear or radial rows in the ectoplasm. In mid and late blastulae the largest albumen vesicles in the micromeres are comparatively larger than those in the macromeres. There are also relatively more vesicles in the micromeres than in the macromeres. The largest vesicles reach a diameter of about 6–7 μ.

At gastrulation the macromeres invaginate to form the endoderm and they carry with them the ectoplasmic zone of albumen vesicles which now lines the archenteron. The dorsal ectoderm cells have mostly massive vesicles (up to 8 μ in diameter) while those in the ventro-lateral cells are smallest in size (Fig. 8). These seem to have more 'satellites' associated with them. The vesicles in the endoderm cells are medium-sized. The greatest amount of albumen is therefore found in the dorsal ectoderm while the ventro-lateral ectoderm has the least. The mesoderm cells and the endoplasmic regions of ectoderm and endoderm, in other words all the interior regions of the embryo, are devoid of albumen. Free albumen is often seen in the archenteron or primitive gut of late gastrulae and this sometimes protrudes through the blastopore or mouth, as a cord or strand. Some of the larger albumen vesicles in both ectoderm and endoderm are distinctly multi-vesicular in appearance—the small vesicles inside having darker (blackish) rims. The small satellites and the smaller albumen vesicles are generally becoming rarer especially in the dorsal region.

In post-gastrulae the dorsal cephalic ectoderm and endoderm, to a lesser extent, are extremely PAS-positive (Fig. 9). The gut is full of free albumen while the large endoderm cells have developed enormous albumen vacuoles which sometimes stain less intensely than the albumen vesicles, and appear granular. Immediately lining the gut are more intensely staining albumen vesicles in the cytoplasm of these cells. The nuclei are pushed towards the inner poles of the cells by the albumen vacuoles. There are 2 or 3 albumen cells projecting into the body cavity behind the visceral mass. The small-celled endoderm in the visceral region also have 1 or 2 small vesicles in the zone in contact with the free albumen. The ectodermal cells of the cephalic vesicle are much flattened and full of extremely large albumen vesicles; while those of the posterior region of the body are much smaller and are found only in the ectoplasmic zone. The anterior part of the mantle, and shell gland ectoderm that has just invaginated, also contain ectoplasmic vesicles which are by far the smallest. Those of the shell gland seem to be associated with numerous satellites. The dorsal ectoderm behind the shell gland and the ventral ectoderm behind the mouth, have little or no albumen. Some of the albumen vesicles have a multivesicular appearance.

The polar bodies, at all stages of development, are PAS-positive. Fine crimson granules and vesicles are distinctly seen within them (Fig. 4). Sometimes they are very numerous. The polar bodies gradually disappear after gastrulation.

The albumen vesicles also contain small amounts of acid mucopolysaccharide (AMP). They stain light purple with basic fuchsin (Curran, 1964), light blue with alcian blue (Zugibe, 1963), and darker blue with Hale's reagent (Pearse, 1961). They are stained purple in the aldehyde fuchsin/alcian blue (AF/AB) sequence (Spicer and Meyer, 1960). The smaller albumen vesicles give more intense reactions than the larger ones, while the albumen vacuoles in the endoderm cells and a few large vesicles in the ectoderm gave the weakest reactions and stain pale blue in the AF/AB procedure. Smaller granules and vesicles are not discernible in early stages due to a diffuse staining of the cytoplasm, but darkly stained satellites are seen associated with large vesicles in advanced embryos. The satellites stain bright blue with AB and purple with AF/AB. Active methylation eliminates AF staining, while sulphation produces an intense uniform purple (blackish) mass. The results indicate the presence of sulphated, and to a lesser extent non-sulphated AMP, in the satellites and albumen vesicles. The albumen vacuoles seem to contain more non-sulphated AMP. A gradual change from the sulphated to the non-sulphated condition is seen during the growth of the vesicles. These observations closely parallel those of vital metachromasy.

Other staining and cytochemical reactions

The albumen vesicles are very feebly stained (or colourless) with Heidenhain's haematoxylin. They could, however, be stained darkly after Ciaccio fixation. It is difficult to distinguish the smaller inclusions. The granules, small vesicles, and albumen vesicles are all unstained with acid fuchsin in mitochondrial preparations. Albumen vesicles are feebly osmiophil in Mann-Kopsch preparations and fairly sudanophil in lipid preparations. Generally, the smaller or newly formed albumen vesicles are more lipid-positive. In centrifuged ova the equatorial band stains dark grey with Sudan black, while the bodies in the hyaloplasm are almost black, and a few are associated with the second maturation asters; satellites are sometimes darkly impregnated with osmium in the ectoderm cells of post-gastrulae. Some of the albumen vesicles are distinctly multivesicular in appearance after osmium impregnation. The vacuoles in the albumen cells (endoderm) have the least amount of lipid. The free albumen in the gut has appreciable amounts of lipid. No doubt, some of the lipid in these inclusions contribute to their PAS reactivity.

Albumen vesicles are feebly calcium-positive. They are also faintly pigmented and give a pale blue reaction with Lillie's test for melanin (Pearse, 1961). The albumen in the egg capsule gives essentially the same chemical reactions as the larger albumen vesicles in the embryo

Electron Microscopy

The electron micrographs obtained show minute vesicular elements at the surface and a few multivesicular bodies of various sizes distributed randomly in the cytoplasm.

The egg surface shows a remarkable accumulation of minute vesicular elements, some of which seem to conglomerate in groups (Figs. 11, 12). These are about 0·05–0·2 μ, or less, in diameter. They are found mostly on the outer vitelline membrane which is rather vaguely defined, and also within it but outside the plasma membrane. A few vesicles are seen distinctly in the peripheral cytoplasm just beneath the plasma membrane (Fig. 11). The cytoplasm immediately below the plasma membrane is generally free of other granules and is very similar to the rest of the ground cytoplasm. Occasionally, one or two mitochondria or a small multivesicular body may be seen very close to the plasma membrane. The condition at the surface strongly suggests that there is some sort of micropinocytotic activity whereby vesicular elements find their way into the cytoplasm. Many examples of micropinocytosis in eggs are reported in Beams (1964). Micrographs of mature ova and 8-cell stages show appreciably more vesicular elements at the surface than those of newly laid immature ova. In the latter, the outer membrane is more closely apposed to the plasma membrane (Fig. 10). The exact mode of origin of these minute vesicles is not clearly understood. Those outside the plasma membrane are very similar to those within it, and whether they are limited by a fine membrane or not is difficult to say. PAS-positive bodies of similar size were seen on the egg membrane in light microscopy.

There are two types of multivesicular bodies, large and small. The smaller ones have a well-defined, limiting, double membrane which enclose minute vesicles very similar to Golgi vesicles (Figs. 15, 18). These bodies are rounded or oval (0·2–0·5 or more in diameter) and many are found in the sub-cortical zone in ova (Figs. 12, 13). These have a remarkable resemblance to the multivesicular bodies seen in the micrographs of *Spisula* (Rebhun, 1960), and of the rat kidney (Novikoff, 1961—Fig. 22). Rebhun has identified these bodies with certain metachromatic granules (β particles) which were first reported in *Barnea* (Pasteels and Mulnard, 1957). These are seen to be associated with cleavage asters and are stratified in the hyaline zone in his micrographs. A few bodies in *Arion* have incomplete outer membranes and some of their contents are found in the cytoplasm (Figs. 15, 17). In one micrograph (Fig. 15) there is also an oval body with one or two vesicles and a circular tuft of membranous elements. This

resembles an autophagic vacuole described by de Duve (1963). These observations indicate that these bodies are related to lysosomes. They also correspond in size to the metachromatic, PAS-positive, acid phosphatase-rich granules seen in light microscopy.

The larger multivesicular bodies (Fig. 14) are about 2 μ or more in diameter and contain numerous vesicles of assorted sizes. Their outer membranes are not so well-defined and are found to be lacking in some places. The smaller vesicles within them are very similar to those seen in the small multivesicular bodies (above), while the larger ones resemble those seen at the egg surface. These probably correspond to the larger metachromatic, PAS- and acid phosphatase positive vesicles seen in light microscopy.

There are also larger oval 'dense bodies' (Fig. 16) in which one sees one or two circular electron lucid areas. This body is filled with an almost uniformly electron dense substance. There are others which are essentially similar in structure to small multivesicular bodies, The vesicles within the latter can be made out with difficulty. These, too, seem to be of lysosomal origin and are perhaps stages in the development of a lipochondrion.

Conclusions and Discussion

From the mass of evidence presented, it is apparent that we are dealing with a heterogeneous group of bodies essentially similar in many respects, and differing markedly from the other cytoplasmic inclusions (Mitochondria, Golgi, yolk, etc.). Broadly speaking, these bodies may be classified into three groups: (*a*) Minute polymorphic bodies (< 2 microns) which correspond to the β particles described in the eggs of many invertebrates (Novikoff, 1961; Beams, 1964) and are related, if not identical, to the lysosomes of de Duve (1963); (*b*) small vesicles of varying sizes (2–4 microns) which are lysosomal in nature, and are comparable to phagosomes; (*c*) large albumen vesicles of assorted sizes which are probably formed by the coalescence or enlargement of the small vesicles.

The lysosomes seem to arise mainly by vesiculation of Golgi lamellae while the phagosomes are probably formed by a process akin to pinocytosis. Both inclusions are associated with each other and are found mainly in the subcortical zone in early ova and are later attracted towards asters during early cleavage. Lysosomes and tiny phagosomes are also associated with large albumen vesicles as 'satellites' in later embryos. These inclusions could be traced during early development and their functional and developmental interrelationships will be discussed.

Micropinocytosis

When the ovum comes down the oviduct, it is already surrounded by albumen and when it is laid the first maturation division has been completed. Electron micrographs of ova at this stage show a fair amount of minute vesicular elements at the surface, which are also detectable by the PAS reaction at the light microscope level. There are also large PAS-positive vesicles and granules, associated with each other, in the peripheral subcortical zone. As the ovum matures, these bodies increase in number (while the vesicles also increase in size) and later become associated with the cleavage aster. There seems little doubt that the vesicular elements at the surface are very similar to minute micropinocytotic vesicles of the type described in many cells, especially eggs (Novikoff, 1961; Beams, 1964). There is also evidence accumulating suggesting that prefabricated protein yolk may be taken directly into the oocytes of certain animals by micropinocytosis (Beams, 1964). What seems to be taken into the ovum of *Arion* is albumen (capsule fluid) which is essentially a proteinaceous yolk-like nutritive substance.

Multivesicular bodies (phagosomes and lysosomes)

Most of the micropinocytotic vesicles are at first submicroscopic in size but later they seem to enlarge slightly and conglomerate to form larger multivesicular bodies. This is clearly seen

in one micrograph (Fig. 12) where a multivesicular body is being formed just outside the plasma membrane. (PAS-positive bodies of comparable size were also seen between the vitelline and plasma membranes.) This body resembles the massive multivesicular body seen in the cytoplasm (Fig. 14). It seems likely that most of the large vesicles in this massive multi-vesicular body are in fact micropinocytotic vesicles, held within a membrane by an electron-lucid material such as a mucopolysaccharide. Similar conclusions have been made by Novikoff (1961) who has studied and reviewed the work done by many others. The large multivesicular body is comparable in size to the PAS-positive vesicles seen in the peripheral cytoplasm in ova. The fact that these bodies first appear close to the cell surface also shows that they have possibly arisen by pinocytotic activity. They have been found to be lysosomal in nature, as they contain acid phosphatase and are limited by a membrane. They are therefore comparable to the body described as a 'phagosome' (de Duve, 1963).

The smaller multivesicular bodies seen in the electron micrographs correspond in size to the PAS-positive, acid phosphatase-rich granules. These, as I have pointed out earlier, are probably identical to lysosomes and are very similar to the particles, first described in *Barnea* by Pasteels and Mulnard (1957), and later in many other eggs (Brachet, 1960; Beams, 1964). Their fine structure resembles very closely the multi-vesicular bodies described in rat ova by Sotelo and Porter (1959) and in the eggs of the surf clam *Spisula* (Rebhun, 1960). Further, the presence of 'unit' outer membranes establishes their identity beyond doubt. The most significant feature of these bodies is that they contain small vesicles which have a remarkable resemblance to those found in the Golgi zone (Fig. 14). Rebhun made similar observations in *Spisula.* It is possible that these vesicles arose by vesiculation of Golgi lamellae. The Golgi also show acid phosphatase activity and are chemically very similar to these bodies. Novikoff and his associates (1961, 1962), after an extensive study of a number of vertebrate tissues, suggest a developmental or functional interrelationship between the Golgi apparatus and the lysosomes. They found a concentration of lysosomes near the Golgi zone and pointed out that lysosomes could arise by vesiculation of Golgi lamellae. If this is so, then the origin of the outer lysosome membrane is not explained.

It must also be pointed out here that there were minute vesicles on the vitelline membrane. These, however, are less electron dense, appear more hollow, and on the whole are larger and more variable in size than those seen in the lysosomes. Hence, it seems unlikely that some of the vesicles in lysosomes arise by pinocytotic activity. Besides, there was no acid phosphatase activity at the cell surface or in the egg albumen.

Dense bodies (lipochondria)

The dense bodies, too, seem to be of lysosomal origin (de Duve, 1963), and are perhaps stages in the development of lipochondria. The larger body is somewhat reminiscent of Text-Fig. 2. drawn by Baker (1963) based on the description and electron micrographs of Ashhurst and Chapman. Recent work in Oxford has shown that the lipochondria are complex glycolipoprotein bodies and may contain enzymes such as acid phosphatase (Baker, 1963). Baker suggests that the lipochondrion may be regarded as an overgrown and modified lysosome. Large oval bodies (1–2 μ in diameter) answering the description of lipochondria have been seen occasionally in early embryos sometimes in the outer zone and in polar bodies. Unlike the metachromatic bodies, they have a very high lipid content and stain orthochromatically with vital dyes and seem to stratify close to the oil cap zone. These perhaps correspond to the 'dense bodies' seen in micrographs. Text-Figure 1 shows the relationship of lipochondria to lysosomes and Golgi.

Intracellular digestion

Lysosomes are thought to be organelles of intracellular digestion (Novikoff, 1961; de Duve, 1963; Allison, 1965). According to de Duve, at least 4 types of lysosomes are involved in this

process: 'storage granules', 'digestive vacuoles', 'autophagic vacuoles', and 'residual bodies'. The small multivesicular bodies perhaps correspond to the 'storage granules', which is the original form of the lysosome. Lysosomes are regarded as tiny, 'suicide bags', filled with powerful digestive enzymes capable of breaking down most of the constituents of living matter. When its membrane is ruptured the enzymes leak out into the cytoplasm and could digest its contents. Bodies with incomplete membranes were seen in *Arion* and some of their contents were found in the cytoplasm. A body resembling an 'autophagic vacuole' was also seen in one micrograph. It had a tuft of membranous elements which could well be parts of some cellular membrane, perhaps a mitochondrion. The grape-like aggregations (or vesicles) that were seen in certain cells of advanced embryos after vital staining and in peroxidase preparations could be likened to 'residual bodies'. These are perhaps later stages in albumen digestion. Groups of uric acid crystals were sometimes seen in similar situations.

The lysosomes were always found to be associated with the phagosomes in the peripheral cytoplasm of the egg. Although such an association was not seen in electron micrographs there is some evidence that these two classes of bodies have coalesced in much the same way as outlined by de Duve (1963). If the large multivesicular body (phagosome) is examined closely it is found to contain smaller vesicles very similar to those seen in small multivesicular bodies (lysosomes). Further, there was a group of four or five lysosomes, all with ruptured membranes, very close to the egg surface where micropinocytotic vesicles were aggregating together (Fig. 12). It is suggested that the small micropinocytotic vesicles come together to form larger groups which then coalesce with lysosomes to form the larger more complex multivesicular bodies, in a manner comparable to the formation of a 'digestive vacoule'. They perhaps acquire their acid phosphatase activity after fusion, and this would explain the lysosomal nature of the phagosome. Further evidence for this view is that they often give complementary though weaker reactions than the lysosomes, which suggests a dilution of the lysosomal contents in their substance. Possible interrelationships between Golgi, lysosomes, and pinocytotic activity are shown in Text-Fig. 1. The apparent fusion of micropinocytotic vesicles with lysosomes, the existence of 'phagosomes', which behave very much like lysosomes, and the occurrence of unusually large pinocytotic vacuoles resembling multivesicular bodies in certain cells, are reported in Novikoff (1961).

It seems likely that in *Arion*, many lysosomes fuse with a single large phagosome. They could also remain at the surface of a phagosome and exert their digestive influence from without or absorb small quantities of albumen and digest it within their membranes. This seems to be the case in later embryos where myriads of 'satellites' are seen close to or on the surface of larger albumen vesicles, which could be regarded as enormous phagosomes or digestive vacoules. Satellites of neutral red were seen by earlier workers around food vacoules in Protozoa (Baker, 1958). Recently, lysosomes have been seen adhering to these food vacuoles (phagosomes) and are thought to be involved in intracellular digestion (Allison, 1965). In *Arion*, the lysosomes are mainly involved in the digestion of albumen (capsule fluid) which is composed chiefly of protein and various polysaccharides. It is a remarkable fact that the albumen vesicles change in their chemical composition during early development. As the phagosomes grow in size they lose their acid phosphatase and peroxidase activity and they become less and less metachromatic and neutral red positive. A gradual decrease in their sulphated acid mucopolysaccharide content was also noted. A change in their physical appearance was also seen. During enlargement, they become more and more homogeneous in texture and sometimes appear granular. Some of them, however, were seen to be distinctly multivesicular at the light microscope level. This suggests that smaller vesicles have come together in the formation of a large albumen globule reminiscent of the fusion of soap bubbles. This and all other staining reactions suggest that the vesicles grow in size, chiefly by a coalescence of smaller vesicular elements. Further, at the early blastula stage, there is a sudden appearance of larger albumen vesicles, first in the micromeres and then in the macromeres. This is preceded by an increased output of albumen intake at the 16-cell stage. The large vesicles are found to be associated with numerous 'satellites' (both lysosomes and tiny phagosomes). The latter eventually decrease in number during gastrulation leaving

enlarged vesicles with fewer associated bodies. In late and post-gastrula stages the vesicles are enormous, having decreased in number and have very few satellites, chiefly lysosomes in mulberry-like groups. All this suggests coalescence of the vesicles to form larger ones.

Pinocytosis

In *Limnaea*, Elbers and Bluemink (1960) have described the intake of albumen in larger gulps by pinocytosis. They have examined blastulae by electron microscopy and found typical pinocytotic vacuoles with granular contents and fine limiting membranes. Pinocytotic activity of this order cannot be ruled out, at least in later embryos of *Arion*. The intake of enormous amounts of albumen in the blastula stage and the presence of more homogeneous and sometimes granular vesicles lend some support to this view. Unlike in *Limnaea*, no conical or hemispherical protrusions, or annular thickenings were seen on the surface of blastulae. Even so, further studies with the electron microscope are necessary to find out whether this form of pinocytosis occurs in the blastulae of *Arion*.

Chemical aspects

The small granules (lysosomes) are chemically similar to Golgi bodies, in many respects. They are rich in acid phosphatase, stain with neutral red, are intensely PAS-positive, contain acid mucopolysaccharide, and have a fairly high content of lipid (probably phospholipid). Unlike Golgi, they show vital metachromacy and are not usually seen in Mann-Kopsch preparations or mitochondrial preparations.

Metachromasy

Metachromasy of lysosomes seem to be due mainly to a comparatively high content of acid mucopolysaccharide. The albumen vesicles have galactogen as well, which is also metachromatic in aqueous solutions of stains (Grainger and Shillitoe, 1951). The chief substances responsible for their metachromasy seem to be acid mucopolysaccharides and galactogen. As the vesicles enlarge they become less and less metachromatic. This would suggest a gradual disappearance of these substances. Indeed, a decrease in the sulphated acid mucopolysaccharide content was found to coincide with this change. As lysosomes and albumen vesicles are constantly in physiological association with one another, one would expect them to show complementary reactions.

Neutral red staining

Lysosomes are now known to take up vital dyes such as neutral red (Allison, 1965). But the affinity of lysosomes and albumen vesicles for neutral red is rather difficult to explain. Neutral red granules of lysosomal origin have also been demonstrated in the mouse exocrine pancreas cells by electron microscopy (Byrne, 1964). Byrne thinks that the dye is segregated in the lysosomes possibly in combination with phospholipid. The lysosomes in *Arion* have been found to stain with sudan black in wax sections and with nile blue in vital preparations. Electron micrographs show well-defined double membranes presumably phospholipid in nature. Like all other cellular membranes it is thought to be essentially lipoprotein with a high phospholipid content (Novikoff, 1961). Even the larger vesicles have membranous coverings, so that it is conceivable that the dye is associated with phospholipid.

This is only partly true in the case of albumen vesicles. The results with Nile blue are remarkably similar to those obtained with neutral red. Nile blue colours fatty acids or phospholipids blue (Baker, 1958). As in the case of metachromasy, there is also a comparable

loss of staining intensity as the albumen vesicles increase in size. This suggests a gradual decrease in the phospholipid content. A similar absorption of lipid was also seen in Mann-Kopsch preparations. It must also be remembered that Nile blue sometimes stains albumen vesicles in shades of purple. This is due to an impurity (Nile red) which stains neutral lipids red (Baker, 1958). These results show that the dyes are not confined to membrane-bound lipid alone and that lipids other than phospholipid may stain as well.

Phagocytic food vacuoles are also known to take up neutral red and in this case the proteolytic enzymes contained within them are thought to be stained (Baker, 1958). This, too, seems an interesting proposition. We have already seen that the lysosomes are chiefly involved in the intracellular digestion of albumen vesicles which are akin to phagocytic food vacuoles. The lysosomes store the enzymes and are most intensely stained, and when they fuse with the pinocytotic phagosomes the enzymes are released and mix with the contents of the latter. The gradual loss of neutral red staining during the growth of the albumen vesicles could then be interpreted as being due to a gradual dilution of digestive enzymes.

Finally, Malhotra (1960) suggests that neutral red staining may be due to the presence of acid mucopolysaccharide. The latter was found in both lysosomes and albumen vesicles. The parallelism seen between the staining of albumen vesicles of varying sizes with metachromatic dyes and neutral red is in accordance with this view. The whole problem is very complicated because all three substances (phospholipid, proteolytic enzymes, and acid mucopolysaccharide) are found in these bodies and perhaps they are all responsible for the staining with neutral red.

Acid phosphatase activity

The identification of a lysosome depends mainly on enzymatic criteria. The presence of acid phosphatase, which led to its discovery, is thought to establish its identity. The lysosomes and phagosomes (small albumen vesicles) contain the enzyme while the large albumen vesicles do not show any activity. Acid phosphatase splits off inorganic phosphate from various phosphate esters which are found in living cells and is of great importance to cellular metabolism.

A certain correlation seems to exist between metachromasy, the ability to stain with neutral red, and the presence of acid phosphatase. This seems to be true in the case of the lysosomes and the phagosomes. The larger albumen vesicles do not show any acid phosphatase activity but still stain with neutral red and are metachromatic, though less intensely. It is possible that the enzyme is gradually diluted during the growth of the vesicles and cannot be detected at the light microscope level. Indeed, Bryne (1964) found acid phosphatase activity in neutral red granules, only in electron micrographs of material previously incubated by Gomori's lead method. No reaction product was seen under the light microscope. She concluded that the amount of precipitated reaction-product is below the threshold of resolution of the optical microscope.

Benzidine peroxidase activity

The identification of benzidine peroxidase as an intrinsic component of lysosomes and phagosomes, perhaps for the first time, is very significant though not surprising. Straus and other workers, in a remarkable series of experiments, injected (intravenously) horse-radish peroxidase into animals, and traced them into bodies in the kidney and liver which were identified as phagosomes and lysosomes (Novikoff, 1961). The injected enzyme (protein) was revealed by the benzidine reaction and first appeared in vacuoles which were apparently formed by a fusion of smaller micropinocytotic vacuoles. These were called 'phagosomes' by Straus and behaved very much like lysosomes. Much later, the enzyme was found in lysosomes (Fig. 15.—Novikoff,

1961). Linearly arranged microcytotic vacuoles have often been seen between the cell membrane and lysosomes in certain cells and it is thought they are the means by which injected enzymes (proteins) find their way into the lysosomes (Novikoff, 1961). A similar if not identical situation seems to exist in *Arion*, where albumen could be compared to the injected protein. The lysosomes and phagosomes show peroxidase activity, without prior injection. This is best seen in centrifuged eggs, where the picture is remarkably similar to that seen after incubation for acid phosphatase. Peroxidases are oxidative (haemo-protein) enzymes which catalyze the transfer of oxygen from hydrogen peroxide (and other peroxides) to a variety of acceptors. The enzyme found in these bodies is presumably concerned with respiration.

Non-enzymatic components

Apart from lipids, the non-enzymatic components of lysosomes and small phagosomes are various polysaccharides. The PAS-reaction was shown to be extremely useful in demonstrating these in wax sections. The PAS-reactive material seems to consist of neutral mucopolysaccharide or mucoprotein or both. There may be traces of glycolipid and phospholipid contributing to this reaction. Both sulphated and non-sulphated acid mucopolysaccharides were found in appreciable amounts and were mainly responsible for the metachromatic reactions of these bodies. The small phagosomes also had galactogen and perhaps traces of calcium. Lysosomes containing PAS-positive material have been found in liver and kidney (Novikoff, 1961) and in the eggs of *Barnea* (Pasteels and Mulnard, 1957).

The large phagosomes give the same reactions as the albumen in the capsule. This fluid is the chief source of nutrient in later embryos. It is remarkable that in *Arion* it is taken up so early in development and by the time the gastrula stage is reached almost the whole of the ectoderm and endoderm are involved in its digestion and utilisation. Albumen is a very complex clear viscous fluid, essentially carbohydrate-protein in nature. Its chief constituents were galactogen, acid and neutral mucopolysaccharides, and mucoprotein. No doubt these substances are essential in the metabolism of the embryo, but the fact that such complex substances as mucopolysaccharides are utilised so early in development is intriguing. It is also noteworthy that appreciable amounts of lipid were absorbed from the albumen. Calcium, as we know, is very important in keeping the cells together during cleavage. It originates from the shell and is transported *via* the albumen to the embryo.

Physical aspects

The lysosomal bodies are easily distinguishable from other cell inclusions on account of their highly polymorphic character, variability in size, and general distribution. The heterogeneous appearance of larger lysosomes and small phagosomes under phase-contrast, and in many other vital and fixed preparations is noteworthy and may be used as a means of detecting them with the light microscope.

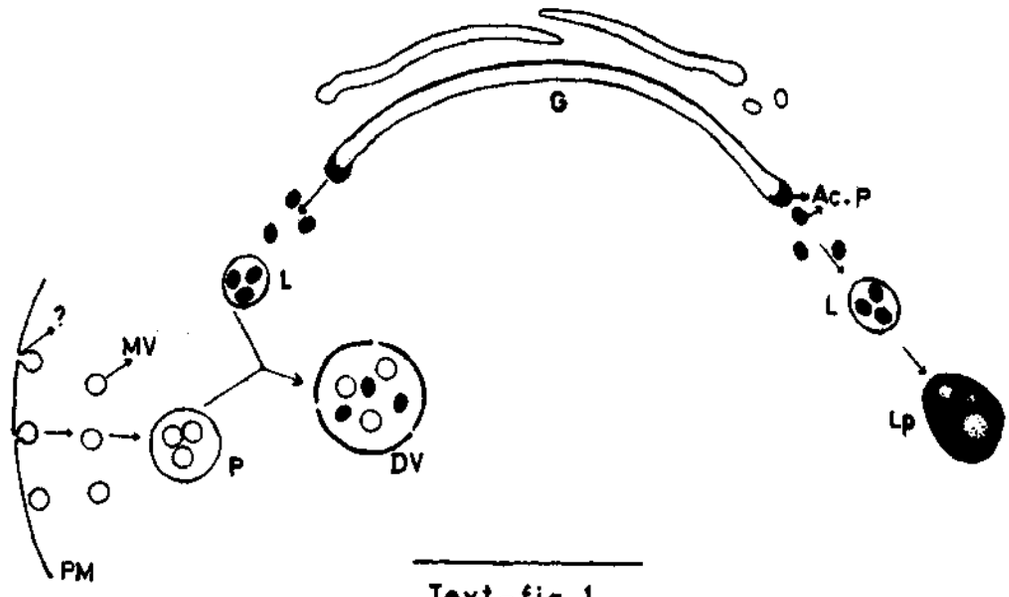
Centrifugation

Another method of identifying these particles is to isolate them in homogenates of cells, by centrifugal fractionation (de Duve, 1963). The lysosomes invariably stratify in the mitochondrial fraction and could be further isolated by centrifuging at very high speeds. A similar, though incomplete stratification, was achieved in the living eggs using ordinary mild centrifugation. The lysosomes and related bodies were found in the mitochondrial zone (hyaloplasm) and can be easily identified. The small phagosomes and associated lysosomes stratify in the equator which is the most centrifugal region of the hyaloplasm. Perhaps this is the first time such a layer has been detected in pulmonate eggs. This again confirms the lysosomal nature of the phagosome and at the same time indicates their higher protein content, because we now know

that the relative amounts of lipid and protein present in an inclusion determines its stratification.

Role in cell division

The association of the metachromatic particles (lysosomes and small phagosomes) with cleavage asters suggest that they may play a role in cell division. This has been also suggested by earlier workers (Brachet, 1960; Rebhun, 1960; Novikoff, 1961). It is also possible that there is some relationship between nuclear, lysosomal, and pinocytotic activity. The utilisation of albumen then seems to be regulated by the nucleus. Further, albumen vesicles and 'satellites' were also seen in close proximity to the nuclei.



Text-fig 1

Role in morphogenesis

It has been suggested that metachromatic granules (multivesicular bodies) may be involved in morphogenesis (Brachet, 1960; Rebhun, 1960; Novikoff, 1961). In *Arion* macromeres of early cleavage stages were seen to have more peroxidase activity while an intensely positive acid phosphatase ring was seen around the blastopore in invaginating gastrulae. There was also an accumulation of PAS-positive bodies in the cells of the shell gland. All these areas are known to be morphogenetically very active and it is possible that these bodies have their part to play in the process of differentiation. Obviously, further work has to be done before definite conclusions are made.

Atrophy of polar bodies

The polar bodies need very special mention. They have been found to contain all the cytoplasmic inclusions present in the ovum. But vital staining and cytochemical tests show that they have more than their due share of lysosomes and related bodies. Most of these bodies seem to have found their way during maturation. When a polar body is pinched off, it takes with it the lysosomal bodies found in the peripheral zone at the animal pole and those, if any,

associated with the maturation aster. On account of this, they are sites of increased lysosomal activity and are intensely metachromatic and neutral red positive, and respond strongly to tests for acid phosphatase, peroxidase, and PAS reactivity. The polar bodies degenerate gradually during early development and by the time the post-gastrula stage is reached, they have invariably disappeared. It seems very likely that the lysosomal bodies play a role in their atrophy. Intracellular digestion and autolysis are thought to be the two chief functions of lysosomes (de Duve, 1963). This seems to be the case in *Arion*. The actual atrophy of the polar body could take place by the rupture of the lysosomal membrane and the consequent release of the lytic enzymes into the surrounding cytoplasm or parts of other cellular membranes could be engulfed and digested within the lysosomes. Lysosomes are also known to rupture following the death of a cell and this is thought to be a sort of built-in mechanism for the self-removal of dead cells (de Duve, 1963). The polar bodies, at least during cleavage, are very much alive and behave exactly like the egg. They take up vital stains and may stratify on centrifugation and often cleave at least once or even twice. Death seems to occur later in development; perhaps during gastrulation. Whether lysosome rupture occurs before or after death, or whether it occurs at all, remains to be seen. It would be interesting to make an electron microscopical study of polar bodies at different stages of development and to determine exactly the sequence of events leading to their final atrophy. It should be pointed out that lysosomes are thought to play a part in the regression of other embryonic structures such as the amphibian tadpole tail, chick Mullerian duct, and the mammalian mesonephros (Novikoff, 1961; de Duve, 1963). A marked rise in lysosomal enzyme activity was seen in such regions and an actual lysosomal digestion of cells occurred in the tadpole tail. Hadek (1965) has found no cytoplasmic organization in the polar bodies of the rabbit egg at the electron microscope level.

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EXPLANATION OF PLATES

FIG. 1. Diagram showing lysosomes, phagosomes, and albumen vesicles as they appear in various preparations. Satellites, mulberry-and grape-like aggregations are also shown.

FIGS. 2–9. Photomicrographs showing the distribution of PAS-positive inclusions in early embryos. Lysosomes, phagosomes, and albomen vesicles stain very intensely while glycogen is finely granular. Figs. 2–3. Fig. 2. V.S. Late ovum showing cleavage spindle (Gendre/PAS)—Lysosomes and phagosomes are in peripheral zone. Fig.3. V.S. 2-cell stage (Bouin/PAS) showing lysosomes and phagosomes being attracted towards asters.

FIGS. 4–5 *a*. Fig. 4. V.S. 8-cell stage (Bouin/PAS) showing lysosomes and phagosomes associated with asters and in polar body. Fig. 5. V.S. Centrifuged 2-cell stage (Helly/PAS) showing lysosomes in hyaloplasm and sosomes associated with phagosomes at the equator. (Note yolk granules also stain after Helly-fixation.) Fig. 5 *a*. Diagrams of centrifuged ova showing acid phosphatase and benzide peroxidase activity.

FIGS. 6–7. Fig. 6. V.S. Early blastula-24-cell stage (Carnoy/Hale/PAS)—Large albumen vesicles are just appearing in the micromeres. Fig. 6 *a*. V.S. Early blastula (above)—Saliva control-showing ectoplasmic zone of micromeres withalbumen vesicles associated withs atellites. Fig. 7. V.S. Mid-blastula (Zenker/PAS).

FIGS. 8–9. Fig. 8. T.S. Late gastrula (Bouin/PAS). Fig. 9. L.S. Post-gastrula (Helly/PAS).

FIGS. 10-12. Electron micrographs of early embryos. Fig. 10. Early ovum at second maturation—portion of egg-surface showing micropinocytotic activity, × 24,000. Fig. 11. Mature ovum soon after maturation —portion of egg—surface showing micropinocytotic vesicles in cortical cytopla × 24,000 Fig. 12. Mature ovum showing an increase in vesicular elements at the surface an ruptured lysosmes, × 24,000.

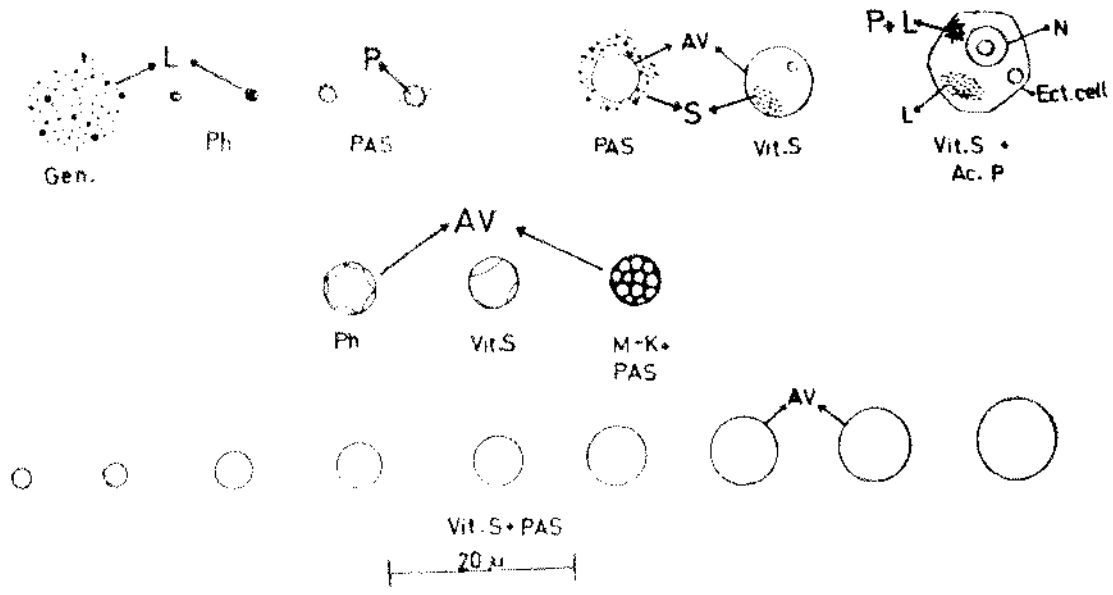

L
Ph
Gen.
P
PAS
AV
S
PAS
Vit.S
P+L
N
O
Ect.cell
L
Vit.S +
Ac. P
AV
Ph
Vit.S
M-K+
PAS
AV
Vit.S+PAS
20 μ

Fig. 1

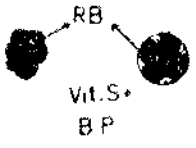
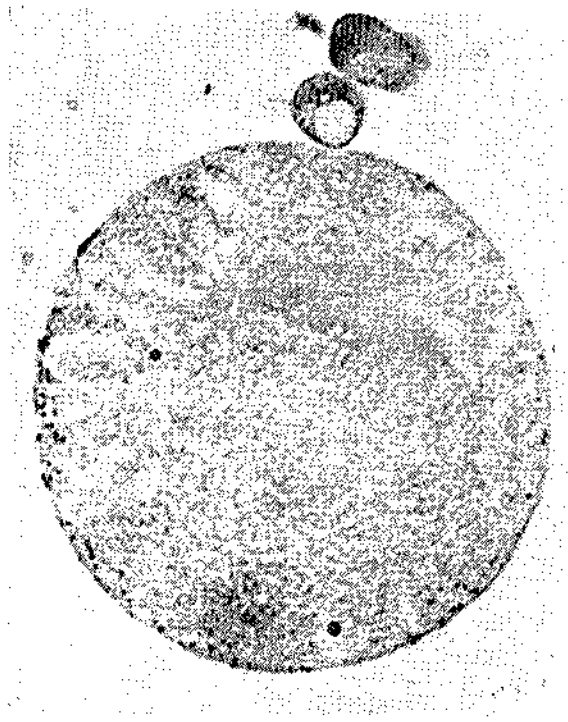

RB
Vit.S+
B P

Fig. 1



50 μ

Fig. 2

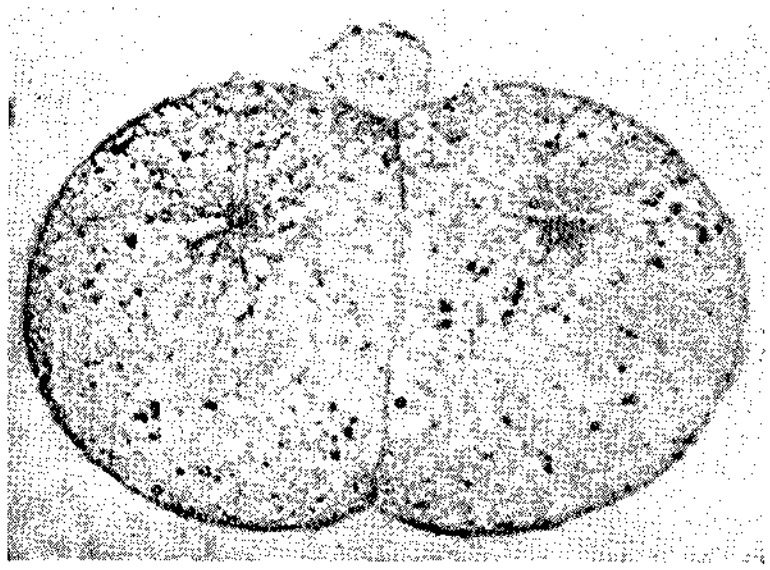
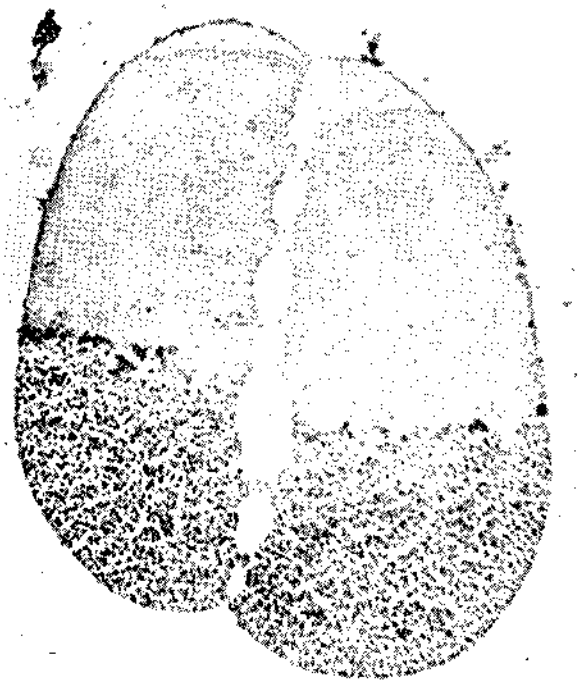


Fig. 3

FIGS. 2-3

50 μ

Fig.4



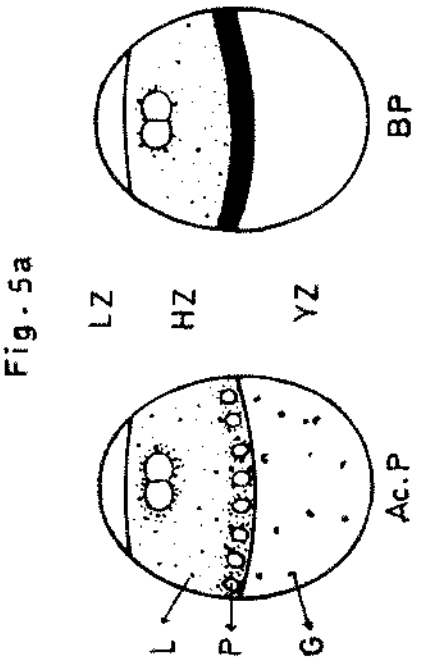


Fig. 5a

Fig. 5

FIGS. 4 & 5 *a*

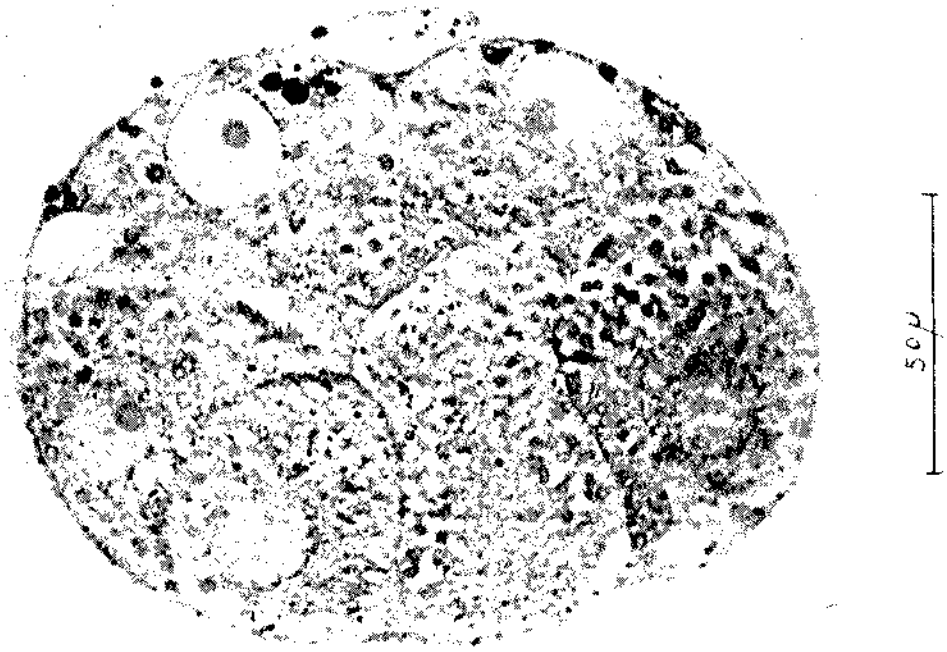


Fig. 6

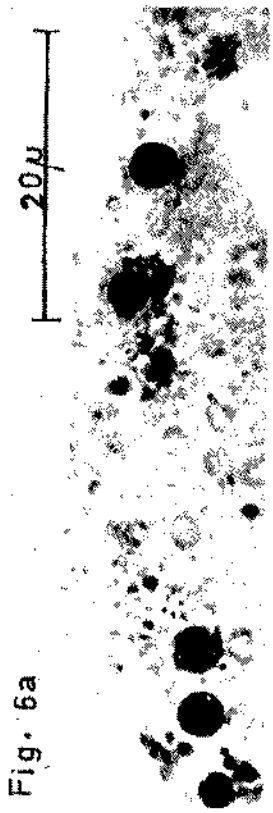


Fig. 6a

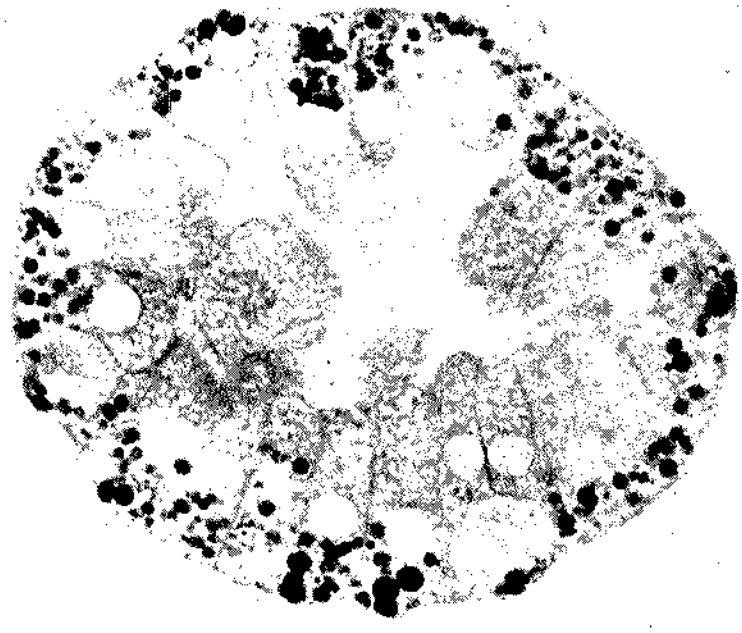


Fig. 7

50μ

Figs. 6–7

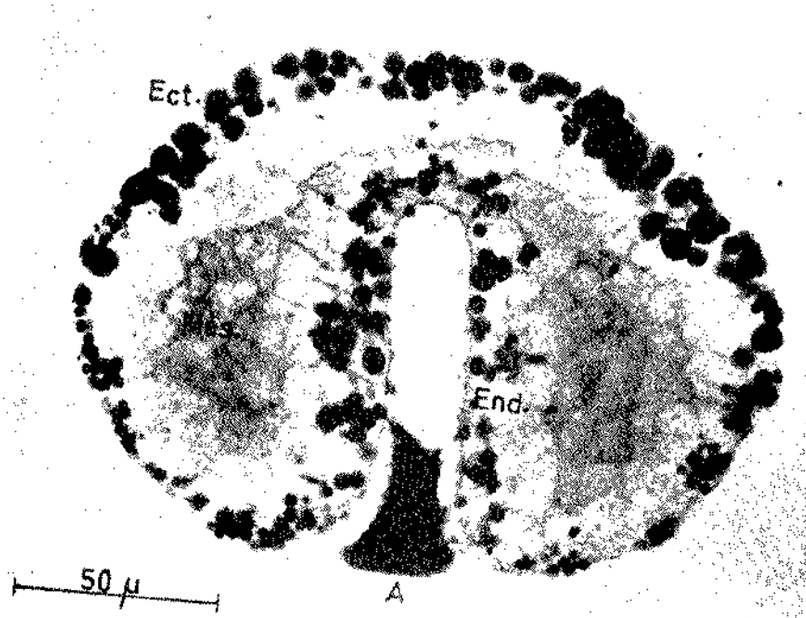


Fig. 8

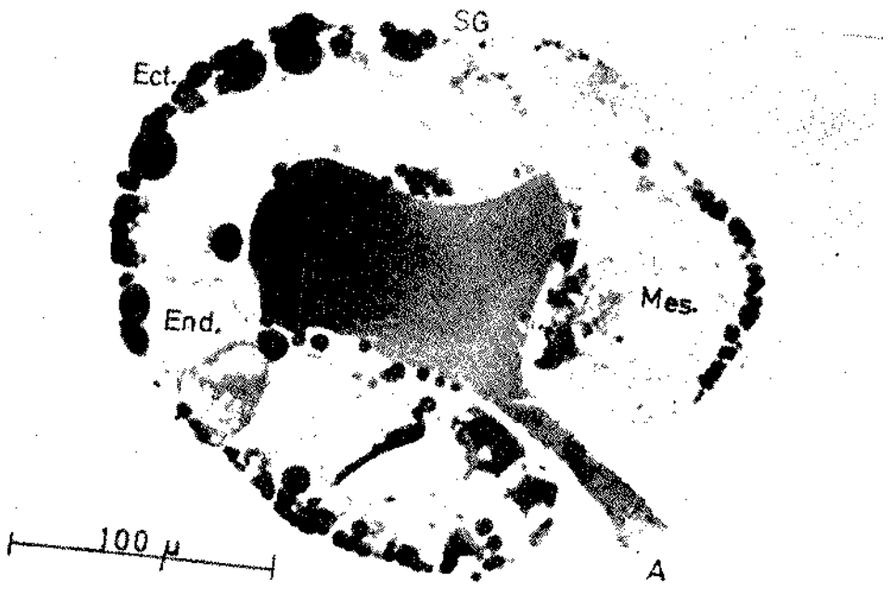
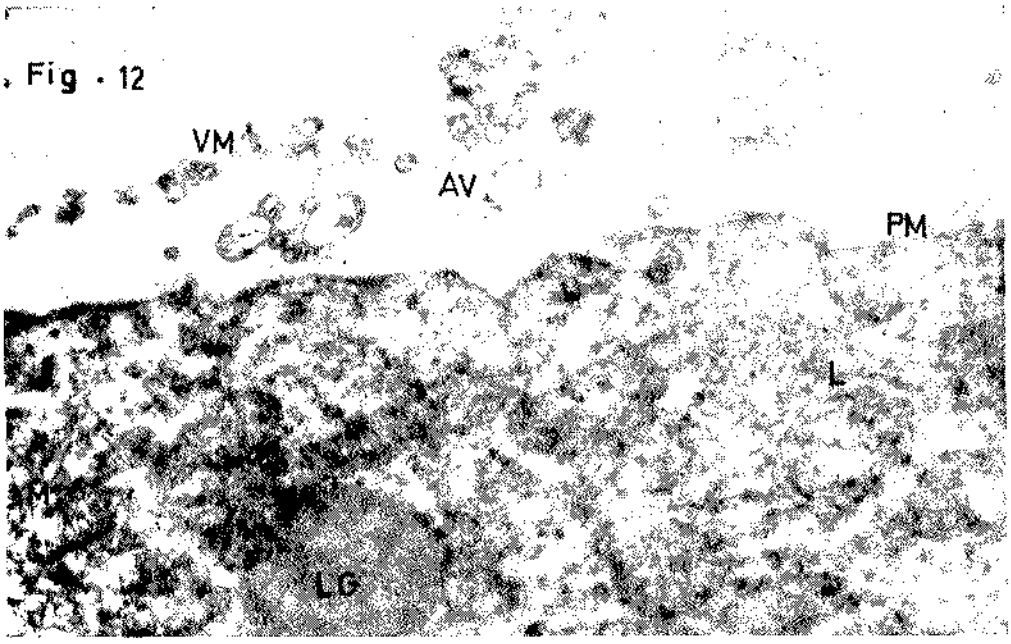


Fig. 9

Fig.10

Fig.11



Figs. 10-12

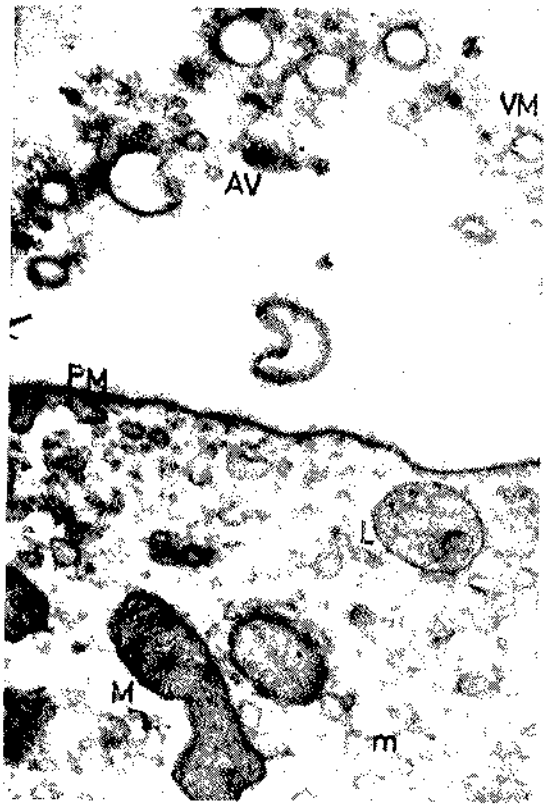


Fig.13

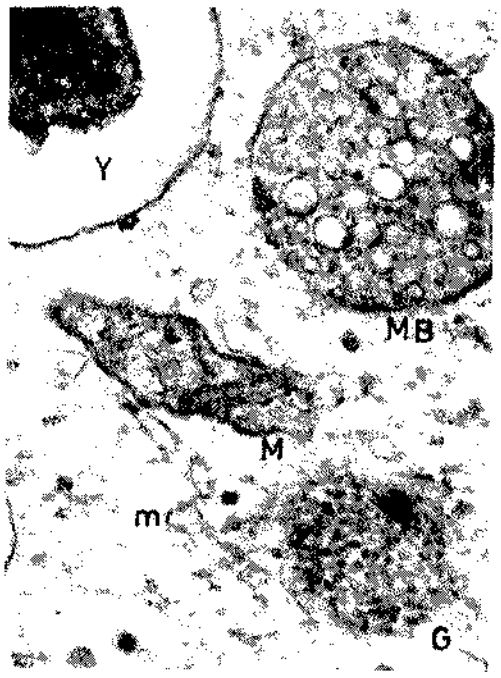


Fig.14

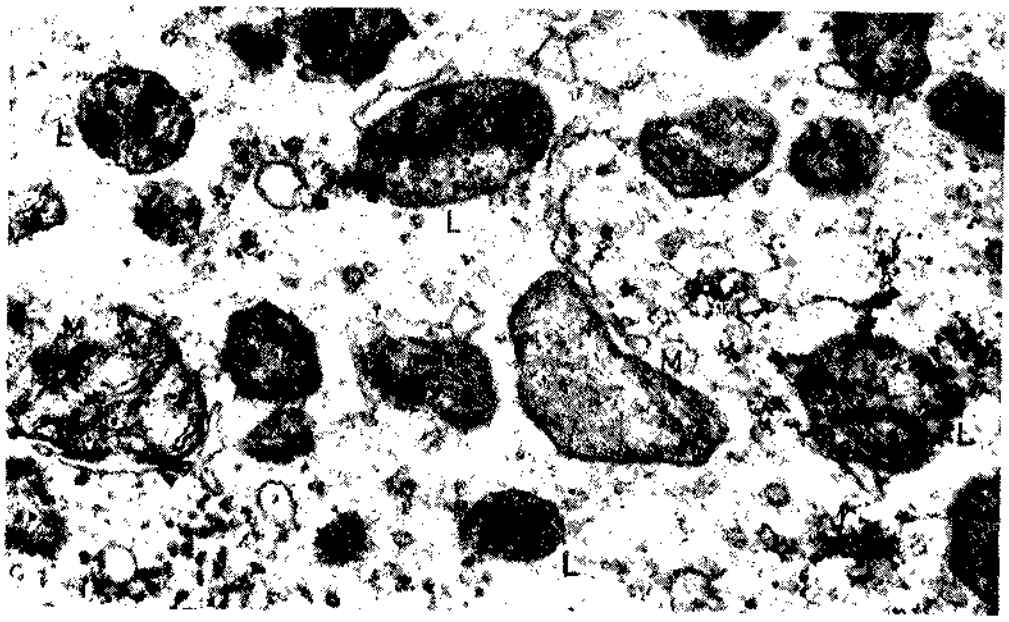


Fig.15

FIGS. 13–15

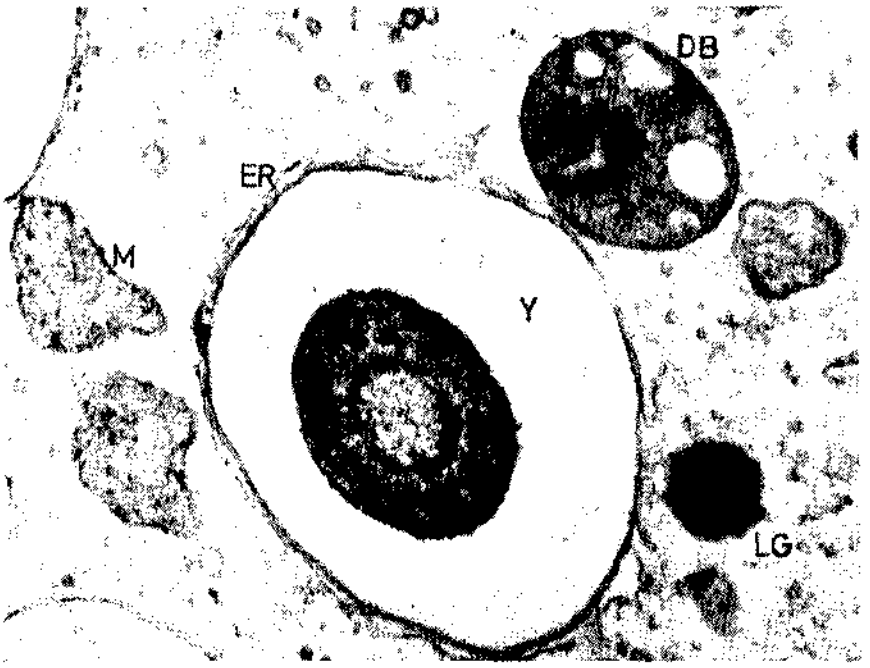
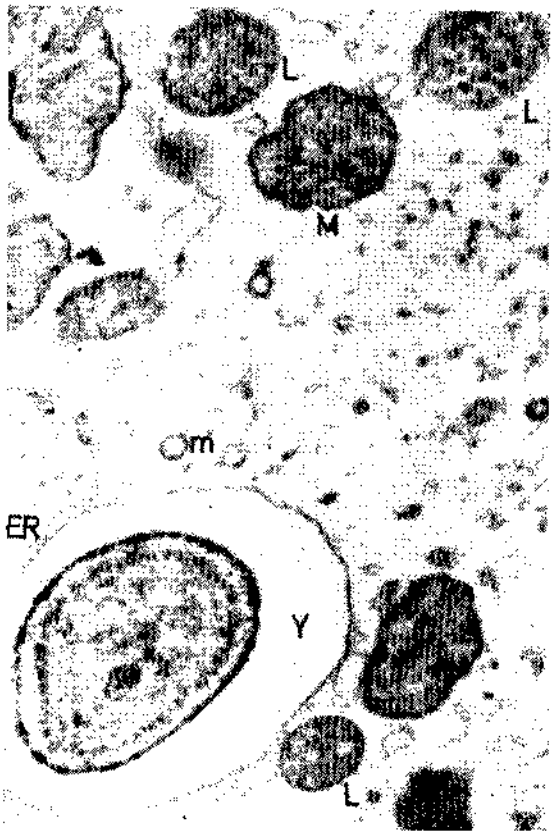


Fig.16

Fig.18

Fig.17



FIGS. 16-18

FIGS. 13–15. Electron micrographs of early embryos. Fig. 13. 8-cell stage—surface of a blastomere showing a further increase in vesicular elements and a lysosome within the plasma membrane, ×24,000. Fig. 14. 8-cell stage showing a massive multivesicular body and a Golgi body, ×30,000. Fig. 15. 8-cell stage showing various appearances of lysosomes, ×30,000.

FIGS. 16–18. Electron micrographs of early embryos Fig. 16. 8-cell stage showing a 'dense' body and a yolk granule, ×30,000. Fig. 17. Early ovum showing a ruptured lysosome and dense mitochondria, × 24,000. Fig. 18. 8-cell stage showing lysosomes, ×30,000.

A, albumen; Ac. P, acid phosphatase activity; AV, albumen vesicle; BP, benzidine peroxidase activity; DB, dense body; DV, digestive vacaole; Ect., ectoderm; End., endoderm; ER, endoplasmic reticulum (ergastoplasm); G, Golgi; Gen., general appearance; HZ, hyaline zone; L, lysosomes; LG, lipid globule; Lp, lipochondrion; LZ, lipid zone; m, microsome (ergastoplasmic vesicle); M, mitochondrion; MB, multivesicular body (large); Mes., mesoderm; M-K, Mann-Kopsch preparation; MI, micropinocytotic invagination; MV, micropinocytotic vesicle; N, nucleus; P, phagosome; PAS, periodic acid Schiff test; Ph, phase-contrast; PM, plasma membrane; RB, residual body; S, satellites; SG, shell gland; VM, vitelline membrane; Vit. S, vital staining; Y, yolk granule; YZ, yolky zone.

APPENDIX

TABLE I

Summary of important staining and cytochemical reactions

	Lysosomes	*Phagosomes*	*Albumen vesicles*
Vital Stain			
Toluidine blue	+++ P	+++ P	+++ to + P
Methylene blue	+++ P	+++ P	+++ to + P
Neutral red	+++ to ++	++/	+++ to +
Nile blue	+++ B	++ B	+++ to + BP
Enzyme Activity			
Acid phosphatase:			
Lead method	++++	++	+/−
Azo method	++++	++	+/−
Benzidine peroxidase	++++	+++	−
Cytochemical Test			
PAS reaction	++++	++++	++++
PAS/pectinase	+++	+++	++
Best	++	++	+++
Best/pectinase	+	+	+/−
Alcian blue	+ +	++	+
Aldehyde fuchsin/Alcian blue	+++ P	+++ P	+++ to + P or +B
Hale	+++	+++	++
Hale/PAS	++++ R	++++ R	++++ R
Lipid Reaction			
Sudan Black B	+++	++	++ to +

++++ = very intense reaction
+++ = strong reaction
++ = moderate reaction
+ = weak reaction
+/− = very weak or uncertain reaction
− = negative reaction

P = purple
B = blue
R = crimson red

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