A comparison of certain isozyme patterns in lobeless and normal embryos of the snail, *Ilyanassa obsoleta*

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**SUMMARY**

A study was made of the emergence of certain enzymes during the embryogenesis of *Ilyanassa*. Lobeless and normal embryos were compared in order to determine the effect of polar lobe removal on subsequent molecular developments. Polyacrylamide gel electrophoresis in capillary tubes, a technique requiring only small numbers of embryos, was used to obtain the isozyme patterns of alkaline phosphatases and of esterases. It was found that lobe removal interfered with the emergence of normal isozyme patterns of alkaline phosphatase and esterase during development. Certain bands of enzyme activity were severely reduced or absent while others appeared to be normal. The results provide further evidence that the influence of the polar lobe on development is of a specific nature.

**INTRODUCTION**

The egg of the marine gastropod *Ilyanassa obsoleta* produces, at first cleavage, a protrusion of the vegetal pole cytoplasm known as the polar lobe. By removing the polar lobe and observing subsequent development, Clement (1952) demonstrated that this portion of cytoplasm contains materials which are necessary for the normal development of the veliger larva. The larva which develops from a lobeless egg has a form quite unlike the normal larva and, although it is capable of producing certain larval structures such as velar cilia, pigment, and endodermal mass, it lacks velum, eyes, foot, external shell, statocysts, operculum, intestine, and heart. Clement (1962) has found that, although the lobe-dependent structures appear late in development, the lobe exerts its influence on these structures during the first few cleavages and, after that, the polar lobe region can be removed without affecting the subsequent development of larval structures.

Lobeless embryos exhibit decreased RNA synthesis which may indicate impairment of the process of gene activation during development (Davidson et al. 1965). It has also been shown that removal of the polar lobe interferes with the protein-synthesizing capacity of the developing embryo (Collier, 1961).

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Do these lowered levels of protein and RNA formation indicate that there is a generalized impairment of synthetic capacity in the lobeless embryo or does lobe removal affect the production of some molecules more than others? The purpose of the present study was to examine these alternatives by comparing the electrophoretic patterns of certain enzymes formed during the development of normal and lobeless embryos.

**MATERIALS AND METHODS**

Adult snails were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, and kept in circulating Instant Ocean synthetic sea water (Aquarium Systems, Inc., Wickliffe, Ohio 44092) at 20 °C. They were fed raw shrimp three times a week.

Methods for obtaining and handling the eggs were those of Clement (1952); see also Costello et al. 1957. Millipore-filtered, pasteurized Instant Ocean was used throughout these experiments in place of sea water, except for calcium-free sea water, which was prepared according to the formula of Shapiro (Cavanaugh, 1964). Uncleaved eggs from a capsule were washed and divided into two groups; one group was kept untreated in Instant Ocean while eggs in the other group were agitated in calcium-free sea water at first cleavage (trefoil stage) to remove the lobes. Agitation was either by hand or by means of a mechanical shaker. Lobeless and normal eggs were then washed and collected as described below for the larvae or they were reared at 20 °C in sea water containing penicillin and streptomycin (50 i.u. each/ml, Microbiological Associates, Bethesda, Maryland) until the controls were yolk-free veligers. The larvae (normal or lobeless) were then immobilized by cooling and the exact number required for later application to one gel (see Results) were placed in a small centrifuge tube, washed several times with ice-cold Instant Ocean and finally rinsed by allowing them to settle quickly through cold distilled water. All excess liquid was removed with a fine pipette and the material was stored at −55 °C. When it was necessary to pool eggs or larvae from several capsules, each capsule of eggs was made to contribute to experimental and control groups in the correct relative proportions.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

In order to minimize the amount of embryonic material required for disc electrophoresis, the polyacrylamide gels were prepared in capillary tubes approximately 1-7 mm × 75 mm. The washed tubes were coated with Siliclad (Clay-Adams, Inc.). They were filled to a height of 5 cm with a de-gassed, small-pore polyacrylamide gel solution, pH 8·9 (Davis, 1964). On top of this separation gel was layered a 2 cm photopolymerizing spacer gel, pH 6·7 (Davis, 1964). Chemicals for the polyacrylamide gels were purchased from Canal Industrial Corporation, Rockville, Md.
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The frozen samples were thawed to 0 °C, 5 µl of the Tris/glycine buffer pH 8.3 used for electrophoresis (Davis, 1964) were added and the material was homogenized at 0 °C with a rotating stainless steel wire loop driven by a dental drill. 2.5 µl of cold 2.2 M sucrose were added and the mixture was applied to the gel. Electrophoresis was carried out in an apparatus consisting of two plexiglas buffer tanks 18 cm long x 6 cm wide x 3.5 cm deep. The reservoir buffer was Tris/glycine pH 8.3 (Davis, 1964) containing a small amount of Bromphenol blue as a marker dye. The material was electrophoresed at room temperature using 0.1 mA of current per gel tube until the marker dye had migrated 3 cm (about 2 h). To remove the gel from the capillary tube, the tube was scored in the middle, immersed in ice-cold water, and, while submerged, was broken without breaking the gel, and the gel was pulled out.

Staining for alkaline phosphatase was accomplished with sodium alpha-naphthyl acid phosphate and 4-aminodiphenylamine diazonium sulfate in a Tris-maleate buffer containing magnesium sulfate (Taswell & Jeffers, 1963). Visualization of non-specific esterases was with 1% alpha-naphthyl acetate and 1% Blue RR salt (Allied Chemicals) in 0.2 M Tris/HCl, pH 7.19 (Johnson & Spinuzzi, 1966). The stained gels were read and photographed. They were then placed on glass slides bordered with tape, covered with 1.5% molten agar, allowed to dry, and removed from the glass. This procedure produced a clear, relatively flat preparation suitable for densitometer scanning and for storage.

RESULTS

In order to compare lobeless and normal embryos with respect to their abilities to develop certain enzyme activities, it was necessary to select for study those enzymes whose isozyme patterns showed well-marked changes during normal development. In cases where isozyme groups changed little from egg to larva, there would be difficulty in distinguishing persistence of a molecule from its turnover. This would complicate analysis of the isozyme patterns in the lobeless larva. Lactate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, alkaline phosphatase, and non-specific esterase were examined in normal eggs and larvae and the latter two enzymes were chosen for more intensive study because their isozyme patterns showed the most pronounced changes.

Fig. 1 shows the emergence of alkaline phosphatase activity during normal development. The egg and early embryo exhibit one faint band of activity. Beginning about day 7 this band increases in intensity and on day 8 another band appears. The final pattern in the veliger consists of two very intense bands of activity. For this study, the eggs from several capsules were pooled on day 1, the day the eggs were laid. These eggs were reared in one dish and each day a sample of the embryos was removed, washed, and frozen. The last sample was collected on day 10 when the veliger larvae were free of visible yolk. When the
Fig. 1. Alkaline phosphatase activity during development. An homogenate of 50 embryos was applied to each gel. 'Var.' means the band varied between the intensity shown and complete absence from the gel.
Fig. 2. Esterase activity during development. Each gel contained a homogenate of 50 embryos.
complete series had been collected, all samples were electrophoresed concurrently. This procedure was repeated three times on three different groups of pooled eggs with similar results.

An identical approach was used in studying esterase activity during normal development and the results are shown in Fig. 2. The egg exhibits only one band of activity. This condition persists until day 6 when the final larval pattern of five bands begins to be established.
Fig. 4. Esterase activity in normal and lobeless eggs (2-cell stage) and 10-day larvae. Comparisons were made on the basis of equal volumes of material (see Text). 'Var.' means the band varied between the intensity shown and complete absence from the gel.
In the foregoing experiments the sample applied to each gel contained 50 embryos so that comparisons in these cases were based on a number of embryos. It was found that 50–100 embryos/gel was optimal for producing readable patterns. The total protein contents of uncleaved eggs and of larvae were found to be comparable with perhaps a slight decline in total protein at the veliger stage. Fifty embryos contained approximately 13 μg protein. In comparing lobeless and normal embryos, three bases were used: (1) equal numbers, usually 100 per gel; (2) equal volume based on the lobe having 29% of the volume of the whole egg (Clement & Tyler, 1967), in which 100 lobeless embryos were compared with 71 normal embryos; (3) equal protein content based on the determination that the lobeless egg has approximately 50% of the protein content of the normal egg (100 lobeless embryos per gel versus 50 normal embryos). The latter two bases, which are the more conservative in demonstrating differences, are presented here.

Figs. 3 and 5 depict the patterns of alkaline phosphatase activity found in normal and lobeless eggs and 10-day larvae. At the time that the controls were good, yolk-free veligers, the lobeless larvae had the typical appearance as described by Clement (1952) and Atkinson (1968). Care was taken to use only typical lobeless larvae which were actively swimming. The pattern of alkaline phosphatase activity obtained from these larvae resembled most closely the pattern in the eggs. In twelve replications of this experiment, using twelve different lots of embryos, the only differences were minor ones of band intensities. Electrophoresis of a mixture of lobeless and normal larvae produced a pattern similar to that of the normal larvae, thereby excluding the possibility that an inhibitor was responsible for the pattern obtained from lobeless larvae.

Figs. 4 and 6 demonstrate the patterns of esterase activity in normal and lobeless eggs and larvae. In the lobeless larvae one band does not appear, one is severely reduced in intensity, one moderately reduced, while two appear unaffected. Differences between Figs. 2 and 5 in the patterns for esterase activity in normal larvae reflect both differences in the amount of sample applied to the gel and the variability of the biological material. In Fig. 6, the material in gel 4 did not migrate as far as that in gels 2 and 3 so that the band at 16 mm in gel 4 corresponds to the band at about 18 mm in gels 2 and 3. The eleven replications of this experiment confirmed this relationship.

**DISCUSSION**

The purpose of this work was to study the nature of the effect that lobe removal has on later synthetic events by studying the appearance of several molecular species during embryogenesis of the lobeless and normal *Ilyanassa* eggs. Preliminary electrophoresis of whole embryo protein indicated that total protein patterns were too complex to analyse confidently. Therefore, it was decided to study the electrophoretic patterns of specific enzymes and to make the working
Fig. 5. Alkaline phosphatase activity in normal and lobeless larvae. Actual gels: (1) blank (no embryos), (2) 71 normal veligers, (3) 50 normal veligers, (4) 100 lobeless larvae.

Fig. 6. Esterase activity in normal and lobeless larvae. Actual gels: (1) blank (no embryos), (2) 71 normal larvae, (3) 50 normal larvae (gel is broken near top), (4) 100 lobeless larvae. Band at 16 mm in gel 4 corresponds to band at 18 mm in gels 2 and 3.
assumption that appearance of a band of enzyme activity indicates formation of a new type of molecule, although other interpretations are possible.

Morrill & Norris (1966) used starch gel electrophoresis to study the appearance and disappearance of several enzymes during the normal development of *Ilyanassa*. Although their findings are not strictly comparable to the present work because of differences in technique, their study gave impetus to the present investigation. Development of procedures using greatly reduced gel size has made it practical to extend this type of work to experimentally modified material such as the lobeless embryos used here. Preliminary experiments indicated that the electrophoretic patterns produced in small gels are comparable to those obtained with standard size gels.

In normal development the appearance of larval patterns of esterase and alkaline phosphatase activities begins about days 6 and 7. At this time the larva has just assumed the veliger shape and is undergoing final differentiation of its larval features such as velum, eyes, shell, foot, heart, and digestive system. Further refinement of larval characteristics and of enzyme patterns continues until day 10, at which time the larva is a typical yolk-free veliger.

In lobeless embryos one band of alkaline phosphatase activity never appears. Although the technique does not make it possible to conclude that the molecule represented by this band is totally absent, there has at least been a drastic change as a result of removing the polar lobe. The other band of activity appears as it does in the egg and could indicate either a persistence of the enzyme from the egg or a low level of turnover not characteristic of the normal larva.

The pattern of esterase activity in lobeless embryos shows that two bands are greatly reduced, one band never develops and two bands are nearly identical to those in normal larvae.

Considering both sets of enzymes, alkaline phosphatases and esterases, the reduction in total protein synthesis observed in lobeless embryos (Collier, 1961) does not appear to be an indication of just a general effect on metabolism. Lobe removal severely affects the emergence of certain bands of enzyme activity, but has little or no effect on others. This is similar to the situation found on the morphological level. Certain larval structures, such as velum, eyes, foot, shell, intestine, and heart are lobe-dependent, while others, such as velar cilia, pigment, esophagus, and stomach, are not. At present it is not possible to relate these deficiencies in enzyme molecules to the missing larval structures. A knowledge of the bases of these isozyme patterns such as is available for lactate dehydrogenase isozymes in other species is needed, as is a histochemical localization of the enzymes in the larval tissues. For example, Cather (1967) has found alkaline phosphatase activity appearing in the shell gland during development.

*Ilyanassa* embryos, especially experimentally altered ones, are difficult to gather in large numbers and, as a result, have not received the attention that they merit on the basis of their interest to embryologists. For example, the
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phenomenon of cytoplasmic localization is well exemplified here in the influence of the polar lobe on development. The present study provides additional evidence for the specificity of this influence. The relatively small amount of material necessary for the present electrophoretic analyses should encourage further and more precise work along these lines.

This study was undertaken while the author was on the tenure of a postdoctoral fellowship from the Division of Child Health and Human Development, National Institutes of Health. The research was conducted in the laboratory of Dr E. M. Johnson, Anatomy Department, University of Florida, and was supported in part by a National Institutes of Health grant (HD-00109) to Dr Johnson. The author is grateful to Dr Gabriel Ceron for his valuable aid in developing the electrophoretic technique used here.

REFERENCES


(Manuscript received 21 December 1970)