Molecular Heterogeneity of Lactic Dehydrogenase During Development of the Snail *Argobuccinum oregonense* Redfield[1]

ERWIN GOLDBERG AND JAMES N. CATHER

Departments of Zoology, North Dakota State University, Fargo, University of Michigan, Ann Arbor and University of Washington Friday Harbor Laboratories

The demonstration that different proteins with similar enzyme activity exist in tissues and the observation that there is addition and loss of these proteins during differentiation, suggest the possibility of a new level of discrimination of molecular events associated with development. At present, we are at the stage of descriptive biochemistry in the developmental system. The recent studies of the intracellular and intercellular localization of these multiple molecular forms of enzymes (Nace et al., '60; J. M. Allen, '61) and the genetic control of their synthesis (S. L. Allen, '61), imply a functional significance requiring elucidation and correlation with developmental events.

The eggs of molluscs have been considered excellent experimental material particularly for the study of mosaic development since the work of Crampton (1896) stimulated E. B. Wilson ('04) to do his classical series on *Dentalium*. These works have been followed by those of Clement ('52, '56, '62) and have formed an excellent experimental basis on which to establish a biochemical analysis of development. Collier ('57a, '57b, '60, '61) has already initiated fruitful biochemical studies on these forms, as has Berg and his co-workers ('58, '59).

The present paper deals with the distribution and modifications of lactic dehydrogenases during the course of normal development in the prosobranch mesogastropod *Argobuccinum oregonese.*

MATERIALS AND METHODS

The eggs of *Argobuccinum* (*Fusitriton*) *oregonense* Redfield were investigated. Adult snails were collected intertidally or in depths to about 65 feet with SCUBA equipment, in the waters adjacent to San Juan Island, Washington. They were taken to the Friday Harbor Laboratories of the University of Washington where the studies were excuted.

As the snail lays her eggs she forms capsules around them. The capsules are deposited in a spiral cluster on aquaria walls over a number of days; the mother remains on the cluster during the developmental period. All stages can be obtained from a mature cluster of capsules with the oldest eggs at the center of the spiral and the youngest at the end. Approximately 4,000 eggs which develop synchronously are deposited in each capsule.

The capsule to be used was cut from the substrate, rolled around on a piece of paper towel to remove excess debris and then placed in a Syracuse watch glass containing pasteurized sea water (Costello et al., '57). The base of the capsule was cut off with small curved scissors and the eggs were removed by pressure from a pair of needles. As soon as the capsular jelly dissolved, the eggs were washed with pasteurized sea water and eggs which appeared to be damaged were removed. The eggs were then staged and a few were fixed and mounted (method of Clement and Cather, '57).

The main sample of the eggs was then washed twice with cold 0.05 M phosphate at pH 7.4 and ground in one milliliter of this buffer in a homogenizer tube, rotating the teflon pestle by hand for five minutes. The homogenate was centrifuged at 4°C

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for thirty minutes at 10,000 × g and the supernatant used for determining lactic dehydrogenase (LDH) activity. Thirty-two capsules representing 12 different developmental stages were studied.

Disc electrophoresis in a polyacrylamide gel, as described by Ornstein and Davis ('61) was used to separate the proteins contained in the extract. With this method about 100 μg of protein in a sample volume of 0.10 ml was resolved in 30 minutes at room temperature (approximately 22°C). Usually six gel columns, each conducting 5 ma, were simultaneously electrophoresed.

At the completion of the run, the gel columns were removed from the cylinders by rimming with a blunt dissecting needle under water. Some of the columns were then fixed and stained for protein by 0.2% Napthol blue black in a 5:5:1 solution of water, methanol and acetic acid. Destaining of the sample was accomplished electrophoretically with 7½% acetic acid. Unfixed gels were rinsed with cold Tris (hydroxymethyl) aminomethane buffer, 0.1 M, pH 8.3, and incubated at 37°C for from one to four hours in a reaction mixture comparable to that described by Appella and Markert ('62) and containing, in final concentration: 0.1 M Tris, 0.036 M L(+) lactic acid sodium, 0.3 mg/ml NAD (nicotine adenine dinucleotide), 0.8 mg/ml nitro blue tetrazolium, and 0.14 mg/ml phenazine methosulfate, all at pH 8.3. Sites of LDH activity on the gel were localized by the precipitation of the reduced tetrazolium salt (purple in color).

In order to compare samples electrophoresed at different times the relative mobility of each band of LDH activity was calculated arbitrarily, by comparing the distance it migrated with the distance migrated by bromphenol blue added as a tracking dye to the system to mark the electrophoretic front. In this system only positively moving ions were separated. It was possible to resolve negatively moving ions by utilizing a different gel and buffer system and reversing the electrodes (Reisfeld et al., '62).

RESULTS

Selected developmental stages of the snail are presented in figures 1–6. Multiple forms of LDH are detectable in all stages studied. In uncleaved eggs, and in cleavage through the mesentoblast stage, five electrophoretically distinct proteins show enzyme activity. There are present two slowly migrating forms of the enzyme moving toward the anode, and a single site of activity corresponding to a negatively moving protein. The anodal components are depicted in figure 7 and the cathodal form in figure 8. The stages studied which show this pattern include uncleaved eggs, four cell, first quartet, third quartet, and post mesentoblast formation, stages. The cathodal component is present in all stages studied.

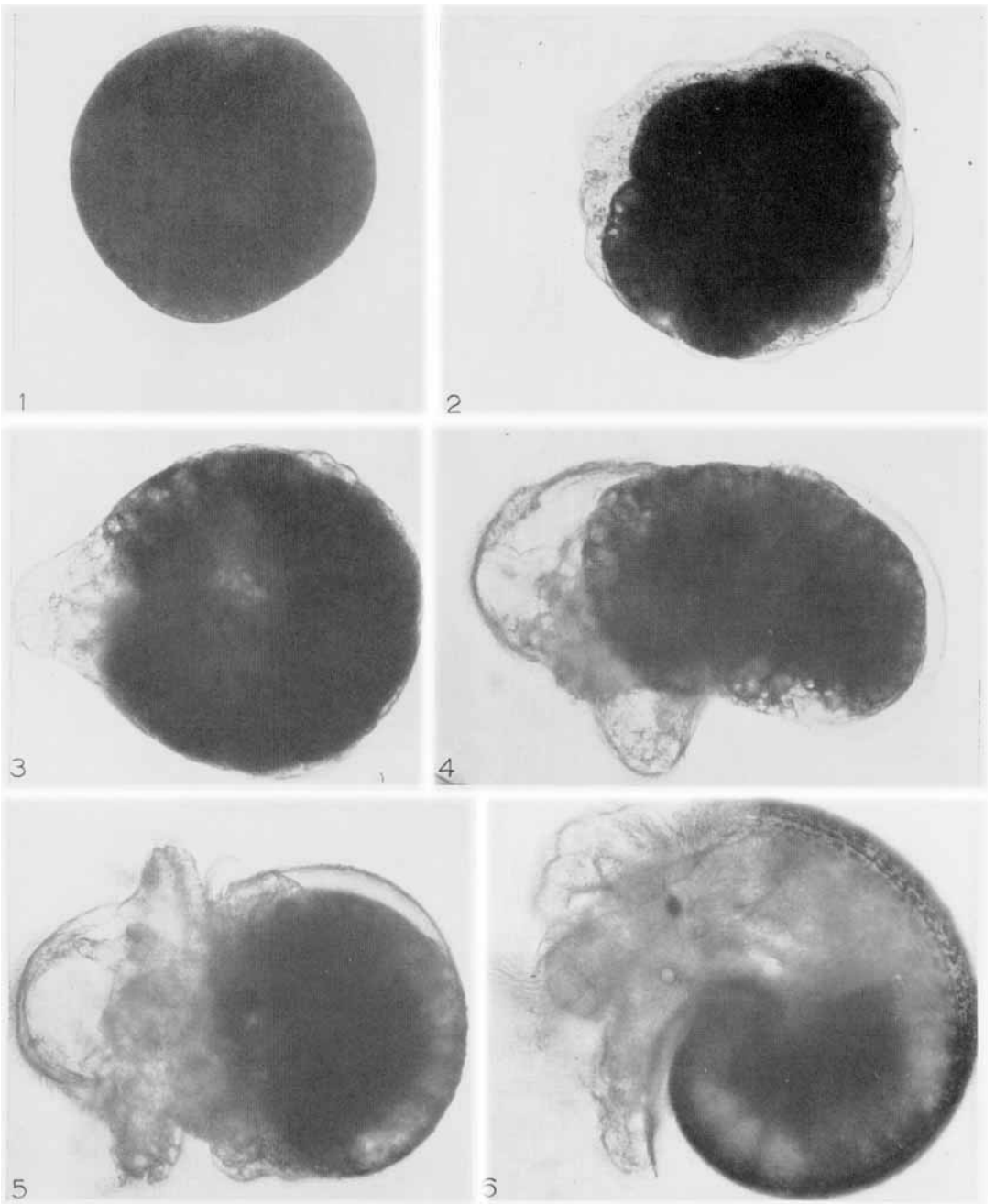
The two most rapidly moving LDH's are lost as blastulation proceeds. This was verified by running nine experiments on blastula stages obtained from different capsules. Therefore, in the blastula, early and late gastrula and head vesicle stages, three sites of LDH activity were detectable on the gel column, namely, the two anodal forms (fig. 9) and the cathodal component.

In the early veliger stage as the foot and shell become well formed a new LDH appears (fig. 10), and the three previously present persist. As development of the veliger progresses to the mid-veliger stage with the differentiation of the endoderm, a second new enzyme moiety becomes apparent (fig. 11), so that there are now present five different molecular forms of the enzyme, including the negative moving one in the larva. This pattern is retained in the late veliger stage when larval differentiation has been completed. Figure 12 summarizes these data, diagrammatically.

The change in LDH distribution is paralleled by the protein pattern as shown by representative samples depicted in figure 13. It is apparent that the extracts contain a number of proteins which do not show LDH activity. The protein staining technique was not sufficiently sensitive to show the negatively moving component. When lactate was omitted from the reaction mixture no reduction of the nitro blue tetrazolium occurred.

DISCUSSION

Molecular heterogeneity of enzymes has become a well documented phenomenon in



Figures 1–6, × 360.

Fig. 1 Uncleaved egg.
Fig. 2 Gastrula.
Fig. 3 Head vesicle formation.
Fig. 4 Early veliger.
Fig. 5 Middle veliger.
Fig. 6 Late veliger.

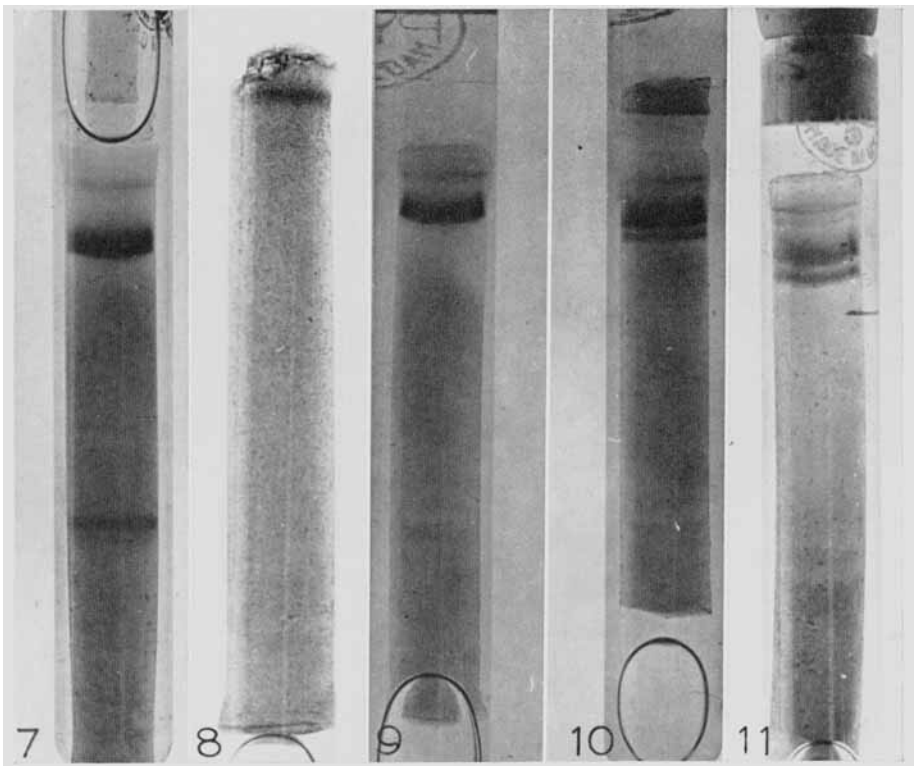


Fig. 7-11 Representative polyacrylamide gel columns stained for lactic dehydrogenase activity, after electrophoresis. The origin is at the top of each gel column in the photograph.

Fig. 7 Extract from uncleaved eggs or early cleavage stages.
Fig. 8 Typical pattern showing negative moving component in all stages.
Fig. 9 Extract from embryos in blastula through head vesicle stage.
Fig. 10 Extract from early veliger stage.
Fig. 11 Extract from mid-veliger and late veliger stages.

recent years (cf. Ann. N. Y. Acad. Sci., '61). Previous studies, notably those by Markert and Møller ('59), Cahn et al. ('62), and Flexner et al. ('60), demonstrated changes in the number and distribution of LDH's associated with tissue differentiation. The latter authors reported that maturation of liver was accompanied by a reduction from four to two, in the number of components of LDH in both rat and guinea pig. Guinea pig cerebral cortex contained four major bands of activity in the adult, two of which were only weakly represented in the newborn animal.

Markert and Møller ('59), who proposed the term *isozyme* for different molecular forms of protein with the same enzymatic specificity, have demonstrated that in swine the embryonic heart contains more LDH isozymes than adult heart, whereas embryonic skeletal muscle contains fewer isozymes than adult muscle. These authors suggest that the final adult pattern is reached by both gains and losses in the isozymic repertory of embryonic tissues and that the pattern in a tissue reflects the state of differentiation of its cells.

Appella and Markert ('61) and Cahn, Kaplan, Levine and Zwilling ('62) separated the LDH's from a given tissue and reported that each form of the enzyme is made up of four very similar polypeptide chains. The polypeptide sub-units can be separated into two classes on the basis of charge. The latter authors propose that the enzyme obtained from heart muscle is a tetramer composed of four identical

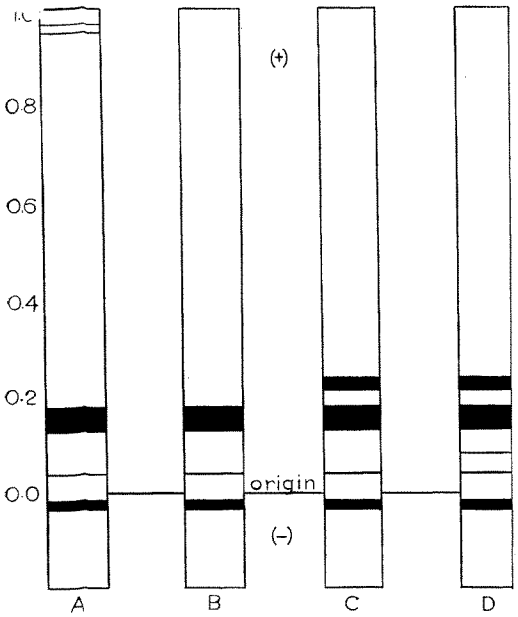


Fig. 12 Diagrammatic representation of the size and location of bands indicating LDH activity, from different developmental stages, as seen on the gel columns. Patterns seen in:
A. Uncleaved through post-mesentoblast formation, stages.
B. Blastula through head vesicle stages.
C. Early veliger.
D. Middle and late veliger.
Numbers refer to relative mobility (see text for explanation).

sub-units, and that from skeletal muscle is also a tetramer of four different but identical sub-units. Therefore, if the two classes of sub-units are present within the same cell, upon re-combination into all possible groups of four they could yield five electrophoretically distinct types of LDH. Evidence is cited by Cahn et al. ('62) which suggests that the synthesis of the two types of sub-units is controlled by two separate genes.

If we accept this hypothesis we can attempt to explain our results as follows: by designating the two classes of polypeptide sub-units as x and y and assuming that they differ in net charge, then in the veliger, we can account for five different molecular species (xxxx, as the negative moving component; (xxxy, xxyy, xyyy; and yyyy as the most positive moving component). On this basis, then, xxxx, xxxy, and xyyy would have to represent the species present from the time of blastulation to veliger formation. The type yyyy appears in the early veliger, and xxyy represents the fifth LDH moiety to show up as differentiation proceeds. The bands of activity on the gel appear to be spaced at approximately equal intervals, as this hypothesis predicts (Cahn et al., '62). If these suppositions are accepted it is necessary to suggest that the two classes of sub-units must be present during the entire developmental period and that their combination to form tetramers within the cell does not occur randomly but rather in some organized fashion. It should be noted that the sub-units themselves do not possess enzyme activity (Appella and Markert, '61). Since evidence

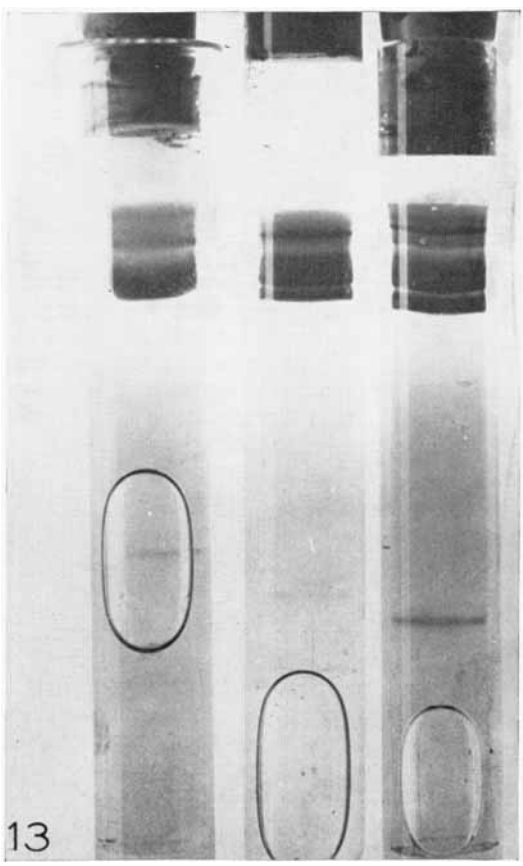


Fig. 13 Polyacrylamide gels stained for protein. The gel on the left of the photograph shows a pattern typical for all stages of development up to the veliger. In the center gel is the protein pattern of the early veliger, and on the right is that of the late veliger stage.

indicates that cytoplasmic RNA is responsible for protein synthesis, then it is possible that each form of the enzyme requires a specific RNA for its synthesis and on the basis of our results we suggest that the appropriate RNA for the synthesis of the various LDH's appears at different stages of development. Collier ('61) has reported that an increase in RNA synthesis in *Ilyanassa* occurs on the fourth day of development, which would be just slightly earlier or correspond to the stage at which we get the addition of our first new LDH. We can speculate that the precursors (in this case as polypeptide sub-units) for synthesis of new protein are available in the developing embryo to be utilized when the synthetic mechanisms are differentiated.

We must still account for the two bands of LDH which disappear just prior to blastulation. It may be that they represent artifacts of analysis since the egg is relatively fragile and seems much easier to homogenize than the later embryos, and therefore mechanical fragmentation of the enzymes may occur. This seems very unlikely and we are more of the opinion that they are a contribution from gonadal tissue to the egg, perhaps representing a combination of two or three of the subunits possessing enzyme activity. Considering this, we could have xxy and xyy for example, which would have net charge differences and also being much smaller molecules would migrate more rapidly than the tetramers in the electrophoresing system. This remains to be established.

It has been suggested previously (Cahn, '62; Kaplan, '61; Markert and Appella, '61), and we concur, that molecular heterogeneity of LDH is of functional significance. J. M. Allen ('61), in a cytochemical investigation, finds that LDH isozymes are distributed in characteristic locations within the cell and suggests that they may respond differentially to changes in physiological environment. This author states that these differential responses have important significance in terms of cellular function, reflecting the action of microenvironmental influences upon enzymes situated in different regions of the cell or, perhaps, in different cells. Certainly such microenvironmental influences are numerous in the dynamic state of protoplasmic constituents during embryogenesis, and the multiple forms of LDH which we observe in *Argobuccinum* may respond to such changes, differentially. Nace et al. ('60) have been able to localize serologically distinct lactic dehydrogenases in different regions of the frog oocyte. Kaplan and Ciotti ('61), Kaplan ('61) and Cahn ('62) have observed differences in the catalytic properties of the LDH's which they suggest are important in regulating the physiological activities of the tissues in which the enzymes are found (Cahn et al., '62).

Further experimentation is in progress to characterize the metabolic processes of *Argobuccinum* in order to elucidate the possible role of the multiple LDH's during development.

SUMMARY

Molecular heterogeneity of lactic dehydrogenase has been studied during development of *Argobuccinum*. Five electrophoretically distinct molecules possessing LDH activity were present in the uncleaved egg and during the cleavage stages. The two most rapidly moving anodal components disappeared as blastulation commenced. A new LDH appeared in the early veliger stage followed by a second new form of the enzyme as differentiation of the larva progressed. In the completely differentiated veliger five LDH moieties, spaced at approximately equal intervals on the gel used for electrophoresis, were detectable. It is suggested that LDH's which disappear are a contribution of gonadal tissue to the oocyte, and that the appearance of the new LDH's is associated with differentiation of appropriate synthetic mechanisms, possibly with the requirement of a specific RNA for each form of the enzyme.

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LITERATURE CITED

Allen, J. M. 1961 Multiple forms of lactic dehydrogenase in tissues of the mouse: their specificity, cellular localization, and response to altered physiological conditions. Ann. N. Y. Acad. Sci., *94:* 937–951.

Allen, S. L. 1961 Genetic control of esterases in the protozoan *Tetrahymena pyriformis*. Ibid., *94:* 753–773.

Berg, W. E., and D. M. Prescott 1958 Physiological studies of differentiation in *Mytilus edulis*. II. Accumulation of phosphate in isolated blastomeres and polar lobes. Exp. Cell Res., *14:* 402–407.

Berg, W. E., and Y. Kato 1959 Localization of polynucleotides in the egg of *Ilyanassa*. Acta embryol. morphol. exp., *2:* 227–233.

Appella, E., and C. L. Markert 1961 Dissociation of lactate dehydrogenase into sub-units with guanidine hydrochloride. Biochem. Biophys. Res. Com., *6:* 171–176.

——— 1962 As cited by E. S. Vesell, and A. G. Bearn. Variations in the lactic dehydrogenase of vertebrate erythrocytes. J. Gen. Physiol., *3:* 553–565.

Cahn, R. D. 1962 Ph.D. dissertation, Brandeis University.

Cahn, R. D., N. O. Kaplan, L. Levine and E. Zwilling 1962 Nature and development of lactic dehydrogenases. Science, *136:* 962–969.

Clement, A. C. 1952 Experimental studies on germinal localization in *Ilyanassa*. I. The role of the polar lobe in determination of the cleavage pattern and its influence in later development. J. Exp. Zool., *121:* 593–626.

——— 1956 Experimental studies on germinal localization in *Ilyanassa*. II. The development of isolated blastomeres. Ibid., *132:* 427–446.

——— 1962 Development of *Ilyanassa* following removal of the D macromere at successive cleavage stages. Ibid., *149:* 193–215.

Clement, A. C., and J. N. Cather 1957 A technic for preparing whole mounts of veliger larvae. Biol. Bull., *113:* 340.

Collier, J. R. 1957a A study of alanyglycine dipeptidase activity during the development of *Ilynassa obsoleta*. Embryologia, *3:* 243–260.

——— 1957b The proteolytic activity of the gastropod egg. Exp. Cell Res., *13:* 122–124.

——— 1960 The localization of some phosphorus compounds in the egg of *Ilyanassa obsoleta*. Ibid., *21:* 548–555.

——— 1961 Nucleic acid and protein metabolism of the *Ilyanassa* embryo. Ibid., *24:* 320–326.

Costello, D. P., M. E. Davidson, A. Eggers, M. H. Fox and C. Henley 1957 Methods for obtaining and handling marine eggs and embryos. Marine Biological Laboratory, Woods Hole.

Crampton, H. E. 1896 Experimental studies on gasteropod development. Arch. Entw.-mech., *3:* 1–19.

Flexner, L. B., J. B. Flexner, R. B. Roberts and G. de la Haba 1960 Lactic dehydrogenases of the developing cerebral cortex and liver of the mouse and guinea pig. Dev. Biol., *2:* 313–328.

Kaplan, N. O. 1961 Mechanism of action of steroid hormones. Pergamon, Oxford.

Kaplan, N. O., and M. M. Ciotti 1961 Evolution and differentiation of dehydrogenases. Ann. N. Y. Acad. Sci., *94:* 701–722.

Markert, C. L., and E. Appella 1961 Physicochemical nature of isozymes. Ibid., *94:* 678–690.

Markert, C. L., and F. Møller 1959 Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. Proc. Nat. Acad. Sci., *45:* 753–763.

Nace, G., T. Suyama and N. Smith 1960 Early development of special proteins. Sym. Germ Cells Devel. Institut. Intern. d'Embryologie and Fondazione, A. Baselli. 564–603.

Ornstein, L., and B. J. Davis 1961 "Disc" electrophoresis. Distillation Products Industries Preprint.

Reisfeld, R., U. J. Lewis and D. E. Williams 1962 Disc electrophoresis of basic proteins on polyacrylamide gels. Abstr. Am. Chem. Soc. Mtgs. 64c.

Wilson, E. B. 1904 Experimental studies on germinal localization. I. The germ regions in the egg of *Dentalium*. J. Exp. Zool., *1:* 1–72.