Malic Dehydrogenase Isozymes: Distribution in Developing Nucleate and Anucleate Halves of Sea Urchin Eggs

Abstract. *Nucleate and anucleate halves of sea urchin eggs have seven and five forms of* L-*malic dehydrogenase, respectively. Fertilization results in reduction of the number of enzyme forms in both halves. The normal complement of seven isozymes in unfertilized eggs appears to be a synthesis of five soluble and four particulate forms. Reduction in the number of isozymes after fertilization takes place principally in soluble forms and appears to be under cytoplasmic control.*

Changes in the number and amount of the multiple molecular forms of lactic dehydrogenase, malic dehydrogenase, and catalase occur during late stages of development (*1*); however, few of these isozymes have been studied during early cleavage stages. Fertilization of the sea urchin egg is followed by reduction of the five to seven molecular forms of L-malic dehydrogenase (MDH) to three by the 64-cell stage (*2*). An intercellular gradient of MDH isozymes was demonstrable at this stage, there being three in small blastomeres and two in the large blastomeres (*3*). The reduction in number of molecular forms of MDH may be unrelated to new protein synthesis, since this reduction is unaffected by treatment of the embryo with actinomycin D or puromycin (*4*); however, other studies have shown that actinomycin D (*5*) and puromycin (*6*) may not inhibit protein synthesis immediately following fertilization.

These studies of changes in MDH isozymes during echinoderm development may be related to physicochemical studies of the mammalian enzyme, which have demonstrated kinetic (*7*), electrophoretic (*8*), and chromatographic (*9*) differences between soluble and mitochondrial forms of MDH. It may now be possible to relate more closely the malate dehydrogenases from mammals and echinoderms, for although it had been widely held that only one form of MDH is present in mammalian mitochondria, multiple isozymic forms of mammalian mitochondrial MDH have been demonstrated (*10, 11*). The electrophoretic mobility and enzymatic activity of these isozymes were altered by iodination (*11*).

The factors responsible for initiating changes in cellular respiration and protein synthesis soon after fertilization are unknown, although their cytoplasmic location seems clear. The isozymes of MDH undergo alteration in this period after fertilization, and the process appears to be influenced principally by cytoplasmic factors since it occurs in fertilized anucleate halves of eggs. Both soluble and particulate MDH occur in several active forms in the sea urchin, and we have compared the patterns in these fractions with the patterns in whole eggs before and after fertilization.

Eggs, sperm, and embryos from *Arbacia punctulata* were collected (*2*) and homogenized in 0.025*M* barbital buffer or in filtered sea water (*12*). The buffered homogenate was centrifuged at 10,000*g* for 30 minutes, and the supernatant fraction was retained. The homogenate in sea water was centrifuged at 22,000*g* for 30 minutes, and a soluble and particulate fraction were thus obtained. The pellet was suspended in 0.025*M* barbital buffer and was homogenized. Nucleate (light) and anucleate (heavy) fractions of unfertilized eggs were prepared by centrifugation (*13*) (Fig. 1). These components were fertilized with normal sperm and permitted to develop in bowls of sea water for 90 minutes. They were then collected by gentle centrifugation and homogenized in 0.025*M* barbital buffer. Only bowls in which at least 95 percent of the eggs were fertilized were retained.

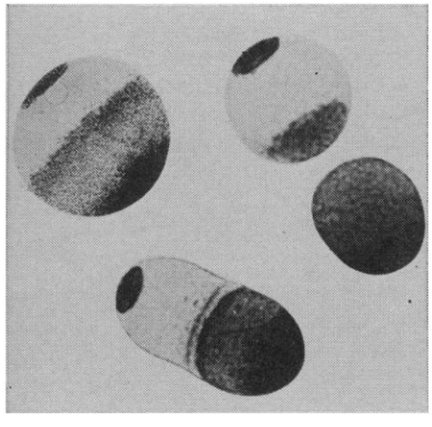

Fig. 1. Photomicrograph of the stages leading to separation of the unfertilized sea urchin egg into nucleate and anucleate halves. The striated egg in the upper left position has the following structures from top to bottom: oil cap, refractive nucleus in the clear area, mitochondrial layer, yolk, and pigment layers. The lower, oblong egg is shown shortly before splitting into nucleate (light) and anucleate halves shown in the upper right corner.

The resulting preparations were assayed spectrophotometrically with a Beckman DB recording spectrophotometer for malic dehydrogenase activity with nicotinamide-adenine dinucleotide (NAD) as coenzyme or with its analog acetylpyridine-adenine dinucleotide (APAD). We used a modification of a method (*4*) in which 0.265*M* tris(hydroxymethyl)aminomethane buffer, *p*H 9.3, is used and KCN is omitted. Isozymic forms of MDH were identified by their electrophoresis (disk) on polyacrylamide gel (*12*). Enzyme activity was assayed immediately after centrifugation, and equivalent amounts of enzyme were placed on all gels being compared. All samples were prepared and assayed the same day. Electrophoresis of nucleated portions of unfertilized eggs results in the separation of seven bands of MDH activity comparable to the seven bands produced by electrophoresis of unfertilized whole eggs (Fig. 2A). Electrophoresis of the more dense anucleate fractions results in five bands; bands one and six are missing (Fig. 2A).

Variability in the staining inten-

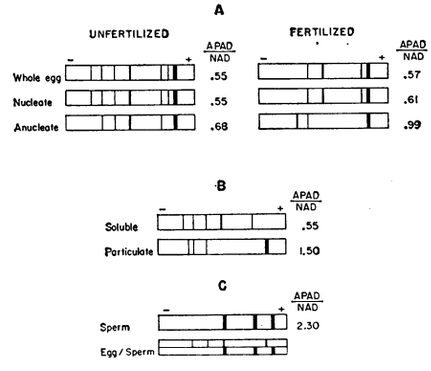

Fig. 2. Pattern of isozymes of L-malic dehydrogenase. (A) Isozyme patterns in unfertilized and fertilized sea urchin eggs; (B) patterns in particulate and nonparticulate fractions from unfertilized eggs; (C) comparison of isozyme patterns in the unfertilized egg and sperm. The ratio APAD/NAD refers to the ratio of L-MDH activity in millimicromoles of reduced acetylpyridine-adenine dinucleotide (APAD) produced per minute to that in millimicromoles of reduced nicotinamide-adenine dinucleotide (NAD) produced per minute assayed as mentioned above.

sity of the first bands that move toward the cathode was noted in both cell fractions. The ratio of MDH activity with APAD to that with NAD (the analog ratio) was 0.55 in the nucleate fraction and 0.68 in the anucleate sample. Malic dehydrogenase activity averaged 47.3 $\times$ 10^4 mμmole of NADH produced per minute per milligram of protein in the nucleate and 11.0 $\times$ 10^4 mμmole per minute per milligram of protein in the anucleate fraction. Approximately two-thirds of the total MDH activity was present in the nucleate portion; one-third was found in the anucleate half.

Fertilized nucleate halves allowed to develop for 90 minutes at room temperature showed a reduction in the number of MDH isozymes from seven to four (Fig. 2A). Comparable changes in whole eggs have been observed (*2*, *12*). The number of MDH isozymes in fertilized anucleate fractions incubated for an equivalent period was reduced from five to three. Staining of the band (No. 2) closest to the cathode was increased. The ratio of MDH activity with APAD to that with NAD was 0.61 in fertilized nucleate halves and 0.99 in the fertilized anucleate samples. These changes in the isozyme pattern and in the analog ratio are reminiscent of the observations of an intercellular gradient in MDH isozymes at the 64-cell stage (*3*).

Electrophoresis of the soluble fraction obtained by centrifugation of the homogenate in sea water at 22,000*g* revealed five bands of MDH activity corresponding to bands one through five derived from the supernatant fraction of whole eggs homogenized in barbital buffer and centrifuged at 10,000*g*. The analog ratio of the soluble MDH was 0.55. The particulate fraction demonstrated the very dark anodal band (No. 7) which was absent in the soluble preparations, and three slow-moving bands close in position to, but not identical with, bands Nos. 2 and 3 were revealed. The analog ratio of the particulate fraction was 1.50 (Fig. 2B). An interesting variation in the particulate band, No. 7, was found when eggs from individual *Arbacia* were examined separately. Approximately one-third of the urchins produced eggs that demonstrated two equally stained bands; two-thirds of the urchins produced eggs with the isozyme of the single deeply stained band described above.

Three forms of MDH were found in *Arbacia* sperm (Fig. 2C) homogenized in barbital buffer. Intensely stained bands corresponding to Nos. 4, 5, and perhaps 7 of the unfertilized egg were produced by sperm samples; this finding indicates that both soluble and particulate forms are present. Tubes of acrylamide gel were prepared with a sliver of glass cover slip placed longitudinally so as to divide the upper gel into two equal portions. Samples of homogenized whole eggs were applied to one side, and samples of homogenized sperm were introduced on the other. The isozyme patterns were compared after electrophoresis of the two in parallel in the same separating gel. The sperm band closest to the cathode and band No. 4 from the egg sample appeared to be identical, since they formed a single continuous band across both halves of the gel; however, the sperm band closest to the anode did not fuse with band No. 7 of the egg sample (Fig. 2C). Malic dehydrogenase activity in these sperm samples averaged 83.0 $\times$ 10^4 mμmole of NADH per minute per milligram of proteins, and the analog ratio was 2.30.

The reduction in molecular forms of MDH within 90 minutes after fertilization in nucleate and anucleate fractions may be related to other cellular changes in protein synthesis and respiration which occur before the first cell division in sea urchin embryos. Reduction in the number of these enzymes in the anucleate fraction appears to exclude nuclear control of these changes and may implicate a stable cytoplasmic factor, such as the cortical granules, unaffected by centrifugation. Although the reduction appears primarily in the soluble forms of MDH, mitochondria are present in both halves (*14*), and the DNA content (*15*) and protein synthetic capacity of these organelles (*16*) necessitates consideration of their influence on changes in the cellular environment. The possibility of genetic variability in the major particulate form of MDH may provide a tool for evaluation of mitochondrial influences on these proteins. However, Roodyn (*17*) was unable to detect synthesis of MDH and other respiratory enzymes in isolated mitochondria that did form structural protein. Study of the sperm isozymes after fertilization, especially of the particulate forms, would be of interest since the fate of sperm mitochondria in the fertilized egg is unknown.

Finally, the large number of different molecular forms of MDH in egg and sperm samples and the intracellular distribution into soluble and particulate forms appear in conflict with the tetramer hypothesis suggested by Moore and Villee (*3*) to explain the initial observation of five forms of MDH in a structural model similar to that proposed for lactate dehydrogenase (*18*). Conformational changes in MDH resulting from the marked alteration in cellular ionic concentration that occurs following fertilization have not been excluded, and recent work by Kitto *et al.* (*11*) and the demonstration of identical subunits by Fondy (*19*) may facilitate a more thorough understanding of the structure of MDH and of the factors responsible for alteration of its isozymic forms.

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20. These experiments were conducted at the Marine Biological Laboratory, Woods Hole, Massachusetts.

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