Reports

Malic Dehyrogenases in Sea Urchin Eggs

Abstract. Unfertilized *Arbacia* eggs have five electrophoretically distinct malic dehydrogenases which differ in their rates with pyridine nucleotide analogs. Both the number of peaks and their relative size change during early embryonic development. A D-malic dehydrogenase which utilizes the 3-acetylpyridine analog of DPN but not DPN itself was found. Electrophoresis on starch gel results in five peaks, all of which differ from the peaks of the L-malic dehydrogenases.

Multiple molecular forms of many types of enzymes have been demonstrated (*1, 2*). Even a single tissue may contain several enzymes which catalyze the same reaction but have different physical, chemical, and kinetic properties. The relationship of this phenomenon to the one gene–one enzyme theory and to the problem of cellular differentiation poses important biologic questions which remain to be solved.

Physicochemical studies of lactic dehydrogenases from beef heart revealed that the proteins are remarkably similar in primary structure and in antigenic properties but do show differences, particularly in charge (*3*). Cahn *et al.* suggested that the five lactic dehydrogenases usually found in vertebrate tissues consist of tetramers of two kinds of polypeptide chains and demonstrated antigenic differences between "heart" and "muscle" polypeptides (*4*). Changes which occur during late embryonic development in the amounts of the multiple forms of enzymes have been demonstrated for lactic dehydrogenase (*5*) and malic dehydrogenase (*6*). Each adult tissue includes several cell types, and the finding of multiple forms of an enzyme in an adult tissue leaves unanswered the question of whether some forms might be present in only one of the several cell types. To avoid this problem, unfertilized eggs, which consist of a single cell type, were used.

Eggs from *Arbacia punctulata* were collected by mild electric shock. They were washed, diluted with filtered sea water, fertilized, and incubated at 20°C. Each bowl of eggs was examined microscopically and only those in which 95 percent or more of the eggs had been fertilized were used. Embryos were collected by brief centrifugation, homogenized in a glass homogenizer in two volumes of 0.025*M* barbital buffer, *p*H 8.7, and centrifuged at 10,000*g* for 20 minutes. The supernatant fraction was mixed to a thick paste with starch granules and inserted in a 1- by 15-cm slit 5 cm from the cathode in a 21- by 33- by 0.3-cm starch gel electrophoresis block. The starch gel block was made by pouring heated hydrolyzed starch (12 g/100 ml of 0.025*M* barbital buffer, *p*H 8.7) of the desired consistency into the electrophoretic tray (*7*). Proteins were separated by electrophoresis for 10 hours at 200 v and 45 ma in 0.025*M* barbital buffer at *p*H 8.7. After electrophoresis, 1-cm sections were cut, eluted with artificial sea water, and centrifuged; the supernatant fraction was retained for assay. Malic dehydrogenase activity was measured in glycylglycine buffer at *p*H 7.4 with a Beckman (model DB) spectrophotometer and a recording potentiometer. Enzyme activity, calculated from the initial linear portion of the curve, was expressed as millimicromoles (mμmole) of reduced pyridine nucleotide.

The supernatant fraction of unfertilized *Arbacia* eggs contains five electrophoretically separable peaks of diphosphopyridine nucleotide (DPN) L-malic dehydrogenase, numbered I to V in order of migration toward the anode. The major peak of DPN L-malic dehydrogenase (peak II), which represents approximately 60 percent of the total activity, shows migration to about 6 cm from the origin. The position and number of these bands were confirmed in other experiments in which the L-malic dehydrogenase activity present in the starch gel block was demonstrated by incubation with nitroblue tetrazolium and phenazine methosulfate in the presence of sodium L-malate (*1*).

When all of the 1-cm fractions from the starch gel were tested for L-malic dehydrogenase activity with pyridine nucleotide analogs, six or seven peaks of activity were found with the 3-acetylpyridine analog of DPN (APDPN) but only three peaks were found with the thionicotinamide analog of DPN (TNDPN). Although peak II with DPN regularly coincided with the major peak of L-malic dehydrogenase activity with APDPN and TNDPN the other peaks did not coincide. The three TNDPN peaks coincide with three of the APDPN peaks but not with the DPN peaks. Ratios of dehydrogenase activity with the analogs obtained from unfractionated extracts do not characterize any of these peaks, not even the major one, peak II, which does have activity with DPN and the two analogs.

Arbacia eggs were found to contain D-malic dehydrogenases which catalyze the dehydrogenation of D-malic acid in the presence of APDPN but which have no activity when DPN is used as the hydrogen acceptor. Electrophoretic separation of the supernatant fraction from the homogenate of unfertilized *Arbacia* eggs, followed by assay of each 1-cm fraction for malic dehydrogenase activity with both D- and L-malate, revealed the presence of five D-malic dehydrogenases having activity with APDPN but none having activity with DPN itself. None of the five D-malic dehydrogenases migrated at the same rate as any of the DPN or APDPN L-malic dehydrogenases. Thus, in these cells there are more than 10 molecularly distinct proteins with malic dehydrogenase activity.

When *Arbacia* embryos are maintained at 20°C the blastula hatches at about 6 hours, the gastrula is formed by 24 hours, and the pluteus stage is reached by 48 hours. Embryos harvested after 6, 12, 24, and 48 hours of incubation were homogenized, and the supernatant fraction was subjected

Instructions for preparing reports. Begin the report with an abstract of from 45 to 55 words. The abstract should *not* repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper.

Type manuscripts double-spaced and submit one ribbon copy and two carbon copies.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each. Submit three copies of illustrative material.

For further details see "Suggestions to contributors" [*Science* **125**, 16 (1957)].

to electrophoresis. Six-hour embryos show only three peaks for DPN L-malic dehydrogenase, I, II, and IV. After 12 hours of incubation, peak V appears, and these four electrophoretically distinct DPN malic dehydrogenases persist until the embryo is 48 hours of age. Band three does not appear in embryo extracts. After the 48-hour stage of development peaks I and II are less distinctive; peak II is spread over more fractions and overshadows peak I. Peak V, usually present in a single 1-cm fraction, occupies three such fractions in the 48-hour embryos.

At each age there are six or seven L-malic dehydrogenase peaks for APDPN and three for TNDPN, with no marked changes as development proceeds. The ratio of APDPN/DPN malic dehydrogenase activity is 0.68 in the supernatant fraction from unfertilized eggs before electrophoresis, 0.56 after the first 12 hours of development, and 2.2 by 48 hours of development. This change with embryonic age is not due to a change in the properties of the protein with the major L-malic dehydrogenase activity, peak II, for the APDPN/DPN ratio of this peak remains relatively constant throughout the period studied. The malic dehydrogenases of the sea urchin differ markedly from those of vertebrate tissues—chick, rat, and man—in which only two electrophoretically distinct proteins have been observed (*6*, *8*).

RICHARD O. MOORE
CLAUDE A. VILLEE
Marine Biological Laboratory, Woods Hole, Massachusetts, and Department of Agricultural Biochemistry, Ohio State University, Columbus, and *Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*

References and Notes

1. C. Markert and F. Møller, *Proc. Natl. Acad. Sci. U.S.* **45**, 753 (1959).
2. N. O. Kaplan, M. M. Ciotti, M. Hamolsky, R. E. Bieber, *Science* **131**, 392 (1960); C. A. Villee, *Biol. Bull.* **119**, 298 (1960); R. O. Moore and C. A. Villee, *ibid.* **121**, 398 (1961); F. Wroblewski, Ed., "Multiple Molecular Forms of Enzymes," *Ann. N.Y. Acad. Sci.* **94**, 655 (1961).
3. C. L. Markert and E. Appella, *Ann. N.Y. Acad. Sci.* **94**, 678 (1961).
4. R. D. Cahn, N. O. Kaplan, L. Levine, E. Zwilling, *Science* **136**, 962 (1962).
5. L. B. Flexner, J. B. Flexner, R. B. Roberts, G. De La Haba, *Develop. Biol.* **2**, 313 (1960).
6. B. Wiggert and C. A. Villee, *Science*, this issue.
7. O. Smithies, *Biochem. J.* **61**, 629 (1955).
8. This research was aided by grants A-4241 and C-2400, from the National Institutes of Health, and grants from the Charles and Marjorie King Fund and the Association for the Aid of Crippled Children.

20 September 1962

Lactic and Malic Dehydrogenases in Human Fetal Tissues

Abstract. The nonparticulate fraction of homogenates of heart, liver, kidney, lung, and cerebral cortex from human fetuses of varying gestational ages was subjected to electrophoresis on starch grains. Five lactic dehydrogenases and two malic dehydrogenases with characteristic rates with pyridine nucleotide analogs were found in all tissues at all ages. Quantitative differences in the relative proportions of these peaks were observed as gestation proceeded.

Multiple molecular forms of lactic dehydrogenase have been observed in human serum (*1*) and adult human heart and liver (*2*). Analyses of several adult human tissues revealed a total of five electrophoretically separable lactic dehydrogenases. Not every tissue had all five of these forms (*3*). Changes in the relative proportions of the molecular forms of lactic dehydrogenase during development have been observed in rat tissues (*4*) and guinea pig tissues (*5*).

Human fetal tissues were obtained at the time of operation during interruptions of pregnancy and were immediately frozen in dry ice and stored at −10°C until analyzed (*6*). In preparation for starch grain electrophoresis, purified potato starch was washed according to the method of Bloemendal (*7*). A starch block, 33 by 7 by 1 cm, provided with flannel wicks to connect with the electrode vessels was prepared, and both block and wicks were wrapped in thin plastic. A starch segment 1 cm wide was cut out of the block 11 cm from the cathode end. A one to one homogenate of 2 to 4 grams of fetal tissue was made in a glass homogenizer with barbital buffer (*p*H 8.6, ionic strength 0.1) that was also used for electrophoresis. The homogenate was centrifuged at 10,000*g* for 1 hour, and the clear supernatant fraction was mixed with dry, washed starch. The resulting paste was placed in the 1-cm slit in the block. Horizontal electrophoresis was continued for 36 hours at 250 v and 30 ma. Platinum electrodes were used; the electrode vessels were divided into two compartments with filter paper wicks to provide fluid bridges. During electrophoresis the starch block was cooled by circulating water at 1°C. After electrophoresis 1-cm fractions were cut and eluted with 0.15*M* NaCl. Lactic and malic dehydrogenase activities were measured in glycine-NaOH buffer at *p*H 10, with a Zeiss (PMQ II) spectrophotometer (*8*). Enzyme activity, calculated from the initial linear portion of the curve, was expressed as millimicromoles (mμmole) of reduced pyridine nucleotide.

Five electrophoretically separable forms of lactic dehydrogenase (LDH) and two forms of malic dehydrogenase (MDH) were found in human fetal heart, liver, lung, kidney, and cerebral cortex. In a few instances, one of the five peaks of LDH was not detected, but in none of the tissues examined was any one of the five peaks absent in all stages of development. The two

Table 1. Percentage of total enzyme activity associated with each electrophoretically separated fraction.

Age*	LDH activity (%) in peak					MDH activity (%) in peak	
	1	2	3	4	5	1	2
				Heart			
13.5	0.7	28.2	31.8	31.8	7.5	—	—
14.5	0.5	18.8	43.9	33.4	3.4	16.8	83.2
16.0	2.5	12.2	34.8	37.8	12.7	30.4	69.6
21.5	2.7	10.0	37.7	37.7	11.9	41.3	58.7
24.0	1.6	4.5	28.5	31.6	33.8	29.4	70.6
Term	7.8	3.9	29.8	40.3	18.2	40.8	59.2
				Lung			
10.0	2.2	36.5	27.4	23.9	10.0	27.4	72.6
11.0	2.2	3.3	44.9	28.5	21.0	51.5	48.5
14.5	1.1	1.4	27.9	49.3	20.3	47.2	52.8
16.0	2.9	0.0	24.6	53.0	19.5	52.5	47.5
Term	37.2	27.8	23.6	6.5	4.9	59.8	37.2
				Liver			
8.4	2.6	19.4	35.6	38.2	4.2	54.3	45.7
10.0	6.6	21.1	34.4	22.2	15.7	51.6	48.4
11.0	4.5	23.2	39.8	27.2	5.3	57.8	42.2
12.0	3.8	39.8	36.4	17.5	2.4	35.5	64.5
14.5	15.3	19.4	40.1	16.6	8.6	36.0	64.0
21.5	19.4	35.9	21.4	17.6	5.7	53.7	46.3
24.0	11.6	5.0	35.2	41.5	6.7	45.2	54.8
Term	4.5	9.9	37.1	37.4	11.1	51.6	48.4

* Crown-rump length in centimeters.