MULTIPLE SUBSTRATE SPECIFICITIES OF SOME DEHYDROGENASE MOLECULES

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Received January 25, 1964

A wide variety of enzymes demonstrating dehydrogenase activity has been described. Generally, these have been considered separate and relatively specific enzymes, although several reports of "non-specific" dehydrogenase activities have appeared. For example, Vesell and Bearn (1957) found in zone electrophoresis of human serum that, of four zones of activity, two showed both malate and lactate dehydrogenase activity, while each of the others appeared specific. Struck and Sizer (1960) showed that beef glutamate dehydrogenase (GDH) was active toward a number of other amino acids. Davies and Kun (1957) showed activity of ox heart mitochondrial malate dehydrogenase (MDH) toward several α-keto dicarboxylic acids.

This report presents evidence that several dehydrogenase activities, previously considered unrelated, reside in the same molecule. The technique employed was starch gel electrophoresis, with dehydrogenase activity being demonstrated in the gel using nitro blue tetrazolium as the terminal electron acceptor. The evidence for the identity of enzymes acting upon different substrates is based upon the identity of the isozyme patterns, using various tissues from normal animals as well as some with naturally-occurring alterations in electrophoretic patterns.

MATERIALS AND METHODS

Animals used were the house mouse, Mus musculus, and the deer mouse, Peromyscus maniculatus. Tissues studied were kidney, heart, brain, liver, and muscle. Tissues were homogenized in distilled water, frozen and thawed twice, and the solutions were cleared by high-speed centrifugation. Vertical electrophoresis was carried out at 4° C.

Each tissue was studied under several different electrophoretic conditions. The pH, type of buffer, ionic strength, voltage, and starch concentration were experimentally varied. Gel buffer systems employed were: potassium phosphate at 0.0025 M (pH 8.5), and 0.02 M (pH 8.5); potassium phosphate-citric acid, 0.01 M (pH 7.0), and 0.006 M (pH 7.0); sodium borate, 0.02 M and 0.03 M (both at pH 8.5). These were all at a starch concentration of 14%. Additionally, starch concentration was varied (10%, 15%, 20%, and 25%), using the phosphate-citrate buffer at 0.01 M.

Eight different substrates were tested: ethyl alcohol, sodium salts of DL-α-glycerophosphate, and of the acids DL-lactic, L-glutamic, L-malic, L-aspartic, β-hydroxybutyric and alanine. The concentrations of the substrates, as well as the buffers, were varied in the incubation mixtures, to provide optimum conditions. Otherwise, the incubation mixtures were identical, and contained sodium cyanide, EDTA, DPN, phenazine methosulphate, and nitro blue tetrazolium (Sigma).

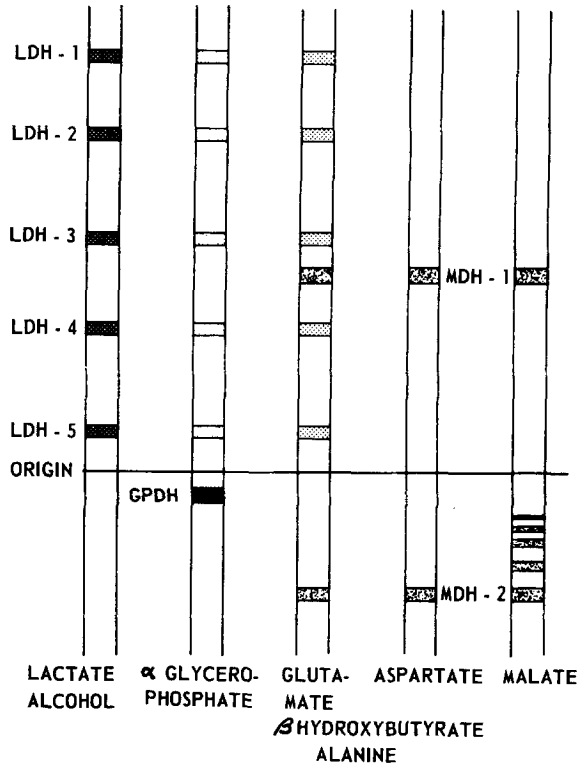
RESULTS

Eight major zones of dehydrogenase activity were observed (Figure 1). These were present under most of the conditions employed, although their positions, intensities, and resolutions varied. These eight bands could be separated into three groups. The first group, most clearly demonstrated with lactate, consisted of five bands. These are the five LDH isozymes which commonly occur in most animal tissues, first described by Markert and Møller (1959), and designated LDH-1 to -5. The second group consisted of two bands, usually most active toward malate, and called here MDH-1 and -2. These correspond with the two isozymes sometimes designated in the literature as S-MDH and M-MDH (soluble and mitochondrial). The eighth band, occurring near the origin, was demonstrated only with α-glycerophosphate (called here GPDH).

Note in Figure 1 that all the bands described above except GPDH exhibited activities on more than one substrate. The other seven isozymes (LDH-1 to -5, and MDH-1 and 2) all appeared with glutamate, β-hydroxybutyrate and alanine. The LDH bands were visible with alcohol and α-glycerophosphate, while the MDH bands appeared with aspartate. Note however that the subbands of MDH-2 were observed only with malate.

In addition to these eight bands, a ninth very prominent band sometimes occurred in the deer mice. This enzyme, called simply DH-1 will be discussed below.

Fig. 1 DEHYDROGENASE ZONES DEVELOPED ON VARIOUS SUBSTRATES. PHOSPHATE - CITRATE GEL BUFFER, pH 7.0, PEROMYSCUS BRAIN.

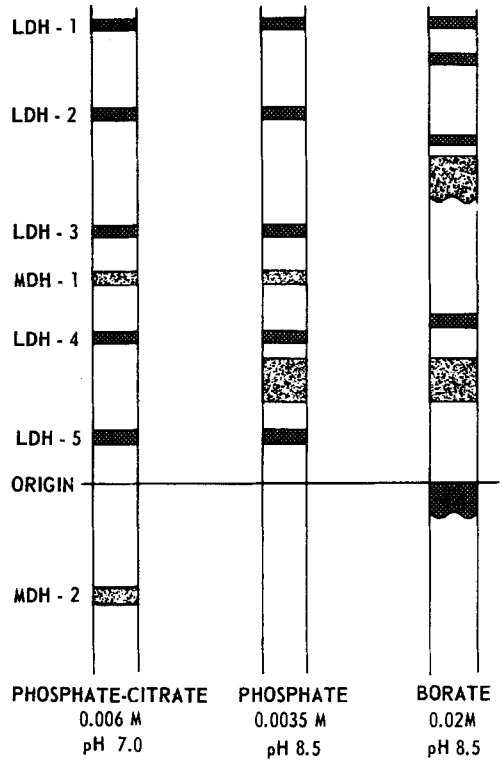


Effects of varying gel conditions

Figure 2 illustrates the changes produced in the LDH and MDH bands when buffer conditions were altered. Three different gel buffers were used: phosphate-citrate 0.006 M (pH 7.0); phosphate 0.0035 M (pH 8.5), and borate 0.02 M (pH 8.5). The most significant effect was on the position of MDH-2 relative to the LDH's. In the phosphate-citrate the MDH-2 occurred cathodal to the origin, whereas in the other two systems it was anodal. Relative positions of the other bands remained constant.

Varying the starch concentrations from 10% up to 25% resulted in a retarded rate of migration of all bands. This technique, described by Smithies (1962), indicates relative molecular weights among the various molecules, the heavier molecules being slowed with increasing starch concen-

Fig. 2 GDH ISOZYME PATTERNS UNDER VARIOUS GEL CONDITIONS.



tration relatively more than the lighter ones. In this study, a plot of relative migration of all the seven GDH bands (5 LDH's and 2 MDH's) against the reciprocal of starch concentration yielded a straight-line relationship. This indicates that all seven of these molecules are of similar molecular weight.

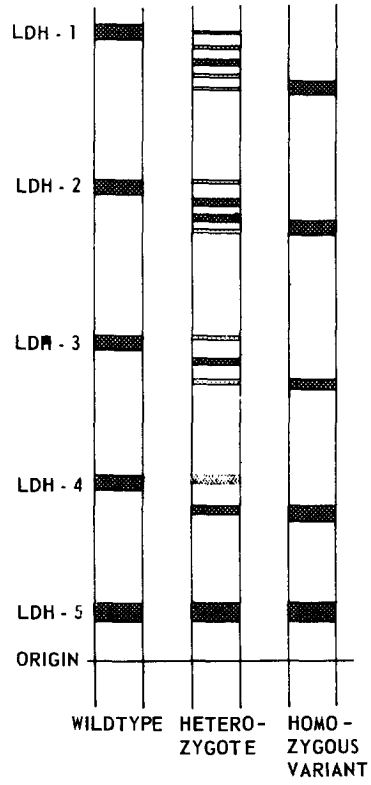
The above relationships, demonstrated by varying the gel conditions, persisted when the various substrates were employed. For example, the shift in relative position of MDH-2 was identical when the substrates were malate, aspartate, glutamate, β-hydroxbutyrate, or alanine. Likewise, all the LDH bands occupied identical positions under identical gel conditions, whether developed on lactate, α-glycerophosphate, glutamate, β-hydroxybutyrate, alanine, or ethyl alcohol.

Naturally-occurring alterations in electrophoretic patterns

Three types of naturally-occurring variations have been utilized in this study, two involving the lactate dehydrogenases, the third involving MDH-1.

A genetically-determined variant in Peromyscus, producing altered mobility of LDH-1 through -4 (Figure 3), has been previously studied in our laboratory (Shaw and Barto 1963). When tissues from the variant animals were developed with the other substrates which ordinarily show activity at the LDH sites (α-glycerophosphate, glutamate, β-hydroxybutyrate, alanine, and ethyl alcohol), the same alterations in isozyme patterns occurred.

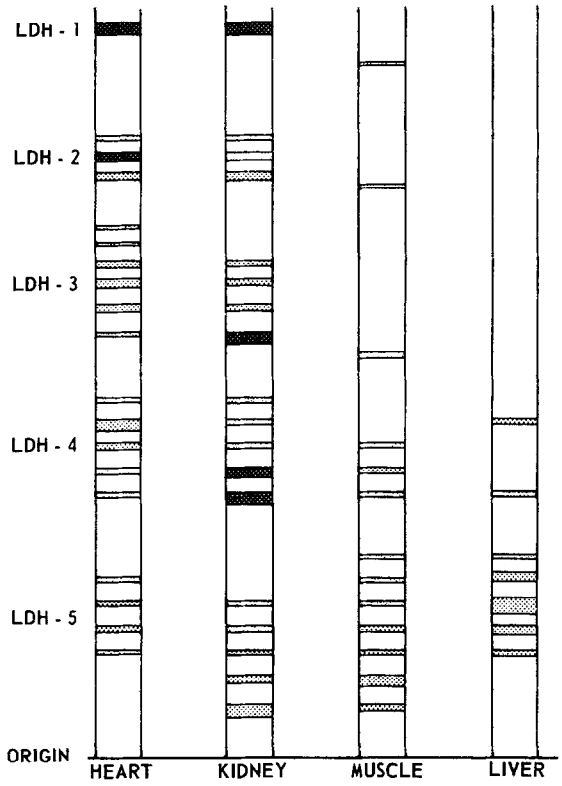
Fig. 3 GENETICALLY DETERMINED LDH VARIANT IN PEROMYSCUS



Another type of naturally-occurring variation in LDH isozyme patterns, first described by Costello and Kaplan (1963), consists of qualitative,

tissue-specific differences seen in the house mouse. Certain of these are illustrated in Figure 4, which compares the LDH patterns of heart, kidney, muscle and liver as seen in the phosphate-buffered gel .003 M, pH 8.5. The sub-bands are thought to result from the occurrence of two or more forms of one of the polypeptide sub-units of which the LDH tetramers are composed. When the gels illustrated in Figure 4 were developed on the other LDH-activated substrates (α-glycerophosphate, glutamate, β-hydroxybutyrate, alanine, and ethyl alcohol), the patterns were, except for quantitative differences, identical.

Fig. 4 TISSUE DIFFERENCES IN LDH PATTERNS,
MUS MUSCULUS PHOSPHATE GEL
BUFFER 0.003M, pH 8.5



A third type of variation, seen in several deer mice, consisted of a slight anodal shift in position of MDH-1. Mating experiments now in progress

will determine whether it is under genetic control. The alteration persisted when the same animals were tested for GDH activity. Other substrates have not yet been tested with this variant.

The isozyme which we have called DH-1 likewise occurred only in certain of the deer mice, and its genetic control has not yet been established. It was located near the cathodal end of the gel at pH 8.5. It does not appear to have been previously described in the literature of dehydrogenase isozymes. It showed activity toward all substrates tested, in approximately the following order of intensity: malate > glutamate > lactate > all others.

DISCUSSION

The demonstration of several enzymatic activities at one electrophoretic site does not constitute proof for multiple activities of the same molecule. In a crude tissue extract, as employed here, many enzymes doubtless migrate to every position along the gel. However, when a number of different electrophoretic conditions are applied, resulting in alteration of isozyme patterns, and the various substrates all reflect these alterations, this strengthens the evidence for multiple activities of the molecules. The demonstration that the patterns produced by variant molecules likewise appear with the several substrates further establishes the existence of multiple activities.

It has been considered that, in the gel electrophoresis procedure, several enzymes might be attached to and migrate with other proteins, thus accounting for their occurrence at the same site. However, this appears unlikely for a number of reasons, particularly the finding of the several activities on the genetically-variant molecules unassociated with a demonstrable variation in tissue proteins.

Additional corroboration, as well as quantification, is being sought in studies of purified enzymes now in progress. However, some of the steps involved in enzyme purification are rather drastic, and may produce cleavages or other alterations, so that the loss of one or another activity would not

necessarily indicate that such activity did not initially reside in the molecule. Such an effect may explain the discrepancy between the present findings and those reported in the literature concerning the specifities and relative molecular weights of GDH (Yielding and Tompkins 1962; Rogers et al. 1963), LDH (Jaenicke and Pfleiderer 1962) and MDH (Davies and Kun 1957; Thorne and Kaplan 1963).

ACKNOWLEDGEMENTS

We thank Dr. Elizabeth Barto, Department of Zoology, University of Michigan, for providing the deer mice, P. maniculatus bairdi.

This study was supported by research grants from the U. S. Public Health Service (MH 07574-01) and the Scottish Rite Foundation for Research in Schizophrenia.

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