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MULTIPLE FORMS OF ENZYMES: TISSUE, ONTOGENETIC, AND SPECIES SPECIFIC PATTERNS*

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The fundamental metabolic activities of organisms are very similar and consequently enzymes catalyzing identical reactions may be found in many different organisms and in many different tissues within an organism. When subjected to a variety of physical,1—4 chemical,5—7 or serological8—17 tests enzymes from different organisms are commonly found to be different from each other even though catalyzing the same chemical reaction. In view of the demonstrated genetic control of protein synthesis18 it is not surprising that differences should exist in the structure of homologous enzymes or proteins synthesized by animals of different species19—24 or even by animals of different genotype within the same species.25—29 Rather surprising, however, is the evidence demonstrating that several enzymes exist in multiple molecular forms not only within a single organism but even within a single tissue. Among the enzymes that have been reported to exist in separate molecular types within a single tissue are esterase,30, 31 ribonuclease,32, 33 pepsin,34 chymotrypsin,35 trypsin,36 lysozyme,37, 38 cytochrome C,39 xanthine dehydrogenase,40 malate dehydrogenase,41 malate dehydrogenase,42, 43, 44 and lactate dehydrogenase.42—49 Likewise, in yeast distinct molecular types of phosphoglyceraldehyde dehydrogenase50 and of enolase51 have been identified. The existence of each of these enzymes as a family of closely related but distinguishable molecular types suggests the need for an extension of the classification of enzymes beyond that based on substrate specificity alone. We propose, therefore, to use the term isozyme to describe the different molecular forms in which proteins may exist with the same enzymatic specificity.

In the present investigation three dehydrogenase enzymes—lactate dehydrogenase (LDH),† malate dehydrogenase (MDH), and isocitrate dehydrogenase (IDH)—have been resolved into physically distinct forms, that is, into isozymes. In addition to the dehydrogenases, three enzymes with broad substrate specifi-
ties—that is, alkaline phosphatase, peroxidase, and esterase—have also been resolved into multiple molecular forms. However, each of these enzymes with broad substrate specificity is probably a family of enzymes the members of which have overlapping but distinct patterns of substrate specificity, and therefore they should not at present be classified into isozymes. The esterases, like the LDH isozymes, do indeed exhibit tissue specific patterns that arise gradually during the course of embryonic development, but the dehydrogenases owing to greater substrate specificity are more suitable material for studying the origin and tissue distribution of closely related but distinct molecular species. In this report the LDH isozymes have been studied principally with special reference to their tissue and species specificity and to their ontogeny.

Partly because of its use as a diagnostic tool in human disease LDH has attracted considerable attention and numerous investigators have demonstrated the occurrence of this enzyme in multiple forms. The work of Wieland and Pfleiderer and of Pfleiderer and Jeckel has been especially noteworthy in demonstrating the species and tissue specificity of the isozymes of LDH. We have been able to confirm their general results. However, the greater resolving power and precision of a combination of electrophoretic and histochemical techniques used in the present investigation have enabled us to amplify and clarify their results. For example, these investigators found one LDH isozyme in pig heart and two in beef heart, whereas we find three and four, respectively. Moreover, comparative electrophoretic mobilities can be more clearly ascertained by the starch gel electrophoresis method used in the present investigation.

Materials and Methods.—One of the best methods for resolving complex mixtures of proteins is zone electrophoresis in starch gels. This method developed initially by Smithies for separating serum proteins has proved valuable in the separation of several enzymes from tissue homogenates. The details of procedure have been set forth previously. By these methods of starch gel electrophoresis about 1 mg of protein in a homogenate volume of 0.02 ml may be resolved in six hours. After electrophoretic resolution of the homogenate the starch block is split horizontally and one half stained with amido black to reveal the location of protein bands. The remaining half is then used for locating enzymatic activity. The identification of each kind of enzyme presents problems peculiar to itself. By the use of conventional histochemical methods several enzymes have been identified directly on the starch gel—namely, esterase, phosphatases, tyrosinases, and peroxidases. The starch strip containing the visualized enzymes has been called a zymogram.

Methods for identifying dehydrogenases directly on the starch gel all proved unsatisfactory and so an indirect method was developed that should have general applicability to any electrophoretically mobile enzyme using DPN or TPN as cofactor. In this method the starch strip, after electrophoresis, is placed in a tray and overlayed to a depth of 2 mm with an agar solution (1.5 per cent) at 45°C containing the reactants. For LDH these are lactate, DPN, hydrazine, diaphorase, methylene blue, and neotetrazolium chloride. Other enzymes require somewhat different reactants. The tray is then covered quickly to exclude oxygen, cooled in an ice bath until the agar gels, and then placed in a dark incubator at 37°C. Within about one hour the location of dehydrogenase activity in the starch is
revealed by the precipitation of a purple formazan dye in the agar directly above the area of the starch containing a dehydrogenase.

A schematic representation of the reactions involved is shown in Figure 1. This method essentially detects DPNH or TPNH—hence its general applicability. The sensitivity can be very great since the accumulated product of a prolonged enzyme reaction is measured. LDH activity of less than a gamma of protein is readily detected.

![Diagram](image)

Fig. 1.—Schematic representation of the reactions involved in localizing LDH isozymes after they have been separated by electrophoresis in a starch gel. A 1.5 per cent solution of agar containing the following reactants is poured over the starch and quickly cooled: sodium-DL-lactate, 0.1 M; DPN, 0.3 mg/ml; hydrazine (neutralized), 0.1 M; diaphorase, 0.6 units/ml; neotetrazolium chloride, 0.5 mg/ml; phosphate buffer pH 7.4, 0.67 M; methylene blue, 0.003 mg/ml. This method detects DPNH or TPNH since these substances reduce neotetrazolium to an insoluble formazan dye.

Once an enzyme has been located on one half of the starch strip the corresponding area of the other half may be cut out, frozen to break up the gel structure, and the enzyme eluted for further tests. An additional, though less satisfactory method in our hands, for locating dehydrogenases is based on the loss of fluorescence under ultraviolet light as DPNH is oxidized to DPN.2 By this method the dehydrogenases show up as dark bands on a fluorescent background.

**Results.**—*Tissue and species specific patterns of LDH isozymes:* Purified, crystalline beef heart LDH was electrophoretically resolved into five distinct protein bands, four of which had enzymatic activity. The band at position III separates into two components after prolonged electrophoresis, but is pictured as occupying a single position in Figure 2. Crude homogenates of beef heart, although containing many different proteins, were resolved into a similar pattern of LDH isozymes.
However, an isozyme from the crude homogenate appeared at position V, and this isozyme apparently was lost during the purification of the crystalline preparation. On the other hand the crystalline preparation contained a minor isozymic component appearing at position IV that was not evident in the crude homogenate. This new component may be a preparative artifact or may result from the concentration of an isozyme that is too dilute to be detected in the crude homogenate.

The LDH isozyme pattern of beef heart was compared with the pattern of isozymes from the heart of sheep, pig, mouse, and rabbit (Fig. 2). Each pattern is unique, though the isozyme at position II is present in each species. Thus far, seven electrophoretically distinct mammalian LDH isozymes have been recognized. Two of these from beef heart are closely adjacent in position III but the remaining five occupy distinctly separated electrophoretic positions. Some tissues contain only one, others as many as five of these isozymes. The apparent relative amount of the different isozymes present also varies considerably between different tissues. An examination of the LDH isozyme pattern of seven different tissues of the pig shows each of these patterns to be tissue specific (Fig. 2). Heart, skeletal muscle, smooth muscle of the pyloric sphincter, diaphragm, liver, kidney, and brain have all been analyzed for their LDH isozymes and each tissue is to some degree different from all the others. It is obvious that tissues differ both in the particular isozymes they contain and in the relative proportions of these isozymes.

**LDH isozyme patterns in embryonic tissues:** Since mature tissues differ from each other in isozymic content these differences must reflect divergent ontogenies. Accordingly embryonic tissues should contain isozymic patterns differing from adult tissues. This expectation was realized in an analysis of isozymic patterns in the tissues of embryonic pigs of 12 cm crown-rump length. A comparison of the isozymic pattern of these embryonic tissues with homologous adult tissues is shown in Figure 2. A photographic reproduction of several zymograms of LDH isozymes is also shown in Figure 3. It is apparent that the final adult pattern is reached by both gains and losses in the isozyme repertory of embryonic tissues. Embryonic heart, an active functioning organ at this embryonic stage, contains a larger number of LDH isozymes than adult pig heart. Skeletal muscle, on the other hand, contains fewer isozymes than adult muscle. Particularly, the single isozyme that migrates toward the cathode at pH 8.6 and is characteristic of adult skeletal muscle is not present at this embryonic stage. Other differences between embryonic and adult tissue, particularly in the relative enzymatic activity disposed in each isozymic band, are shown in the zymograms of Figure 2. Characteristic of several embryonic pig tissues is the presence of an isozyme at position III. Curiously, this isozyme later disappears in adult pig tissues although adult tissues of other species retain an isozyme at this position.

The substrate specificity of the LDH isozymes of beef heart was tested qualitatively with eight substrates: lactate, β-phenyllactate, α-hydroxybutyrate, α-hydroxy-N-caproate, α-hydroxyvalerate, α-hydroxyisovalerate, α-hydroxyisobutyrate, and α-hydroxy-β-methylvalerate. All these substrates were oxidized in the presence of the LDH isozymes though not at the same rate. However, all of the LDH isozymes were alike in the relative catalytic efficiency displayed toward these different substrates. Thus no difference in pattern of substrate specificity was apparent.
Fig. 2.—Diagrammatic representation of the intensity, size, and location of LDH isozymes from several tissues as seen in zymograms made by the procedure outlined in Figure 1. Electrophoretic resolution was achieved in six hours at a voltage drop of 6 V/cm and at a pH of 8.6. Under these conditions all but one of the isozymes migrated toward the anode.
**Multiple forms of other enzymes:** In addition to LDH several other dehydrogenase enzymes have been investigated by the same techniques to determine whether they too exist in isozymic forms. Isocitrate dehydrogenase from pig heart was resolved into three isozymes and malate dehydrogenase into at least two. In assaying for IDH, TPN and a TPN diaphorase from spinach leaves were employed. These different dehydrogenase isozymes (whether of LDH, MDH, or IDH) all proved to be electrophoretically distinct. Glutamate dehydrogenase appeared to be electrophoretically immobile in the starch gel since all activity was confined to the area in which the sample was inserted.

Multiplicity of molecular forms, of course, is not confined to the dehydrogenases. Enzymes with broad substrate specificities may also be resolved into separate molecular types, but these electrophoretically separated molecular species probably represent distinct enzymes rather than isozymic forms of the same enzyme. Further work on their substrate specificity is needed to clarify their status. Previously Markert and Hunter demonstrated the tissue specific patterns of esterases in the mouse and revealed the existence of more than ten electrophoretically distinct esterases. In the present investigation alkaline phosphatase from tissue homogenates of mice was resolved into three phosphatases that occurred in tissue specific patterns. These enzymes were visualized on the starch gel by the histochemical techniques used for esterases except that α-naphthyl-phosphate was used as substrate. A commercial preparation of horseradish peroxidase was resolved into ten bands all showing peroxidase activity as judged by the benzidine test. At a pH of 8.6 seven of these peroxidases migrated toward the cathode and three moved toward the anode.

**Discussion.—Gene-enzyme relationships:** The genetic control of the synthesis of
specific proteins has been repeatedly demonstrated and in a few cases changes in the specific properties of a protein have been correlated with corresponding changes in a gene. For example, several varieties of adult and fetal hemoglobin have been identified and shown to be under genetic control with heterozygous individuals producing the two alternative types of hemoglobin. The existence of isozymes raises the question of whether each of these also is controlled by a separate gene or whether they are all modifications of a single gene product. However, it is implausible to consider the multiplicity of isozymes as reflecting a heterozygous genetic constitution since the many animals examined thus far could scarcely have been so uniformly heterozygous. Indeed the mice were from highly inbred colonies. Nevertheless, a multiple gene basis for isozymes is suggested by the existence of independent genes for the synthesis of fetal and adult hemoglobin. Especially relevant is the fact that these genes for hemoglobin synthesis function at different periods during ontogeny. Certain isozymes also appear to be characteristic of specific stages of development.

The genetic basis for isozyme synthesis cannot be settled at present and must await the discovery of isozymic differences in organisms that can be subjected to genetic analysis. However, it is worth noting that each species has its own characteristic isozymic pattern, presumably reflecting the genetic makeup of the species. Perhaps significance can be seen in the fact that the LDH isozymes of the mammalian species examined occupy only a limited number of electrophoretic positions. That is, although all of the species possess some isozymes in common they differ in the possession of others and in the relative proportions of those present. These overlapping but distinct patterns of isozymes in genetically different animals are amenable to either of two genetic interpretations: each isozyme may be attributed to a single gene, with species differences due to the particular genes possessed, or alternate forms of a gene may produce somewhat different constellations of isozymes, each species then having a uniquely characteristic gene for synthesizing its own array of LDH isozymes.

Isozymes and cellular differentiation: Regardless of the genetic basis for isozyme synthesis it is clear that the genetic potential is realized through the processes of cellular differentiation. Each tissue has its own characteristic pattern of isozymes and this pattern changes during embryonic development until the adult configuration is reached. Embryonic tissues, depending upon the one selected, may possess either more or fewer isozymes than corresponding adult tissues, and the relative amounts of the isozymes also vary in accord with the stage of development. This tissue specificity of isozyme pattern may be based on populations of different kinds of cells with each single cell producing only a single LDH isozyme or alternatively a single cell may produce several isozymes. No evidence is yet available to distinguish critically between these alternatives but the second one seems more probable in view of the observation that embryonic pig heart contains a larger number of isozymes than the adult heart (the reverse is true of skeletal muscle). Moreover, the relative proportions of the various isozymes in embryonic and adult tissues is conspicuously different. If changing cell populations are to account for changing isozymic patterns then these shifts in cell populations would have to be much greater than direct observations of embryonic development would suggest. Such drastic changes in cell populations seem unlikely. More plausible is the hypothesis that
the isozymic pattern of a tissue reflects the state of differentiation of its cells.

If isozymic multiplicity is viewed as a function of cellular differentiation rather than as a rigid expression of genetically specified multiple enzyme-forming sites, then several hypotheses may be considered. During the course of changing cellular metabolism the same enzyme-forming site may produce a variety of closely related but distinguishable protein molecules depending upon the raw material made available to it, or perhaps the protein-forming mechanism itself is subject to slight structural variations with a consequent variation in its products. After an enzyme molecule has been synthesized it is integrated into the structure of the cell. Various modes of attachment within the cell may account for changes in reactive groups on the molecule, thus changing the net molecular charge and hence the electrophoretic mobility of the molecule. Electrophoretic mobility is a function of molecular size as well as charge and the several isozymes might be interpreted as reflecting merely different aggregates or polymers of a single molecular species. However, the monodisperse behavior in the ultracentrifuge of crystalline preparations, containing several isozymes, seems to exclude this possibility.

**Differences among isozymes:** In addition to distinguishing LDH isozymes on the basis of their electrophoretic properties efforts have also been made to distinguish them by their enzymatic behavior. LDHs from different species, from beef heart and rabbit skeletal muscle, were shown by Kaplan et al.6 to reduce analogues of DPN at different relative rates. Likewise Pfleiderer, et al.,7 showed that pig heart, rabbit skeletal, and rat skeletal muscle LDH all show different degrees of inhibition with 1-fluoro-2,4-dinitrobenzol. It is now clear that these two investigations were dealing with mixtures of isozymes but even so the differences observed must reflect differences in individual isozymes. In two investigations individual LDH isozymes were separated from the tissues of a single animal and shown to be different. Wieland and Pfleiderer2 found a correlation between degree of sulfite inhibition and electrophoretic mobility. Vesell and Bearn3,4 resolved by starch paste electrophoresis three LDH fractions from human blood. These fractions were eluted and shown to each have a distinct pH optimum. However they responded alike to two inhibitors, EDTA and PCMB.

The fact that difference in enzymatic behavior, even though slight, can be shown to distinguish isozymes raises the question of the sharpness of the boundary separating a family of isozymes from distinctly different enzymes. It seems unlikely that further research will reveal a continuum of enzymatic properties ranging from one enzyme to the next. However, there may be considerable variation in the degree of uniformity characterizing different families of isozymes. Some may be essentially identical enzymatically, others may diverge so much as to more properly be described as distinct enzymes (esterases for example). Nevertheless, the concept of enzymes existing as families of isozymes seems useful and significant today especially in view of the tissue-specific patterns of these isozymes.

**Serological specificity of isozymes:** In an attempt to characterize homologous enzymes from different sources investigators have frequently resorted to serological methods. Generally, antisera prepared against an enzyme from one species will not cross-react with homologous enzymes from distantly related species.9,10 However, some cross-reaction is commonly observed when the species are closely related (see Cinader8 for general discussion). For example, Gregory and Wróblewski,11
using antisera obtained from chickens injected with rabbit muscle LDH, demonstrated varying degrees of cross-reaction between these antisera and LDHs obtained from five different mammalian species. A similar cross-reactivity was shown by Henion and Sutherland\textsuperscript{12} for phosphorylases extracted from the tissues of the dog, rabbit, and cat. These investigators also demonstrated that the phosphorylases from different organs of the same animal are immunologically different though cross-reactive in varying degrees. Serological distinctions have also been shown among alkaline phosphatases extracted from different human tissues (and also from tissues of the dog).\textsuperscript{56} Wide variation was noted in the degree of cross-reaction between heterologous antisera and the different tissue phosphatases. The results of these tests suggested that human serum phosphatase was a mixture of types found singly in other tissues.

These results with the LDH, phosphorylase, and phosphatase antisera can perhaps be interpreted in the light of isozymic patterns as merely reflecting the degree to which the different tissues share a common repertory of isozymes. Failure to obtain complete cross-reaction may merely indicate the existence of additional isozymes in the tested tissue that were not present in the immunizing extract. This seems particularly probable as an explanation of partial cross-reactions obtained with enzymes from different tissues of the same species. Whether electrophoretically homologous isozymes from different species are serologically identical has not yet been demonstrated, but work on this problem is now proceeding in this laboratory.

The generality and clarity of the picture presented by the existence of patterns of isozymes that are tissue and species specific and that also change during embryonic development poses significant problems for our understanding of the genetic and embryological regulation of enzyme synthesis. We have not as yet gained any insight into the mechanism by which isozymes arise but any concept of cellular differentiation will have to account for them.

Summary.—(1) A search of the literature reveals that many different enzymes have each been resolved by physico-chemical techniques into several distinguishable molecular types. We propose to call these molecular types, isozymes.

(2) From tissue homogenates three dehydrogenases (LDH, MDH, IDH) were resolved into their component isozymes by zone electrophoresis in starch gels. The dehydrogenase isozymes were then localized on the starch by a new method that should be applicable to any electrophoretically mobile enzyme using DPN or TPN as a cofactor.

(3) Lactate dehydrogenase obtained from the tissues of several different species was electrophoretically separated into several molecular types (isozymes) each with the same substrate specificities. The pattern of LDH isozymes is species and tissue specific and changes during the embryological differentiation of the tissue.

(4) Esterase, peroxidase, and phosphatase were also resolved into separate molecular types by electrophoresis but each of these enzymes is probably a family of enzymes the members of which have characteristic but overlapping substrate specificities.

(5) Genetic, embryological, physiological, and serological considerations are presented in an effort to account for specific patterns of isozymes.
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† The following abbreviations have been used: LDH, lactate dehydrogenase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; DPN, diphosphopyridine nucleotide; TPNH, reduced diphosphopyridine nucleotide.

INTERACTION BETWEEN FORMALDEHYDE AND TOBACCO MOSAIC VIRUS

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In preparation for a study of the uptake of formaldehyde by polio virus, the interaction between formaldehyde and tobacco mosaic virus was investigated. This reaction has been variously observed, e.g., in terms of biological inactivation,1–4 alterations in electrophoretic pattern,5, 6 ultraviolet absorption,5 and reactions7, 8 of protein or amino groups, but no information is available as to the extent of the intermolecular combination. This gap in our knowledge is due to the small amount of formaldehyde reacting and the analytical difficulty in measuring this small quantity in the presence of a huge excess of formaldehyde.

Materials and Methods.—This problem has been approached with the aid of carbon-14 labeled formaldehyde (specific activity ≈ 3.5 mc/mmole), from which stock solutions of formaldehyde varying in total concentration (C0) from 50 μg HCHO/ml to ≈ 40,000 μg HCHO/ml were prepared by addition of unlabeled reagent grade formaldehyde. One-ml portions of these solutions were added to 1 ml solution of a purified masked strain of TMV8 in 0.2 M pH 7.0 phosphate buffer containing 2 mg virus/ml in 15-ml Pyrex centrifuge tubes (stoppered by polythene stoppers). After brief agitations, the tubes were immersed in a thermostated water bath for incubation. Order and rate of addition of components proved to be immaterial. Excess formaldehyde was removed by coagulation of virus by 5 ml of 30 per cent (NH4)2SO4, centrifugation, and careful decantation of supernatant fluid. Liquid adhering to the walls of the tube was removed by careful wiping with filter paper. The virus pellet was resuspended in salt solution, and the washing cycle repeated five times. This washing procedure required 5–6 hours and was effective in removing excess formaldehyde. Washed virus was suspended in 3 ml of H2O, and virus content determined by its ultraviolet absorbance.

Aliquots (1 ml) of virus solutions were placed in 11/2-in. stainless steel planchets to which were added 1 ml of a dimedon9 reagent (1 mg/ml) in very dilute pH 4 acetate buffer (1 mg/ml). After 24 hrs of standing, the water was removed by absorption over P2O5. The residues were redistributed with methanol which was