SPECIAL ARTICLE

Isoenzymes in Clinical Diagnosis

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SERUM ENZYMES are useful tools for determining the location and severity of many diseases. They are protein catalysts, some of which enter the serum from damaged tissues. Because they are catalysts, they are more easily detected than many other substances. Amylase released from the diseased pancreas, and phosphatase released from several tissues have been studied for many years and have established the usefulness of serum enzyme tests.

Unfortunately, many of the best studied and most easily detected enzymes occur in more than one organ. Furthermore, some organs like liver and skeletal muscle contain high concentrations of many enzymes and frequently cause confusion in diagnosis based on enzymes.

In one respect, however, the wide distribution of some enzymes is illusory: the enzyme activity is widespread, but the specific protein catalyst may vary from tissue to tissue. This was recognized early in the case of phosphatases. Bone, prostate, and red cells are rich in proteins catalyzing the hydrolysis of phosphates, but the major phosphatases from these tissues differ in pH optima and susceptibility to inhibition by a variety of chemicals. Different enzyme molecules that catalyze the same reaction are called "isoenzymes," "isozymes," or "multiple molecular forms." Their discovery has encouraged further searches for organ-specific catalytic proteins.

The acid and alkaline phosphatases are not usually designated as isoenzymes. This term was first applied to enzymes that catalyzed the same reaction but differed from one another in electrophoretic or chromatographic properties. For the purposes of this review, the definition of isoenzymes will be broadened to include all sets of enzymes that catalyze a given reaction, regardless of the nature of the differences among them. By this definition, acid and alkaline phosphatases are isoenzymes, and the recently described electrophoretic forms within each of these groups are also isoenzymes.

Characteristics of Isoenzymes

Enzymes that catalyze the same reaction may differ from one another in many ways, ranging from small variations in secondary structure to broad differences in amino acid sequence and molecular weight. These criteria are listed in table 1. At one extreme are enzymes with marked differences in structure, but a common substrate. The esterases and the peptidases are probably in this class. At the other extreme are molecules that are identical in all respects but their degree of denaturation. Separatory procedures are now so sensitive that multiple forms of enzymes may appear, which merely reflect artifacts of preparation or handling of the specimens. Relatively minor manipulations may introduce differences caused by the folding of enzyme protein chains, the aggregation of chains into polymers, the addition or removal of lipids, bound metals, or deamidation of carboxyl amide groups. For clinical studies, the isoenzymes with the greatest value are those that differ in a fundamental way, which per-
sists despite the influence of serum contaminants, storage, or analytic technics.

**Detection of Isoenzymes**

Two groups of phosphatase were originally detected by their different pH optima and called “acid” and “alkaline” phosphatase. Further “isoenzymes” of phosphatase were differentiated by susceptibility to inhibition by tartrate and other chemicals. Different pepsins were separated by differences in solubility. Since these early examples, methods of differentiation have increased. The most widely used are technics of separation by electrophoresis or chromatography. The basic methods of isoenzyme detection and measurement are listed in table 2.

Figure 1A is an example of lactic dehydrogenase isoenzymes (LDH), separated by electrophoresis and detected by a staining reaction specific for this enzyme. Figure 1B shows an analogous pattern for creatine kinase. Once separated, the isoenzymes may prove to have different catalytic properties. For example, the muscle and heart varieties of LDH behave differently toward various concentrations of their common substrate, as shown in figure 2. These differences, like different susceptibility to pH, inhibitors, and specific antibodies, may be utilized for detecting and measuring the various enzyme forms.

The isoenzymes that are currently most convenient for clinical diagnosis are those which can be differentiated by simple assays using small amounts of enzyme, and various conditions of heat treatment, pH, substrate concentration, or inhibitors. Such assays do not require preliminary separation of the proteins by chromatography, etc. The heart and muscle forms of LDH are examples of isoenzymes detectable by such chemical properties. In fact, the demonstration of experimental data like that shown in figure 2 is an indication that such methods are applicable. These curves indicate at a glance the conditions of assay that will differentiate the two forms (shown by the vertical dotted lines), one condition measures both forms equally well, the second condition specifically inhibits one of the two forms. Such an assay is precise and quantitative. If more than two isoenzymes are present, however, this type of test may not give as complete a picture of the spectrum of isoenzymes as electrophoresis or chromatography. Finally it may be possible to adapt separatory procedures to a sort of chemical assay. For example, isoenzymes that adhere relatively firmly to materials used in chromatography can be removed from assay mixtures by the “batch” addition of gel to the specimen. In this way, the gel is used instead of a chemical inhibitor.

**Origin of Isoenzymes**

The isoenzymes that differ in amino acid composition, such as the isoenzymes of LDH, probably represent the results of ancestral mutation and gene duplication: a single gene

### Table 1

**Differences in Isoenzymes**

A. Physicochemical
   1. Differences in secondary and tertiary structure, (folding of polypeptide chains), e.g., ref. 4
   2. Different degrees of polymerization to dimers, tetramers, etc. 5

B. Immunochemical
   1. Different reactivity with specific antibodies. 6

C. Chemical
   1. Variations in degree of deamidation of carboxylamide groups or acetylation of amino groups. 7, 8
   2. Variable combinations with carrier proteins, carbohydrates, coenzymes, prosthetic groups, or lipid. 9–11
   3. Different degrees of activation or inactivation by hydrolytic cleavage of terminal peptides, oxidation or reduction of coenzyme or sulfhydryl groups.
   4. Varying degrees of amino acid differences. 12

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*Circulation, Volume XXXII, December 1965*
corresponding to a given enzyme gave rise to two or more genes and two or more different enzymes. Those isoenzymes that differ in prosthetic groups, secondary structure, or state of polymerization may have arisen by other routes. In some cases, further evolution or environmental influences caused separate genes to become expressed to varying degrees in different organs. This resulted in the organ-specific isoenzyme patterns under discussion. There are other instances, notably malic and TPN-linked isocitric dehydrogenases, in which the isoenzymes are found in many tissues but are localized in different subcellular compartments, such as mitochondria and cytoplasm.

Genetic and evolutionary factors have given rise to differences in other well-studied proteins which share a common function, such as hemoglobins, haptoglobins, and gamma globulins. Another example is the hormone-pair oxytocin and vasopressin, which share common properties and probably arose from a single ancestor like the vasotocin of lower forms. It has long been recognized that some such “iso” proteins vary from species to species, from race to race, or from person to person. The coexistence of multiple forms of enzymes or other proteins within the same individual but localized in various organs is the feature that makes them useful in clinical diagnosis.

The existence of two different genes for functionally related enzymes may give rise to more than two isoenzymes. This results from the fact that some enzymes are composed of

| Table 2 |
| Methods for Detection and Measurement of Isoenzymes |

A. Physicochemical
   1. Separation by electrophoresis, \(^{18}\)
   2. Separation by chromatography, \(^{19}\)
B. Immunochemical
   1. Combination with, or inhibition by specific antibodies, \(^{6}, 20\)
C. Chemical
   1. Rate of reaction under various conditions of pH, \(^{3}\) temperature, \(^{21}\) inhibitors, \(^{16}\) coenzyme analogues, \(^{22}\) or substrate concentration, \(^{23}\)

Figure 1
Isoenzymes separated by starch-gel electrophoresis. A shows the lactic dehydrogenases from young rat tissues, separated and stained according to the method of Fine et al. \(^{24}\) B shows the creatine kinases from the same tissues separated and stained according to the methods of Eppenberger et al. \(^{25}\)

Circulation, Volume XXXII, December 1965
ISOENZYMES IN CLINICAL DIAGNOSIS

Figure 2

Inhibition of two chicken isoenzymes of lactic dehydrogenase by pyruvate. The lines A and B indicate two concentrations of pyruvate which could be used to determine the relative amounts of the two isoenzymes (or their subunits) in a single sample, (from Cahn et al.)

subunits, analogous to the component chains of hemoglobin or gamma globulin. A single gene accounts for the structure of a single subunit, but the intact enzyme can be composed of more than one subunit, and molecular hybrids can occur. Thus, the five common isoenzymes of LDH are the products of only two different genes, producing two kinds of subunits, which can combine in five different ways to produce intact tetrameric enzyme molecules. This is illustrated diagrammatically in figure 3. The gene for heart-type LDH produces one kind of subunit, the H subunit, and the gene for muscle-type LDH produces M subunits. The complete enzyme contains four subunits. In heart, the predominant tetramer is H4, in skeletal muscle it is M4, and, in most other tissues, molecular hybrids of H and M predominate. Thus, five isoenzymes result from only two different genes. Enzymes that are dimers could have three isoenzymes as the result of two different genes (fig. 1B). The cell can thereby expand the genetic complement into a wide spectrum of isoenzyme patterns.

Genetic processes have given rise to further diversity of LDH isoenzymes in some individuals, resulting in more than five forms of this enzyme. These appear to be mutations affecting the gene for one of the two subunit types.

Physiologic Significance of Isoenzymes

The possible physiologic significance of isoenzymes is illustrated by lactic dehydrogenase. This enzyme catalyzes a reaction which, in the direction pyruvate \( \rightarrow \) lactate, enables glycolysis to provide energy in the absence of oxygen. This reaction is important in tissues such as skeletal and uterine muscle when energy from glycolysis is required during times of reduced oxygenation. The isoenzymes of LDH in these tissues are rich in muscle-type (M) subunits, which have the property of functioning at high concentrations of pyruvate (fig. 2). Thus, these tissues can utilize the above reaction when pyruvate cannot be oxidized. On the other hand, the isoenzymes of LDH found in heart are rich in H subunits and are inhibited by high concentrations of pyruvate. This inhibition may retard the reaction pyruvate \( \rightarrow \) lactate and promote the shunting of pyruvate to oxidative pathways of the Krebs cycle. In this way, the LDH isoenzymes in heart muscle favor more complete utilization of available energy in glucose. Furthermore, the heart isoenzymes are better suited to oxidize lactate; this may permit the heart to extract lactate from the arterial blood and use it as a metabolic fuel in addition to glucose.

The other tissues of the body make intermediate demands on glycolysis and oxidation, and their LDH is of intermediate type, con-
taining molecular hybrids of M and H subunits, and reacting in an intermediate way with pyruvate. The fine adjustment of metabolism, which can be aided by various proportions of H and M subunits in LDH, is illustrated in table 3. The two zones of the kidney vary in oxygen tension because of the countercurrent circulatory system: the medulla is anaerobic relative to the cortex.31 They also vary in their dependence on anaerobic glycolysis for energy: the medulla utilizes anaerobic pathways more extensively than does the cortex.32 Finally, there is a parallel variation in the proportion of subunits in the LDH from these zones: the anaerobic medulla contains muscle type, and the oxidative cortex contains mostly heart-type enzyme.30 This difference in metabolic and isoenzyme pattern from cortex to medulla may prove useful in the diagnosis and localization of renal disease by tests on blood or urine.

In addition to the differences observed in tissues of adult organisms, the proportions of the two kinds of LDH subunits can be made to vary further by changes in environment. In the uterus, estradiol induces preferential synthesis of M subunits ("preparing" the organ for its anaerobic, muscle-like adult function during parturition).33 In tissue culture, changes in oxygen tension alter the synthesis of the subunits to different degrees.34 As might be predicted, the synthesis of M subunits is favored by anaerobic conditions. These observations reinforce the concept that isoenzyme differences serve a physiologic purpose.30

It has been shown that the proportion of M subunits in tumors is higher than in normal tissues. However, the difference is only relative and has not proved adequate by itself for the diagnosis of cancer.35

Differences in isoenzyme properties which might correlate with physiology have also been described for malic dehydrogenase, glutamic dehydrogenase, and phosphofructokinase. The malic dehydrogenase isoenzymes differ in their resistance to high concentrations of malate, depending on their subcellular site of origin, that from mitochondria resisting high malate better than that from the cytoplasm.36, 37 This is consistent with the fact that malate is oxidized primarily inside mitochondria. Glutamic dehydrogenase isoenzymes vary in catalytic activity. The less active form, which can be produced in vitro by high concentrations of ATP, probably predominates when oxidation of glutamate for energy is unnecessary and ATP levels are high. Oxidation of glutamate by the more active form of enzyme is favored when energy is needed and ATP is low.38 Finally, phosphofructokinase from heart is more resistant to ATP inhibition than the enzyme from other tissues.39 This correlates with the high levels of ATP in heart compared to other tissues.

The isoenzymes of glutamic dehydrogenase, which differ in molecular weight as well as catalytic properties, are composed of varying numbers of subunits. Furthermore, the isoenzymes are interconvertible, and the conversion appears to be under metabolic and endocrine control.40 This may represent the use of isoenzymes by the cell for a constant fine adjustment of enzyme activity. Although the majority of isoenzymes are not so readily interconvertible, many more examples of interconvertible forms will probably come to light. If such interconversions were to occur among

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Comparison of Oxygen Supply, Oxygen Utilization, and Glycolysis with Composition of LDH Isoenzymes in Zones of the Kidney from Various Species</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxygen tension; (human) (mm. Hg), (31)</th>
<th>Glycolysis; (dogs), (al. CO₂ released from medium/ mg. dry wt./ hr), (32)</th>
<th>Oxygen utilization; (dogs), (-QO₂), (32)</th>
<th>M subunits in LDH; (rat), per cent, (36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medulla</td>
<td>20-60</td>
<td>5.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Cortex</td>
<td>90</td>
<td>1.4</td>
<td>14.1</td>
</tr>
</tbody>
</table>

Circulation, Volume XXXII, December 1965
ISOENZYMES IN CLINICAL DIAGNOSIS

multiple forms, all of which were fairly stable, the resulting isoenzyme patterns would provide accurate reflections of the momentary physiologic (or pathologic) states in tissues.

**Difficulties in Interpreting Isoenzyme Patterns**

All cells share the same genes and are theoretically capable of producing the same proteins, including isoenzymes. Although there are obvious marked differences in the localization of some proteins from tissue to tissue, the differences in enzymes and isoenzymes are likely to be relative rather than absolute. This is the feature that has long obscured interpretation of standard serum enzyme tests. Use of isoenzymes decreases this confusion somewhat, because it increases the number of variables that are subject to analysis. In the absence of absolutely organ-specific enzymes or isoenzymes, the greater number of proteins improves the diagnostic potential of this type of test.

It may become possible to incorporate tests for many relatively specific “iso-” proteins into one over-all test by the use of antisera. Since a classical means of identifying isoenzymes is immunologic, it should be possible to develop a series of organ-specific or tissue-specific antisera. If it were possible to detect the very small antigen-antibody reactions that would result when pathologic samples were exposed to organ-specific antisera, this kind of test would serve as a simultaneous screening procedure for a sum of many immunologically distinct isoenzymes and other antigens.

**Table 4**

*Selected List of Mammalian Enzymes with Multiple Molecular Forms (Isoenzymes)*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Usual number of isoenzymes</th>
<th>Isoenzymes differ in tissue or organ localization</th>
<th>Isoenzymes differ in catalytic properties or stability</th>
<th>Subunits proved or indicated</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic dehydrogenase</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>51, 52, 53*</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>54, 55*</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>&gt;2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>59</td>
</tr>
<tr>
<td>Aldolase</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>56</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>20, 57</td>
</tr>
<tr>
<td>Aminopeptidase</td>
<td>&gt;3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>14, 58, 59*</td>
</tr>
<tr>
<td>Fructose 1, 6 diphosphatase</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>60</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>10, 61, 62*</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>63, 64, 65</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>&gt;3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2, 6, 16, 66*, 67*</td>
</tr>
<tr>
<td>Esterase</td>
<td>&gt;3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>68, 69*</td>
</tr>
<tr>
<td>Amylase</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>70</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>38, 71</td>
</tr>
<tr>
<td>Pepsin</td>
<td>4</td>
<td>(+)</td>
<td></td>
<td></td>
<td>17, 72*</td>
</tr>
<tr>
<td>Isocitric dehydrogenase (TPN)</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>27</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>&gt;2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>26, 37</td>
</tr>
<tr>
<td>Glutamic-oxalacetic transaminase</td>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td>19, 73</td>
</tr>
<tr>
<td>Histidine-oxalacetic transaminase</td>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>&gt;3</td>
<td>+</td>
<td></td>
<td></td>
<td>19, 75*</td>
</tr>
<tr>
<td>Galactokinase</td>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td>76</td>
</tr>
<tr>
<td>Carboxic anhydrase</td>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td>77</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td>78</td>
</tr>
</tbody>
</table>

*Reports directly related to clinical medicine.
The preceding section described changes in isoenzyme patterns that may be caused by many physiologic or environmental conditions in vivo. Recent evidence also indicates that changes may occur depending on the time of day, in accordance with a "biological clock" mechanism. Furthermore, because they are different molecules, isoenzymes are handled differently by the body after they are released into the circulation. The various transaminases are cleared from the circulation at different rates, and the clearance of lactic dehydrogenase in mice is affected by a virus. As mentioned in the first section of this review, many changes in isoenzyme pattern can be induced by heat, acid, and solvents. Finally, the isoenzymes that result from various combinations of subunits often can be dissociated and reassociated in vitro. Because of this recombination, a sample that has been frozen and thawed may exhibit a different isoenzyme pattern from the fresh sample.

Thus, physiologic, pathologic, and physico-chemical factors bear upon the isoenzyme pattern during its passage through the cell, circulation, and laboratory. At the very least, conditions of collection, storage, separation, and assay must be standardized before isoenzyme comparisons are valid.

Summary

The recent literature attests to the increasing number of enzymes for which isoenzymes or subunit structure or both are known. Several symposia and reviews have been published within the past few years. Some enzyme activities for which isoenzymes have been described are listed in table 4. Only those found in mammals are included. They are ranked in rough order of apparent usefulness in clinical diagnosis, with the enzymes displaying tissue localization and catalytic differences listed first. Also, the number of molecular forms and the presence or absence of known subunit structure is indicated.

Clinical studies have been made using several of the isoenzymes listed in table 4, and references denoting these studies are marked with an asterisk. Data presented in the table indicate that valuable clinical information might derive from study of other isoenzymes, notably phosphofructokinase, for which tissue differences and adaptable catalytic differences exist. The guidelines set forth in this review can be used for investigating still other isoenzymes, such as those studied in lower forms. In brief, the study of isoenzymes, like the study of enzymes and other organ-specific chemicals, presents an opportunity for great specificity and accuracy in localizing and following disease processes.

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