Clinical Significance of Enzyme Activity Measurements

J. Henry Wilkinson

Additional Keyphrases  diagnostic enzymology  myocardial infarction  liver disease  organ or tissue specificities of enzymes  release of enzymes into, and removal from, blood  precision of enzyme tests  anomalous results

During the past 12 years or so we have all been performing large numbers of enzyme tests and the present occasion seems appropriate for an attempt to assess the status of enzyme tests in diagnosis. On occasion most of us have obtained results which did not quite fit the clinical diagnosis and in some cases an explanation for the anomalous results can be postulated. In others the puzzle remains to be solved. I shall discuss some of these, but I would first like to review the development of diagnostic enzymology and to summarize the present position.

Enzyme Tests in Diagnosis

Except for the digestive enzymes, the phosphatases were the first serum enzymes to find widespread diagnostic applications. These, as is well known, are elevated in bone and liver diseases. Convenient procedures, notably those of Bodansky (1) and of King and Armstrong (2), made this test widely available even before World War II. It is remarkable that although this test has been used for nearly 40 years, interest in it is still growing, as shown by the number of requests received by clinical laboratories and by the increasing number of publications on the subject in the clinical and biochemical literature. Figure 1 shows the number of serum alkaline phosphatase determinations requested at the University of Pennsylvania during the years I worked there.

The introduction of the phosphatases was an important development, but what might be described as the modern phase of clinical enzymology followed the finding by LaDue et al. (5) in 1954 that the AspT activity in serum is transiently elevated after an episode of myocardial infarction. This observation found immediate applications in cardiology but its significance extended far beyond this speciality. The demonstration that intracellular enzymes may be released into the circulation from damaged tissues has been confirmed by numerous investigators. The activities of about 20 enzymes are now more or less routinely determined for diagnostic purposes, not only in myocardial infarction, but in diseases of the liver, skeletal muscle, bone, and other tissues, as well as in certain congenital and acquired anemias and various manifestations of malignant disease.

Not surprisingly, this outburst of activity has led some enthusiasts to make rather exaggerated claims, often due to inadequate methodology or to a rather naive and oversimplified philosophy. I should therefore like to consider the rationale of enzyme tests; some of the factors involved are listed in Table 1.

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1 Abbreviations used: AspT, aspartate transaminase (glutamicoxaloacetic transaminase); l-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1; LDH, lactate dehydrogenase; l-lactate: NAD oxidoreductase, EC 1.1.1.27; CK, creatine kinase; ATP, creatine phosphotransferase, EC 2.7.3.2; AlaT, alanine transaminase (glutamic-pyruvic transaminase); l-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2; GDH, glutamate dehydrogenase; l-glutamate: NAD oxidoreductase (deaminating), EC 1.4.1.2.
The enzyme tests most widely used at the present time are listed in Table 2 according to their organ or tissue specificities. Relatively few serum enzymes are specific for damage in a single tissue; acid phosphatase is possibly the most specific of the tests commonly used, and under certain conditions an enhanced value is diagnostic of metastasizing carcinoma of the prostate. The distribution of enzymes varies from tissue to tissue, and careful selection of two or more tests will usually enable the source of the serum enzymes to be accurately defined. Simultaneous determination of several serum enzymes indicates that those of moderate or low specific activity may in certain circumstances be of diagnostic value. The value of one such test, LDH, has been greatly enhanced by the discovery of its existence in multiple forms (isoenzymes) (4). These can be separated by electrophoresis or by chromatography. Figure 2 shows that tissues can broadly be separated into three groups according to whether they contain the electrophoretically fast-, intermediate-, or slow-migrating LDH isoenzymes.

Myocardial infarction. Duration of increased enzyme activity in certain diseases is frequently of diagnostic importance. After an episode of myocardial infarction, for example, the activity of CK in serum is markedly increased during the first 12 to 48 h, while the AspT reaches a peak after about 24 to 36 h and returns to normal within two to six days. Enhancement of LDH activity is less pronounced but more prolonged, but the LDH1 isoenzyme activity, characteristic of heart muscle, is enhanced much more, and usually does not return to normal in less than 10 days. Serial determination may thus aid differential diagnosis. Typical values observed in myocardial infarction are shown in Figure 3.

Limitations of enzyme measurements in diagnosis of myocardial infarction are listed in Table 3.

Liver disease. Of the many serum enzymes showing increased activities in liver disease, the transaminases and alkaline phosphatase appear to be the most useful. The massive increase in the activities of AspT and AlaT in serum that coincides with the onset of jaundice is almost pathognomonic of virus hepatitis. Values up to 50 times the normal upper limit are common, but the activities usually fall rapidly, and during the second week the transaminases are much less helpful, because moderate elevations occur in other liver diseases.

The relatively high AlaT and isocitrate dehydrogenase \([\text{threo-D,-isocitrate} : \text{NAD oxidoreductase} (\text{decarboxylating}), \text{EC 1.1.1.41}]\) activity in the liver helps to differentiate hepatic diseases from those of other organs rich in AspT. In general, the "liver-specific" enzymes, such as sorbitol dehydrogenase, are insensitive and generally do not help much in differential diagnosis, though some investigators have found ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine

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**Table 1. Factors Governing the Use of Enzyme Tests in Diagnosis**

- Enzyme distribution in tissues
- Intracellular localization
- Release of enzymes from damaged tissues
- Alterations in membrane permeabilities
- Clearance of enzymes from serum
- Duration of elevation of serum enzyme activities
- Serial serum enzyme patterns
- Correlation with results of nonenzyme tests

**Table 2. Organ or Tissue Specificities of Serum Enzyme Tests**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Principal sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Enzymes of high specificity</td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>prostate, erythrocytes</td>
</tr>
<tr>
<td>Alanine transaminase</td>
<td>liver</td>
</tr>
<tr>
<td>Ornithine carbamoyltransferase</td>
<td>liver</td>
</tr>
<tr>
<td>Arginase</td>
<td>liver</td>
</tr>
<tr>
<td>Sorbitol dehydrogenase</td>
<td>liver</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>liver</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>hepatobiliary tract</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>liver</td>
</tr>
<tr>
<td>Amylase</td>
<td>pancreas, salivary glands</td>
</tr>
<tr>
<td>Lipase</td>
<td>pancreas</td>
</tr>
<tr>
<td>B. Enzymes of moderate specificity</td>
<td></td>
</tr>
<tr>
<td>Aspartate transaminase</td>
<td>liver, heart, skeletal muscle</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>liver, heart</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>skeletal muscle, heart</td>
</tr>
<tr>
<td>C. Enzymes of low specificity</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>bone, liver, intestinal mucosa, placenta, kidney</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>all tissues</td>
</tr>
</tbody>
</table>

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Fig. 1. Increase in the number of requests for serum alkaline phosphatase at the William Pepper Laboratory of the University of Pennsylvania from 1965 to 1969.
was the basis of the retention theory, the evidence for which has been reviewed by Gutman (5). According to this hypothesis alkaline phosphatase was produced, by the osteoblastic cells of bone and was excreted in the bile. Much clinical and experimental evidence in support of the retention theory was obtained, beginning with the demonstration by Armstrong et al. (6) that ligation of the bile duct in the dog caused a massive increase in the serum enzyme, which disappeared when the ligature was removed.

The theory, however, did not explain the increased activity found during pregnancy and in hepatitis and other nonobstructive forms of liver disease; when alkaline phosphatase was found to be present in several other tissues, particularly the intestinal mucosa, the placenta, and finally the liver itself, a powerful impetus was given to the search for an alternative theory. The finding that tissue alkaline phosphatases from different tissues could be separated by electrophoresis provided a means for the further investigation of this problem (Figure 4).

Unfortunately, the principal liver and bone isoenzymes have similar and overlapping electrophoretic mobilities, but Hodson et al. (7) were able to show that the normal serum enzyme contained an appreciable moiety that had the mobility of the liver enzyme.

The final coup de grâce to the retention theory came as the result of the work of Posen et al. (8), in Australia, who found that infused placental alkaline phosphatase was cleared from the sera of patients with biliary obstruction at the same rate as from the sera of normal subjects. A possible explanation for the greater serum alkaline phosphatase activity during biliary obstruction is foreshadowed by the findings that in the liver the enzyme is associated with the cell membrane, and that in experimental bile duct ligation there is increased enzyme synthesis. This was first suggested in 1964 by Sebesta et al. (9), but clear demonstration was recently obtained by Kaplan and Righetti (10). It seems possible, therefore, that increased pressure in the bile canaliculi exerts some effect on the cell membrane inducing synthesis of the enzyme, which causes some of the enzyme to find its way into the circulation. This
theory can now only be regarded as tentative; the mechanism of the proposed process remains to be determined.

The recent history of the serum alkaline phosphatase underlines the fact that most of the enzyme tests in use today are purely empirical. They are none the less useful, despite our lack of understanding of the mechanisms involved in the release of intracellular enzymes into the circulation and their removal from it. Nevertheless, it is a safe prediction that during the next decade much effort will be devoted toward putting clinical enzymology on a more rational basis. A number of reports have already appeared that point the way ahead.

Release of Enzymes into the Circulation

I have already listed some of the factors we must consider in using enzyme tests in diagnosis, and I would now like to review some of these in detail. An obvious factor is the distribution of enzymes in damaged tissues. A high enzyme activity in serum directs attention toward tissues rich in that particular component. Thus, high AspT and AlaT activities suggest liver involvement, since this organ is rich in these enzymes. Conversely, liver disease could not ordinarily be the cause of a high serum CK or LDH, since the liver is a poor source of these enzymes.

Cellular enzymes in serum. During recent years, a number of investigators have studied the incidence of enzymes originating in the cell-sap or mitochondria that appear in the serum in various diseases. The rationale of this approach is that cell-sap enzymes are more likely to be released into the circulation than the mitochondrial enzymes in reversible inflammatory processes characterized by increased membrane permeability. In necrotic conditions, however, destruc-

Fig. 4. Human tissue alkaline phosphatase isoenzymes, separated by acrylamide gel electrophoresis (kindly provided by A. C. Pollard and A. W. Walker)

From left: liver, bone, kidney, intestine, liver, lung, liver. The sample slot is at the top and the anode at the bottom.

tion of large numbers of cells will be followed by the appearance of mitochondrial enzymes in the serum (Figure 5).

In the years which followed the introduction of the transaminases as diagnostic tests, there were frequent references in the literature to the appearance of increased serum activities as being indicative of cell necrosis. Experience suggests, however, that the release of enzymes is much more complex.

Study of these processes has been greatly stimulated by the discovery that AspT occurs in markedly different forms in the cell-sap and the mitochondria, while AlaT is almost wholly confined to the soluble fraction of the liver. This led de Ritis (11) to suggest the use of the AspT-AlaT ratio as a means of distinguishing predominantly inflammatory lesions from necrotic processes. Values less than unity were indicative of the former, while a further rise in AspT activity suggested widespread necrosis. Unfortunately, our methods are not always sufficiently precise to permit the use of such ratios, and they have not found extensive application. I shall return to the problem of enzyme methodology later. Schmidt and coworkers (12) devised a very simple chromatographic method for separating the supernatant and the mitochondrial AspT in serum. At the appropriate pH, the mitochondrial enzyme is positively charged and on electrophoresis migrates towards the cathode, whereas the cell-sap form migrates toward the anode.

Table 4. Cytoplasmic (C-) and Mitochondrial (M-) AspT in the Sera of Patients with Liver Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mean serum activities, U/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-AspT</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td></td>
</tr>
<tr>
<td>1-2 weeks</td>
<td>375</td>
</tr>
<tr>
<td>3-4 weeks</td>
<td>141</td>
</tr>
<tr>
<td>5-8 weeks</td>
<td>34</td>
</tr>
<tr>
<td>Active cirrhosis</td>
<td>84</td>
</tr>
<tr>
<td>Cirrhosis with ascites</td>
<td>97</td>
</tr>
<tr>
<td>Cirrhosis, acute exacerbation</td>
<td>231</td>
</tr>
</tbody>
</table>

* Reproduced by permission from Schmidt et al. (15).
Schmidt's group (13) applied their chromatographic technique to the serum enzymes in a variety of liver diseases and demonstrated the presence of significant amounts of the mitochondrial enzyme in several, especially in severe hepatitis (Table 4). Using an electrophoretic method, Boyde (14) has found both forms of AspT in the sera of patients with myocardial infarction, a condition characterized by extensive necrosis.

This group has also pioneered measurement of the serum GDH in liver disease (15). This enzyme is confined to the mitochondria. It has a molecular weight of about $2 \times 10^6$ and consequently is too large to penetrate a slightly damaged membrane. GDH can scarcely be detected in the sera of healthy individuals, hence the occurrence of a significant amount suggests liver cell necrosis. The Schmidt index, $(\text{AspT} + \text{AlaT})/\text{GDH}$, has therefore been introduced to aid the differential diagnosis of liver disease. A value greater than 50 indicates hepatocellular damage; one less than 10 is associated with malignant invasion of the liver.

The criticisms which can be made of the de Ritis transaminase ratio apply, of course, with equal force to the Schmidt index. The precision of our methods is sufficient to enable the index to discriminate between typical cases, but is scarcely adequate for use in complicated cases.

Inflammation. It seems probable that in inflammatory states the loss of soluble enzymes (and other proteins) through an impaired membrane might act as a stimulus to increased synthesis. Certainly the amounts of transaminase released into the circulation during a moderate bout of hepatitis account for a substantial proportion of the total content of the liver (10 to 50%). Consequently, the possibility of increased enzyme synthesis in slightly damaged cells is an attractive hypothesis, but until recently there was little evidence to support it. However, the demonstration of increased alkaline phosphatase production in hepatobiliary diseases indicates that this suggestion must be borne in mind in connection with the transaminase and other enzymes.

Removal of Enzymes from the Circulation

We have also to consider what happens to enzymes once they have reached the circulation. As shown in Figures 6, 7, and 8, they frequently disappear at remarkably rapid rates. It is well known that in hepatitis and after myocardial infarction serum enzymes are cleared at different rates (Figure 3). From these examples it can be seen that Ck is removed much faster than AspT, which in turn disappears more rapidly than AlaT. Thus variation in the rates of clearance has an important bearing on the use of enzyme tests in diagnosis, and the cases illustrated indicate the importance of the time factor in relation to a disease process.

It must be admitted, however, that at the present time we do not know much about the fate of serum enzymes. As long ago as 1960, Strandjord et al. (17) showed that excretion in the urine or in the bile was a minor factor in the clearance of isocitrate dehydrogenase and LDH, since in experimental animals the disappearance rates for these enzymes were unaffected by hepatectomy, splenectomy, or nephrectomy. Except in renal disease, only minor amounts of most serum enzymes are excreted. The principal exception is amylase, which, owing to its relatively low molecular weight (about 45,000), is readily filtered by the kidney.

Among the possible mechanisms whereby serum enzymes may be removed are intravascular inactivation either through inhibition by small molecules, for which there is some evidence, or by antigen-antibody autoimmune reactions. The latter appear unlikely since a second episode of myocardial infarction soon after the first causes enzyme activities in serum to increase as much as on
the first occasion. Another possibility is that serum enzymes might find their way into the small intestine, where they would be digested like any other proteins and their constituent amino acids would return to the amino acid pool.

Yet another means is suggested by some recent work by Vesell et al. (18) on the relative rates of synthesis and destruction of LDH, in rat heart and liver. This isoenzyme is characteristic of the liver, which was found not only to synthesize it faster than the heart, but also to destroy it more slowly. It is clear therefore that tissues have built-in mechanisms for degrading enzyme protein and it seems likely that highly vascular organs might well play a significant role in removing enzymes from the plasma.

The turnover rates of serum enzymes have recently been discussed in greater detail by Posen (18).

Precision of Enzyme Tests

I mentioned earlier that the precision of some of our enzyme procedures leaves much to be desired. Even the best of the standard methods tend to have a coefficient of variation of about 5% for replicate analyses on the same specimen in the same laboratory by different technicians on different days. This means that in one of 20 samples, the error might be greater than ±10%. This is of particular significance when enzyme ratios are used as diagnostic tests, since if such errors are in opposite directions for the numerator and denominator, the ratio might be in error by 20% or more. At the present time, therefore, the use of enzyme ratios might on occasion be misleading.

Instrument and reagent manufacturers, including the sponsors of this lecture, are of course aware of this problem and have introduced a number of new techniques to improve the precision of routine enzyme tests. Although kinetic methods are undoubtedly more reliable than two-point assay systems, since the course of the reaction can be followed, they require more elaborate instrumentation and make greater demands upon the competence of the technicians. They are more difficult to perform in the large numbers now requested, but several new instruments and methodologies have recently become available that suggest that in future we shall be able to get more precise results.

Anomalous Results for Enzyme Activity

Despite careful attention to methodology, we occasionally encounter a result which conflicts with our previous experience, and which in isolation is difficult to interpret. My associates and I have described one such case, a patient in whose

Fig. 7. Serum LDH activities in a patient with pernicious anemia during therapy with vitamin B12

Fig. 8. Serum enzyme activities in a patient with idiopathic myoglobinuria

The biological half-lives for each enzyme are shown. (Results kindly provided by D. A. Arvan and Eleanor Griffith)
serum we detected the presence of additional zones of activity between LDH1 and LDH2 and between LDH2 and LDH4 (20). Several investigators have reported extra bands in the serum or tissues of patients with neoplastic diseases, but no explanation had hitherto been offered. After the death of the patient, samples of liver taken at autopsy showed a marked excess of the fast isoenzymes, which is different from the usual liver isoenzyme pattern. This distribution is also quite different from that frequently found in malignant tissues, when a shift toward the slow components is commonly observed.

One sample of the liver tumor showed a pattern of five evenly spaced isoenzymes, four of which migrated faster than the normal isoenzymes. We therefore suggested that the abnormal LDH is composed of an abnormal M subunit elaborated by the tumor. The even spacing of the five components strongly supports this view. The extra bands found in the serum would appear to be hybrids of the normal H subunit with the abnormal M component. A similar explanation probably could account for the additional bands found by earlier workers.

I should like to end my lecture by referring to another study of anomalous serum enzyme activities that we investigated. Some years ago Drs. Emerson, Withycombe, and I (21) were intrigued by the increase in the serum LDH values in patients who underwent therapeutic dialysis (Figure 9), a phenomenon that I had the opportunity to investigate further at the University of Pennsylvania. Similar observations were made by Ringoir (22) in Belgium, and in this country Morgan et al. (23) demonstrated that dialysates of uremic serum contained an inhibitor of LDH. At the University of Pennsylvania my colleagues, Dr. Ludwig and Miss Senesky, had prepared a series of fractions of bath fluid by counter-current extraction and, in collaboration with these investigators (24), Mr. Fujimoto and I studied their inhibitory action on rabbit-muscle LDH (Figure 10). Three peaks of inhibitory action were observed and, since it had previously been shown that urea and oxalate were potent inhibitors of the enzyme, we determined the contents of these substances in the ten combined fractions. Figure 11 shows that urea was concentrated in fraction 2 while oxalate was found in fractions 4 and 5. That these substances were responsible for the inhibitory effects of these fractions was shown by the fact that both fractions lost their inhibitory action (Table 5) when urea and oxalate were removed. Fraction 2 was incubated with urease in a current of air and oxalate was removed from the combined fractions 4 and 5 either by extraction with t-butyl phosphate, or by precipitation with calcium chloride.

While we obtained strong evidence for urea and oxalate as the sources of the inhibitory effects of fractions 2 and 4 + 5 respectively, we were less successful in identifying the inhibitors in fractions 8 and 9, though we established that they were associated with a neutral extract. An extract at pH 2 contained a number of indole acids, but neither these nor the acid extract inhibited the enzyme. An alkaline extract at pH 11 was similarly devoid of inhibitory action.

The neutral extract was shown by thin-layer chromatography to contain, among other components, 4-aminimidazole-5-carboxamide, which had previously been detected in uremic sera by Asatoor (25). This compound inhibits the enzyme at concentrations exceeding 10 mmol/liter, but unless its inhibitory action is potentiated by other
substances, it seems unlikely that it can account for the whole of the inhibitory effect of fractions 8 and 9.

Another example of an unexpected result leading to advances in our knowledge is the study by Berk et al. (26) of a patient with an enhanced serum amylase activity, who excreted little enzyme in the urine. This led to the recognition of the state of macroamylasemia, in which the serum enzyme appears to occur in a polymerized state.

During this lecture I have concentrated on deficiencies in our knowledge. Where do enzymes come from? What happens to them after they reach the plasma? How can we explain anomalous results? It is vital that we should continue to ask these and other questions and to take every opportunity to find some of the answers. When unexpected results are observed and confirmed, we must endeavor, in collaboration with our clinical colleagues, to find an explanation.

That our empirical use of serum enzyme tests nevertheless has given results of diagnostic value is shown by the increase which has taken place over the past few years. Figure 12 shows that during my time at the University of Pennsylvania the total workload increased 2.5 times while the number of enzyme tests increased fourfold.

I wish to express my appreciation of the co-operation of numerous laboratory and clinical colleagues at the University of Pennsylvania, especially Drs. Howard Rawnsley and Dean Arvan. Some of the work described was supported by grant no. AM 10447, USPHS.

References


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Table 5. Effect of Removal of Urea and Oxalate from Uremic Dialysate Fractions on Their Inhibition of LDH

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dialysate fraction</th>
<th>Activity, % of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>2 (after removal of</td>
<td>65  67</td>
</tr>
<tr>
<td></td>
<td>urea with urease)</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>2 (after removal of</td>
<td>57  ...</td>
</tr>
<tr>
<td></td>
<td>urea with urease)</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>(4 + 5)</td>
<td>94  ...</td>
</tr>
<tr>
<td></td>
<td>(LDH4) (4 + 5) (oxalate pptd. with CaCl2)</td>
<td>87  ...</td>
</tr>
<tr>
<td>Heart</td>
<td>(4 + 5)</td>
<td>67  54</td>
</tr>
<tr>
<td></td>
<td>(LDH4) (4 + 5) (oxalate extracted with tributyl phosphate)</td>
<td>115  96</td>
</tr>
</tbody>
</table>