Enzyme assays in liver disease

R. J. WIEME AND L. DEMEULENAERE

From the Laboratory for Protein Chemistry, Medical Department, Academic Hospital, University of Ghent, Belgium

It is in the field of hepatology that enzyme assays are performed most frequently for diagnostic purposes. Their interpretation requires knowledge of many factors that influence the serum level of the particular enzymes assayed. Therefore, these factors are discussed briefly in relation to liver disease before considering important developments such as the value of estimating D-glutamyltransferase (EC 2.3.2.1)¹ and the isoenzymes of alkaline phosphatase (EC 3.1.3.1). Finally, the application of these criteria to specific diagnostic problems is considered.

General Considerations

The concentrations in normal human liver of the most important enzymes for the diagnosis of liver disease are given in Fig. 1; they are based on published data with the exception of those for D-glutamyltransferase which are the results of just two analyses which we performed to complete this survey. It is clear that the diagnostic value of an

¹Also known as γ -glutamyltranspeptidase.

Fig. 1 Concentration of important enzymes in liver, expressed in international units per gram fresh liver tissue. LD = lactate dehydrogenase, MD = malate dehydrogenase, As-AT = aspartate aminotransferase, AI-AT = alanine aminotransferase, AP = alkaline phosphatase, GMD = glutamate dehydrogenase, SD = sorbitol dehydrogenase, ALD = ketose-phosphate aldolase, GMT = D-glutamyltransferase, and OCT = ornithine carbamoyltransferase.

enzyme cannot be ascertained from its concentration in the liver.

In pathological conditions these concentrations may be very different. For example, the liver isoenzyme of lactate dehydrogenase (LD_b, EC 1.1.1.27) decreases rapidly after a single hepatotoxic dose of carbon tetrachloride, so that a second dose given 24 hours later has no marked effect on the serum level (Wieme and van Maercke, 1961). Conversely, the liver content of alkaline phosphatase was found to increase sevenfold within 12 hours of ligating the common bile duct in the rat, whereas aspartate aminotransferase (EC 2.6.1.1) decreased by 25% (Kaplan and Righetti, 1969). The increase in alkaline phosphatase was inhibited by cycloheximide, suggesting that for at least some liver enzymes increased synthesis is a factor in the response to biliary obstruction, as emphasized previously by Polin, Spellberg, Teitelman, and Okumura (1962). Induced synthesis of D-glutamyltransferase also seems to occur easily.

Another important factor influencing serum levels in disease is the localization of an enzyme within the liver cell (Fig. 2). Some enzymes are found in more than one site, each site containing different iso-

Fig. 2 Schematic representation of the intracellular localization of important liver enzymes. Glutamate dehydrogenase and ornithine carbamoyltransferase are found in mitochondria, alkaline phosphatase and D-glutamyltransferase in microsomes (abbreviations as in Figure 1).

enzymes. Aspartate aminotransferase, for example, is found both in the cell sap and in the mitochondria, whereas alanine aminotransferase (EC 2.6.1.2.) is confined to the cell sap. Slight cell damage tends to release only the enzymes in the soluble fraction of the cell whilst necrotic lesions, which also affect the mitochondria, release both types. It has been suggested that the ratio of serum aspartate to alanine aminotransferase can be used as an index of the severity of liver cell damage (de Ritis, Coltorti, and Guisti, 1955), values less than 1 indicating reversible cell damage not involving the mitochondria, as in early infective hepatitis, and values greater than 1 indicating more extensive cell damage as in the acute exacerbations in the course of liver cirrhosis. According to Schmidt and Schmidt (1962) and Laudahn (1963), a better index of cell necrosis is given by the ratio of the sum of the serum levels of these transferases to the level of the purely mitochondrial enzyme, glutamate dehydrogenase (EC 1.4.1.3). In acute hepatitis, for example, the very high levels of the aminotransferases produce a very high ratio despite the increase in glutamate dehydrogenase levels. However, the marked increase of serum glutamate dehydrogenase, sometimes found in severe cases of myocardial infarction in which liver cell necrosis is absent, suggests that the mitochondria are very sensitive to hypoxia. The usefulness of this method of recognizing necrosis is therefore limited.

It has long been customary to try to distinguish between parenchymal and biliary tract enzymes (Batsakis, Kremers, Thiessen, and Shilling, 1968). In our opinion such a distinction is not clear cut and under certain circumstances can even be misleading. Difficulties arise, whether the enzymes are considered on a functional or an anatomical basis. Thus, when a T-tube draining a completely obstructed common bile duct was clamped for eight hours, the only serum enzyme which increased (Fig. 3) was the mitochondrial enzyme glutamate dehydrogenase (Schultz and Schmidt, 1967). The anatomical variability is illustrated by species differences in the location of glutamate dehydrogenase. In the rat this enzyme is located mainly in the walls of the bile canaliculi, but in the rabbit it occurs mainly in the parenchymal cells (Naftalin, Child, and Morley, 1969a). Clinically, a distinction between biliary and parenchymal enzymes is of dubious value because all liver enzymes tend to participate in the reaction to impairment of bile flow. The distinction is further confused by the existence of a third potential source of enzymes in the liver, namely, primary or metastatic tumour (see alkaline phosphatase).

A meaningful interpretation of changes in serum enzymes must also take into account their turn-over

Fig. 3 At time zero a tube draining the obstructed common bile duct in a patient was clamped. After eight hours, the pressure was released. P = hydrostatic pressure, GMD = serum glutamate dehydrogenase as a percentage of the initial value. The serum levels of alkaline phosphatase and aminotransferase did not change. (From Schultz and Schmidt, 1967.)

rates in the blood. Little is known about the precise mechanism of their disappearance from the plasma, though it seems clear that for the vast majority of enzymes there is no significant loss through the gastrointestinal, renal, or biliary tracts (Massa and Köbler, 1969; Lorentz and Jaspers, 1970; Lorentz, Jaspers, and Adlung, 1970; Posen, 1970). The rate of disappearance from the blood stream is proportional to the concentration of the enzyme, but varies with the nature of the enzyme. It is best expressed as the biological half-life, and is related to certain of the properties *in vitro* of the enzyme such as its lability and solubility. For example LD₅, which is the most labile isoenzyme *in vitro*, has the most rapid turn-over *in vivo*; similarly the enzymes found in the insoluble fraction such as mitochondrial malate dehydrogenase (EC 1.1.1.37) and aspartate aminotransferase also have short biological half-lives.

In a recent survey, Posen (1970) discussed the difficulties encountered in determining these values. They are especially great for liver enzymes because the organ itself plays an important role in determining the half-life. Studies after hepatectomy are useful in this respect, but obviously cannot be made in man. Figure 4 shows recent data from Bär and Ohlendorf (1970) which, though approximate, are in accord with clinical observations. They explain, for example, one difference between serum alanine aminotransferase and LD₅; whereas the former never declines abruptly, LD₅ typically appears in the serum in short bursts, so that the ratio of the two enzymes is found to change from day to day. The explanation is, of course, the very short half-life of LD₅ (10 hours), levels of which

Fig. 4 Biological half-life values of important liver enzymes in the plasma. LD_1 and LD_5 are at the extremes of the scale. CK = creatine kinase (other abbreviations as in Figure 1).

therefore reflect more closely what is going on in the cell at the time the blood sample is taken than do levels of the aminotransferase (half-life, about 50 hours) which may relate to an event that occurred some time before. The biological half-life also has an important effect on the magnitude of the rise in the serum, since the longer the half-life the greater the accumulation of the enzyme in the serum.

D-Glutamyltransferase

Although the clinical usefulness of assays of this enzyme was described 10 years ago (Szewczuk and Orlowski, 1960; Szczeklik, Orlowski, and Szewczuk, 1961), interest in it was limited until the development of a stable, soluble and safe substrate, γ -glutamyl-p-nitroanilide (Goldbarg, Pineda, Smith, Friedman, and Rutenburg, 1963; Orlowski and Meister, 1963). A number of laboratories have since reported their experience, the reports being generally favourable (Rutenburg, Goldbarg, and Pineda, 1963; Kammeraat, 1970; Villa, Dioguardi, Agostoni, Idéo, and Stabilini, 1966; Lukasik, Richterich, and Colombo, 1968; Szasz, Rosenthal, and Fritzsche, 1969a and 1969b; Zein and Discombe, 1970). Our own experience extends to more than 600 determinations. Its behaviour resembles both alkaline phosphatase and the aminotransferases, which is apt to be confusing. We have often experienced difficulty in

finding an explanation for pathological values. The correlation with alkaline phosphatase has not been as close as that reported by Idéo and Dioguardi (1970) and the correlation with leucine aminopeptidase (EC 3.4.1.1) is likewise very loose.

In hepatitis the serum D-glutamyltransferase rises at most to about four times normal at the time when aminotransferases reach their peak, but tends to increase further when these begin to return to normal. It also increases in the recovery phase of acute myocardial infarction (Agostini, Idéo, and Stabilini, 1965), a phenomenon difficult to explain because the enzyme is absent from the heart muscle (Naftalin *et al*, 1969a). It is found in kidney, pancreas, liver, and spleen, and—according to our observations—also in placenta, in the ratio 10.0:8.3:3.9:1.5:1.0. The enzyme is thus far from being liver specific, and increased serum levels are found, as expected, in acute pancreatitis (Naftalin, 1970).

A number of properties render it eminently attractive in the clinical laboratory. These include its stability in serum samples, its absence from red cells, the large range of variation combined with the ease of estimation by spectrophotometry (Szasz, 1969) or colorimetry (Naftalin, Sexton, Whitaker, and Tracey, 1969b). It seems likely to become important in biochemical screening.

Lactate Dehydrogenase (LD) Isoenzymes

Isoenzyme studies, although regarded as being of great diagnostic value (Wilkinson, 1970a and b) are not widely used in clinical chemistry because their determination by electrophoresis is relatively difficult and cumbersome. In fact there are now techniques which are quite simple, which are based on the differences in isoenzymes revealed by different conditions of assay, and which are readily adapted to the average laboratory ('class II' methods, Wieme, 1966). If a mixture contains two isoenzymes, at least two sets of conditions are needed. If the total activity of such a mixture under standard and under selective conditions of assay respectively is represented by Y and Y', and the contribution of each isoenzyme by A and A' and B and B' respectively, then the degree of activation or inhibition of the two isoenzymes under the selective conditions may be expressed by their respective inhibition or activation constants:

$$a = \frac{A'}{A} \text{ and } b = \frac{B'}{B} \quad (1)$$

The contribution of the isoenzymes for any mixture of unknown composition can be calculated from the expression:

$$A = \frac{bY - Y' \cdot}{b - a}$$

while that of isoenzyme B is

$$B = Y - A.$$

Solution of these equations is particularly simple when $b = 0$, ie, when one isoenzyme is completely inhibited by the selective conditions. Clearly no solution is possible if both isoenzymes are affected to the same degree, ie, when $a = b$.

A typical example of a class II method is the use of 2-hydroxybutyrate as a special substrate for LD, in conjunction with a standard LD determination (Elliot and Wilkinson, 1961). This can reveal the ratio between A and B subunits in a LD mixture if it is assumed that such subunits do not change their properties when combined with each other into tetramers of diverse composition. The test cannot, however, give information on all five LD isoenzymes in serum, and it does not, strictly speaking, assay LD₁ and LD₅ individually. Nevertheless, we find it useful for the diagnosis of myocardial infarction. When used in the investigation of liver disease it reveals only gross changes in LD₅.

For assay of all five isoenzymes it would be necessary to solve five equations obtained under different conditions of assay. It is simpler to use 'class I' methods which depend on the physical separation of the components followed by their identification, usually by means of their enzymatic activity under conditions which should be as non-selective as possible. Electrophoretic separation is at present the most satisfactory, its main disadvantage being that it is difficult to automate.

For separation of LD isoenzymes any electrophoretic technique may be used since the separation is extremely easy, though quantitative assay of the isoenzymes is more difficult. Cellulose acetate is satisfactory, but we prefer agar gel. It can be dried into a thin film that is easily stored, and four samples may be separated on a single slide in 15 minutes. The pherogram can then be read within an hour. Our present method for enzyme location is different from that previously used (Wieme, 1968). It is more sensitive and highly reproducible, and is described in detail elsewhere (Wieme, 1970).

The value of such analyses to our own clinicians is evidenced by the fact that in 1970 we performed 5,212 routine LD isoenzyme analyses, compared with 1,715 in 1965, the number of beds and their

occupancy remaining unchanged. The clinicians now recognize that simple assay of total LD activity in serum is an incomplete investigation. This is especially true in some cases where total serum LD is normal, with masking of significant changes in the liver isoenzyme LD₅ which, as this is the smallest component of normal serum, may have no detectable effect on the total serum LD activity.

Isoenzymes of Alkaline Phosphatase (AP)

The more experience we have of differentiating the isoenzymes of AP, the more we believe that, as with LD, determination of total AP should be supplemented routinely by isoenzyme studies, because it is not always possible on clinical grounds to select the cases requiring such studies. So long as the isoenzyme system was thought to be rather simple, the use of class II methods seemed appropriate (FitzGerald, Fennelly, and McGeeney, 1969). These methods include the L-phenylalanine sensitivity of intestinal (Fishman, Green, and Inglis, 1963) and placental AP (Fishman, Inglis, and Ghosh, 1968a), the sensitivity to L-homoarginine of bone and liver AP (Fishman and Sie, 1970), the heat stability of placental AP (Neale, Club, Hotchkis, and Posen, 1965), and the use of urea which inhibits the bone isoenzyme slightly, but strongly inhibits the bone enzyme (Posen, 1967; Horne, Cornish, and Posen, 1968). However, it is now known that human serum contains at least five isoenzymes, so that for the complete study the use of class I methods becomes necessary, especially electrophoretic separation. This, however, is difficult. We use starch gel electrophoresis on a large scale for the differentiation of the phenotypes of placental alkaline phosphatase, and for the sake of completeness we examine in this way all sera requiring AP differentiation. However, we are now convinced that we obtain most information from simple agar gel separations at 20 V/cm for 45 minutes. Under these conditions bile, liver, bone, placental, and intestinal AP are clearly separated in this order of decreasing mobility. The separation is at least as good as that obtained with the more complex polyacrylamide procedure (Kaplan and Rogers, 1969; Canapa-Anson and Rowe, 1970). For locating the enzymes we continue to use a technique described previously (Wieme, 1965), but with agarose replacing Noble agar in the substrate layer. Liver and bone alkaline phosphatase are much better separated in agar gel than in starch gel. However, these two tend to associate in such a way that when both are present in nearly equal concentration, a single but broader band is formed with an intermediate mobility. Some typical pherograms are presented in Figure 5. Slight differences occur in the position of

*Since $Y = A + B$ $bY = bA + bB$, and $bA = bY - bB \dots (2)$ since $Y' = A' + B'$ then from (1) above

$Y' = aA + bB$, and $aA = Y' - bB \dots (3)$ subtracting (3) from (2)

$$A = \frac{bY - Y'}{(b-a)}$$

Fig. 5 Separation of serum alkaline phosphatases in a few typical cases. Agar gel electrophoresis, barbitone buffer, pH 8.4, μ 0.05, 20V/cm for 45 min at 12°C.

Numbers indicate respectively the bile (1), liver (2), bone (3), placental (4), and intestinal (5) isoenzymes. Ia = increased bone fraction (Paget's disease); Ib = normal; Ic = normal; IIa = increased liver fraction (hepatitis); IIb = combined increase of bone and liver fractions; IIIa and b = placental alkaline phosphatase from pregnant women (serum heated at 65° for 5 min so that other enzymes are destroyed); IVa and b = bile, liver and intestinal fractions (liver malignancy on cirrhotic base).

placental AP, corresponding to the various phenotypes which are better revealed by starch gel electrophoresis.

It is excessively rare to find all five fractions together in the same sample of serum. Placental AP is present only in pregnant women. The presence of intestinal AP seems related to liver cirrhosis and also to the ABO secretor status, higher levels being found in individuals of blood groups O and B (Beckman, Björling, and Heiken, 1966; Stolbach, Krant, Inglis, and Fishman, 1967) though the concentration in the intestinal tissue is not related to blood groups (Schreffler, 1966).

Electrophoresis is of value in differentiating the liver and the bone enzyme, and even more so in demonstrating the presence of the bile enzyme (de Jong and Haije, 1970). In this case agar gel is superior to both starch gel and polyacrylamide gel, since the bile enzyme does not penetrate into the latter media owing to its high molecular weight. The presence of both liver and bile isoenzymes in an anicteric patient

points to an impairment of the bile flow which we call 'focal block'. The macromolecular bile components are refluxed back into the blood which they cannot leave again. The low molecular weight components such as bilirubin are, however, again extracted from the blood and secreted in zones where the bile flow is not blocked (Hill and Sammons, 1967). The most frequent cause of this type of obstruction is the presence of malignant primary or secondary tumour in the liver.

There is another interesting change in serum alkaline phosphatase in malignancy, namely, the appearance in the serum of an isoenzyme that cannot be distinguished from placental AP, called Regan isoenzyme after the patient in whom it was first demonstrated. In this patient with a bronchial carcinoma the isoenzyme was found both in the serum and in the tumour (Fishman, Inglis, Green, Anstiss, Gosh, Reif, Rustigan, Krant, and Stolbach, 1968b). On starch gel electrophoresis Regan isoenzyme moves as a rapid band, but its main characteristic is its resistance to heating at 65°C for 5 minutes. It was found in the serum of 27 out of 500 patients with various malignant diseases (Stolbach, Krant, and Fishman, 1969), though the serum of 10 had to be concentrated threefold for demonstration by electrophoresis (Fishman, Inglis, Stolbach, and Krant, 1968c). Warnock and Reisman (1969) found a similar enzyme in extracts of eight out of 10 hepatomas and also in the serum of some of these patients; however the serum enzyme was also found in two controls out of 60, namely, cases of chronic hepatitis and fatty liver respectively. The enzyme differed slightly from the Regan isoenzyme, being less thermostable. Another alkaline phosphatase which was heat-labile and insensitive to L-phenylalanine was extracted from a bronchial carcinoma (Timperley, 1968); appropriate studies on serum were not performed in this case though total serum alkaline phosphatase was increased.

Other Isoenzyme Systems

Other systems exist, including those of ketose 1-phosphate aldolase (EC 4.1.2.7),¹ aspartate aminotransferase, glutamate dehydrogenase, and malate dehydrogenase. The latter is probably the most useful clinically.

Malate dehydrogenase occurs in the form of cytoplasmic and mitochondrial isoenzymes. Reports on their electrophoretic differentiation must be viewed with caution because of two possible artefacts. First, oxaloacetate, used as substrate, is labile and breaks down to pyruvate; thus LD bands may also be revealed. Secondly, the migration of the

¹Also known as aldolase or fructose 1-phosphate aldolase.

pancreas and biliary tract there is no great increase of leucine aminopeptidase. Here our findings differ from those of Rutenburg's group (Pineda *et al*, 1960). Serum alkaline phosphatase, on the other hand, can be extremely high in all cases of obstruction irrespective of the site of obstruction. Our experience with D-glutamyltransferase is limited but we find it a useful addition to the conventional group of liver function tests because any increase tends to be very large.

In patients with liver metastases most help is probably obtained from alkaline phosphatase isoenzymes. Not only is the serum level regularly increased early in these cases, but electrophoresis reveals characteristic elevation of both the liver and the so-called bile fractions (Fig. 5). In an anicteric patient this change makes it essential to search for a primary cancer.

Inflammatory liver disease is fairly easily explored by enzyme analysis. The value of the aminotransferases in the diagnosis of viral hepatitis is now well established. Serum lactate dehydrogenase is useful in following the course of the disease as it is the first enzyme to return to normal during recovery. This should be progressive and smooth, and repeated fluctuations occurring after the third week suggest some complication, especially if unrelated to physical exercise. Repeated determinations of serum LD₅ are especially valuable for follow up in chronic active hepatitis. The fluctuations are of brief duration compared with those of the aminotransferases. This, together with the appearance of slow γ -globulins, is very typical for the disease.

In viral hepatitis interesting changes also occur in D-glutamyltransferase, although most authors regard this as a biliary tract enzyme. In the first few days of the disease there is only a moderate increase, but it tends to rise to high levels when the conventional liver enzymes return to normal. Typical changes, seen in a woman with protracted cholestatic hepatitis treated with 15 mg prednisolone daily, are illustrated in Figure 8. When the alkaline phosphatase, alanine and aspartate aminotransferases become normal, D-glutamyltransferase rises from normal to a very high level. Once again it is seen that there is no close correlation between alkaline phosphatase and D-glutamyltransferase.

The application of enzyme analysis to the detection of toxic effects on the liver, especially of drugs, is a rapidly expanding field (Christian, 1970). However, apparent changes in the serum level of enzymes accepted as related to liver disorders should not be taken at face value. It is necessary to establish that the drug or a metabolite does not interfere with the assay. A classical example of such an artefact is the interference of a metabolite of erythromycin estolate

Fig. 8 Changes in serum enzymes in a patient recovering from epidemic hepatitis. D-glutamyltransferase rises when other serum enzymes tend to normalize.

with the colorimetric determination of aspartate aminotransferase (Sabath, Gerstein, and Finland, 1968). Moreover an increase, even if genuine, could be due to a non-hepatic contribution, such as aspartate aminotransferase from muscle (Knirsch and Gralla 1970), or to an adaptive response to the drug. When the effect of drugs is cytolytic or cholestatic, the usual criteria for interpreting enzyme changes apply although, as seen before for ornithine carbamoyltransferase, these are not valid in all cases.

The aminotransferases are widely used and are perhaps relied upon too much when checking drug toxicity. In our opinion LD₅ (Selmeci, Pósch, and Sós, 1968) and ornithine carbamoyltransferase should not be neglected, while D-glutamyltransferase not only seems to be a very sensitive marker for cholestasis or cellular regeneration, but also has been reported as showing marked changes following moderate doses of ethanol (Zein and Discombe, 1970).

Conclusion

We have seen that consideration of the properties of enzymes permits a more sophisticated approach to the interpretation of their assays in liver disease. Assay of an enzyme that increases in plasma in all kinds of liver disease, such as D-glutamyltransferase, no longer seems without merit; indeed the latter, because it increases in so many conditions often to high levels, seems to us to be eminently suited for inclusion in chemical profiling. Differentiation of alkaline phosphatase isoenzymes constitutes another important advance. It is obvious that the combinations of all these data into a meaningful conclusion is to be left to the well programmed computer.

Our investigations of AP isoenzymes were supported by grant no. 1095 from the Foundation for Medical Research in Belgium.

References

- Agostini, A., Ideo, G., and Stabilini, R. (1965). Serum γ -glutamyl transpeptidase activity in myocardial infarction. *Brit. Heart J.*, **27**, 688-690.
- Bär, U., and Ohlendorf, S. (1970). Studien zur Enzymelimination. I. Halbwertszeiten einiger Zellenzyme beim Menschen. *Klin. Wschr.*, **48**, 776-780.
- Batsakis, J. G., Kremers, B. J., Thiessen, M. M., and Shilling, J. M. (1968). Biliary tract enzymology: a clinical comparison of serum alkaline phosphatase, leucine aminopeptidase, and 5'-nucleotidase. *Amer. J. clin. Path.*, **50**, 485-490.
- Beckman, L., Björling, G., and Heiken, A. (1966). Human alkaline phosphatases and the factors controlling their appearance in serum. *Acta genet. (Basel)*, **16**, 305-312.
- Canapa-Anson, R., and Rowe, D. J. F. (1970). Electrophoretic separation of tissue-specific serum alkaline phosphatases. *J. clin. Path.*, **23**, 499-508.
- Christian, D. G. (1970). Drug interference with laboratory blood chemistry determinations. *Amer. J. clin. Path.*, **54**, 118-142.
- Demeulenaere, L. (1963). Fermentdiagnostiek in de hepatologie. IV. Transaminasen GO en GP, Alkalische Fosfatase en Leucine-Aminopeptidase in het serum bij angiocholitis. *T. Gastro-ent.*, **6**, 179-191.
- Demeulenaere, L., Rottiers, R., van Egmond, J., and Lacrès, R. (1968). Aspects particuliers du diagnostic enzymologique en hépatologie. *Acta gastroent. belg.*, **31**, 747-757.
- Elliott, B. A., and Wilkinson, J. H. (1961). Serum α -hydroxybutyric dehydrogenase in myocardial infarction in liver disease. *Lancet*, **1**, 698-699.
- Fishman, W. H., Green, S., and Inglis, N. R. (1963). L-phenylalanine: an organ specific, stereo-specific inhibitor of human intestinal alkaline phosphatase. *Nature (Lond.)*, **198**, 685-686.
- Fishman, W. H., Inglis, N. R., and Ghosh, N. K. (1968a). Distinctions between intestinal and placental isoenzymes of alkaline phosphatase. *Clin. chim. Acta*, **19**, 71-79.
- Fishman, W. H., Inglis, N. R., Green, S., Anstiss, C. L., Gosh, N. K., Reif, A. E., Rustigian, R., Krant, M. J., and Stolbach, L. L. (1968b). Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. *Nature (Lond.)*, **219**, 697-699.
- Fishman, W. H., Inglis, N. R., Stolbach, L. L., and Krant, M. J. (1968c). A serum alkaline phosphatase isoenzyme of human neoplastic cell origin. *Cancer Res.*, **28**, 150-154.
- Fishman, W. H., and Sie, H. G. (1970). L-Homoarginine: an inhibitor of serum 'bone and liver' alkaline phosphatase. *Clin. chim. Acta.*, **29**, 339-341.
- FitzGerald, M. X. M., Fennelly, J. J., and McGeeney, K. (1969). The value of differential alkaline phosphatase thermostability in clinical diagnosis. *Amer. J. clin. Path.*, **51**, 194-201.
- Goldberg, J. A., Pineda, E. P., Smith, E. E., Friedman, O. M., and Rutenburg, A. M. (1963). A method for the colorimetric determination of γ -glutamyl transpeptidase in human serum; enzymatic activity in health and disease. *Gastroenterology*, **44**, 127-133.
- Hill, P. G., and Sammons, H. G. (1967). An interpretation of the elevation of serum alkaline phosphatase in disease. *J. clin. Path.*, **20**, 654-659.
- Horne, M., Cornish, C. J., and Posen, S. (1968). Use of urea denaturation in the identification of human alkaline phosphatases. *J. Lab. clin. Med.*, **72**, 905-915.
- Idéo, G., and Dioguardi, N. (1970). Gammaglutamyl transpeptidase as a diagnostic aid. *Lancet*, **2**, 1036-1037.
- de Jong, M., and Haije, W. G. (1970). De betekenis van de bepaling van de alkalische-fosfatase-activiteit in serum. *Ned. T. Geneesk.*, **114**, 759-762.
- Kammeraart, C. (1970). Serum gamma-glutamyl transpeptidase: een gevoelige indicator van hepatobiliaire aandoeningen. *Ned. T. Geneesk.*, **114**, 1814-1816.
- Kaplan, M. M., and Righetti, A. (1969). Induction of liver alkaline phosphatase by bile duct ligation. *Biochim. biophys. Acta (Amst.)*, **184**, 667-669.
- Kaplan, M. M., and Rogers, L. (1969). Separation of human serum alkaline-phosphatase isoenzymes by polyacrylamide gel electrophoresis. *Lancet*, **2**, 1029-1031.
- Kirsch, A. K., and Gralla, E. J. (1970). Abnormal serum transaminase levels after parenteral ampicillin and carbenicillin administration. *New. Engl. J. Med.*, **282**, 1081-1082.
- Laudahn, G. (1963). Bestimmung der Glutaminsäure-Dehydrogenase-Aktivität im Serum bei verschiedenen inneren Krankheiten. *Klin. Wschr.*, **41**, 618-619.
- Lorentz, K., and Jaspers, G. (1970). Untersuchungen zur biliären Elimination von Enzymen. I. Verhalten von Coeruloplasminder Acylcholinhydrolase, Benzoylcholinhydrolase, alkalischen Phosphatase, Ornithin-Carbamyl-Transferase, Glutamatdehydrogenase und Glucose-6-Phosphatdehydrogenase. *Klin. Wschr.*, **48**, 215-218.
- Lorentz, K., Jaspers, G., and Adlung, J. (1970). Untersuchungen zur biliären Elimination von Enzymen. II. Verhalten von Lactatdehydrogenase, Hydroxybutyratdehydrogenase, Creatin-Phosphokinase, Glutamat-Oxalacetat-Transaminase und Glutamat-Pyruvat-Transaminase. *Klin. Wschr.*, **48**, 218-220.
- Lukasik, S., Richterich, R., and Colombo, J. P. (1968). Der diagnostische Wert der alkalischen Phosphatase, der Leucaminaminopeptidase und der Gamma-Glutamyl-Transpeptidase bei Erkrankungen der Gallenwege. *Schweiz. med. Wschr.*, **98**, 81-83.
- Massarati, S., and Köbler, H. (1969). Über die Elimination der cytoplasmatischen Glutamat-Oxalacetat-Transaminase (GOT) in Intestinaltrakt, Galle und Urin. *Arch. klin. Med.*, **216**, 285-302.
- Naftalin, L. (1970). Serum gamma-glutamyl transpeptidase as a diagnostic aid. *Lancet*, **2**, 829.
- Naftalin, L., Child, V. J., and Morley, D. A. (1969a). Observations on the site of origin of serum gamma-glutamyl transpeptidase. *Clin. chim. Acta*, **26**, 297-300.
- Naftalin, L., Sexton, M., Whitaker, J. F., and Tracey, D. (1969b). A routine procedure for estimating serum gamma-glutamyl transpeptidase activity. *Clin. chim. Acta*, **26**, 293-296.
- Neale, F. C., Clubb, J. S., Hotchkis, D., and Posen, S. (1965). Heat stability of human placental alkaline phosphatase. *J. clin. Path.*, **18**, 359-363.
- Orlowski, M., and Meister, A. (1963). γ -Glutamyl-p-nitroanilide: a new convenient substrate for determination and assay of L- and D- γ -glutamyltranspeptidase activities. *Biochim. biophys. Acta (Amst.)*, **73**, 679-681.
- Pineda, E. P., Goldberg, J. A., Banks, B. M., and Rutenburg, A. M. (1960). Serum leucine aminopeptidase in pancreatic and hepatobiliary diseases. *Gastroenterology*, **38**, 698-714.
- Polin, S. G., Spellberg, M. A., Teitelman, L., and Okumura, M. (1966). The origin of elevation of serum alkaline phosphatase in hepatic disease. *Gastroenterology*, **42**, 431-438.
- Posen, S. (1967). Alkaline Phosphatase. *Ann. intern. Med.*, **67**, 183-200.
- Posen, S. (1970). Turnover of circulating enzymes. *Clin. Chem.*, **16**, 71-84.
- de Ritis, F., Cortotti, M., and Giusti, G. (1955). Attività transaminasi del siero umano nell'epatite virale. *Minerva Med.*, **1**, 1209-1209.
- Rottiers, R., and Demeulenaere, L. (1966). Valeur de la détermination de l'ornithine-carbamyl-transférase (CCT) dans les affections hépatiques. *Acta gastro-ent. belg.*, **29**, 821-830.
- Rutenburg, A. M., Goldberg, J. A., and Pineda, E. P. (1963). Serum gammaglutamyl transpeptidase activity in hepatobiliary and pancreatic disease. *Gastroenterology*, **45**, 43-48.
- Sabath, L. D., Gerstein, D. A., and Finland, M. (1968). Serum glutamic oxalacetic transaminase. False elevations during administration of erythromycin. *New. Engl. J. Med.*, **278**, 1137-1139.
- Schmidt, E., and Schmidt, F. W. (1962). Methode und Wert der Bestimmung der Glutaminsäure-Dehydrogenase-Aktivität im Serum. Ein Beitrag zur Bedeutung der Untersuchung von Enzym-Relationen im Serum. *Klin. Wschr.*, **40**, 962-969.
- Schreffer, D. C. (1966). Relationship of alkaline phosphatase levels in intestinal mucosa to ABO and secretor blood groups. *Proc. Soc. exp. Biol. (N.Y.)*, **123**, 423-427.
- Schultz, C., and Schmidt, E. (1967). Gallengangsverschluss und Enzymaktivitäten im Serum. *Klin. Wschr.*, **45**, 162-163.
- Selmei, L., Pösch, E., and Sós, J. (1968). Drug-induced changes in serum lactic dehydrogenase (LDH) activity and isoenzyme pattern in rats. *Life Sci.*, **7**, 951-955.
- Stolbach, L. L., Krant, M. J., Inglis, N. I., and Fishman, W. H. (1968). Correlation of serum L-phenylalanine-sensitive alkaline phosphatase, derived from intestine, with the ABO blood group of cirrhotics. *Gastroenterology*, **52**, 819-827.
- Stolbach, L. L., Krant, M. J., and Fishman, W. H. (1969). Ecto-

- production of an alkaline phosphatase isoenzyme in patients with cancer. *New Engl. J. Med.*, **281**, 757-762.
- Szasz, G. (1969). A kinetic photometric method for serum gamma-glutamyl transpeptidase. *Clin. Chem.*, **15**, 124-136.
- Szasz, G., Rosenthal, P., and Fritzsche, W. (1969a). Die Gamma-Glutamyl-Transpeptidase-Aktivität im Serum bei hepatobiliären Erkrankungen. *Dtsch. med. Wschr.*, **94**, 1911-1917.
- Szasz, G., Rosenthal, P., and Fritzsche, W. (1969b). Gamma-Glutamyl-Transpeptidase, Leuzinaminopeptidase und alkalische Phosphatase. *Schweiz. med. Wschr.*, **99**, 606-608.
- Szczeklik, E., Orlowski, M., and Szewczuk, A. (1961). Serum gamma-glutamyl transpeptidase activity in liver disease. *Gastroenterology*, **41**, 353-359.
- Szewczuk, A., and Orlowski, M. (1960). The use of α -(N-gamma-DL-glutamyl)-aminonitriles for the colorimetric determination of a specific peptidase in blood serum. *Clin. chim. Acta*, **5**, 680-688.
- Timperley, W. R. (1968). Alkaline-phosphatase-secreting tumour of lung. *Lancet*, **2**, 356.
- Villa, L., Dioguardi, N., Agostoni, A., Ideo, G., and Stabilini, R. (1966). Prognostic value of serum γ -glutamyl transpeptidase activity in liver disease. *Enzymol. biol. clin.*, **7**, 109-114.
- Warnock, M. L., and Reisman, R. (1969). Variant alkaline phosphatase in human hepatocellular cancers. *Clin. chim. Acta*, **24**, 5-11.
- Wieme, R. J. (1965). *Agar Gel Electrophoresis*, p. 163. Elsevier, Amsterdam and London.
- Wieme, R. J. (1966). Improved agar support for electrophoresis of LDH isoenzymes. *Clin. chim. Acta*, **13**, 138-140.
- Wieme, R. J. (1968). Advances in diagnostic isoenzymology. In *Proceedings of the 6th International Congress on Clinical Chemistry, Munich, 1966*, vol. 2: *Clinical Enzymology*, pp. 21-46. Karger, Basel and New York.
- Wieme, R. J. (1970). LDH isoenzymes. In *Methoden der enzymatische Analyse*, edited by H. Bergmeyer, Verlag Chemie, Weinheim. pp. 557-560.
- Wieme, R. J., and van Maercke, Y. (1961). On the fifth (electrophoretically slowest) serum lactic dehydrogenase as an index of liver injury. *Ann. N.Y. Acad. Sci.*, **94**, 898-911.
- Wilkinson, J. H. (1970a). Clinical applications of isoenzymes. *Clin. Chem.*, **16**, 733-739.
- Wilkinson, J. H. (1970b). Clinical applications of isoenzymes. In *Topics in Medicinal Chemistry*, vol. 3, pp. 25-55. Wiley, New York.
- Zein, M., and Discombe, G. (1970). Serum gamma-glutamyl transpeptidase as a diagnostic aid. *Lancet*, **2**, 748-750.