

Progress report

Alkaline phosphatase

This review is selective and reflects the author's personal interest in the application of laboratory techniques to clinical problems; other aspects of the subject have been well covered in recent reviews.^{1,2,3,4,5,6}

Structure

The alkaline phosphatases are zinc metallo enzymes^{7,8} and it is known that zinc has a functional role in the catalysis;⁹ magnesium or cobalt are also required for enzyme activity.¹⁰

Purified placental¹¹ and intestinal¹² alkaline phosphatases contain approximately 15% nitrogen, and the amino-acid compositions of both enzymes are known.^{13,14} Hydrolysis of bacterial alkaline phosphatases has yielded amino-acid sequences around the active centre suggesting a structural as well as a functional analogy with other esterases; the sequence found near the reactive serine residue in the alkaline phosphatase of *E. coli* is similar to the sequences found at the active centres of trypsin and chymotrypsin.^{15,16} The covalent binding of inorganic phosphate to the serine in the active site is a reaction common to the alkaline phosphatases and an intermediate phosphoryl-enzyme is formed during their action.¹⁷

Like a number of enzymes, including caeruloplasmin¹⁸ and pancreatic ribonuclease B,¹⁹ alkaline phosphatase (AP) is a glycoprotein^{20,21} but the carbohydrate content of alkaline phosphatase has been the subject of conflicting reports. Ahmed and King found little or no carbohydrate in human placental AP,¹¹ whilst Ghosh reported appreciable amounts of carbohydrate in the purified enzyme.²² Purified intestinal AP contains substantial amounts of hexose and hexosamine²³ but no sialic acid.²⁴ Alkaline phosphatases from other tissues have, however, been shown to contain bound sialic acid; for instance, the structurally different but functionally similar isoenzymes of human kidney arise as a result of variations in the amount of bound sialic acid.²¹ The neuraminic-acid-containing nature of human placental AP has been demonstrated by chemical means and the sialic acid residues shown to occupy terminal positions away from the active centre of the enzyme.²⁵ Removal of sialic acid residues from AP, as from other glycoprotein enzymes, affects the electrophoretic mobilities of the protein but does not impair enzymatic activity. The role of the carbohydrate units in glycoprotein enzymes is at present not understood but they are known to exist with a heterogeneous complexity which may represent specific genetic characteristics;²⁶ there is no evidence that carbohydrate units participate in catalysis.

Many studies have reported differences in the chromatographic or electrophoretic properties of alkaline phosphatases in serum or in crude tissue homogenates, rather than in purified preparations; in such studies, specific as well as non-specific binding can occur which may account for the degree of

heterogeneity observed. However, studies on highly purified preparations of human placental AP¹³ have shown two major molecular weight variants on Sephadex G-200: the electrophoretically slower was obtained in crystalline form and had a molecular weight of over 200 000, whilst the faster moving variant had a molecular weight of 70 000. The high molecular weight variant could be converted into the lower molecular weight variant by storage for about four months, whilst the process could be reversed by equilibrium dialysis; kinetic studies indicated no difference between the two variants. *E. coli* and human placental alkaline phosphatases are dimers, each monomeric subunit of placental AP having a molecular weight of 58 000.²⁷ Between pH 4.7 and pH 10.3 human placental alkaline phosphatase appears as a homogeneous entity with the molecular weight of the native enzyme; however, above pH 10.3, a monomer-dimer equilibrium appears to be present, and at pH 2.3 the enzyme was also heterogeneous, with the formation of both low molecular weight compounds and high molecular weight aggregates.²⁸

Functions

Robison, the first to suggest an association between AP and calcification in cartilage and bone,^{29,30,31} originally explained calcification as a hydrolysis of a phosphoric acid ester, the calcium salt of which is easily soluble in water, releasing phosphate ions. He assumed that blood is normally in equilibrium with the inorganic constituents of bone and that the supersaturation necessary for the precipitation of bone salt is produced locally in cartilage and osteoid tissue by the alkaline phosphatase at these sites from the phosphoric esters present in blood. This simple hypothesis failed to explain why some tissues calcified and others, which contained large amounts of the enzyme, did not. Robison later conceived of two factors operating in calcification: first the phosphatase mechanism causing a supersaturation of phosphate ions in the matrix fluid of calcifying cartilage and, secondly, a 'local' factor favouring the deposition of calcium phosphate from supersaturated solutions, whether this state of supersaturation in the cartilage matrix be caused by enzymatic hydrolysis or by a supersaturated solution diffusing into the cartilage from outside.^{32,33} The role of alkaline phosphatase in calcification was considered to be of little significance by some later workers³⁴ but was re-emphasized by others;³⁵ more recent work has suggested that alkaline phosphatase plays a key role not only in calcification but also in bone resorption, by removing the layer of pyrophosphate which covers the mineral surface of bone and which has been shown to retard the rate of deposition of calcium and phosphate onto this surface.³⁶

These contributions throw no light on the possible functions of alkaline phosphatase at other sites in the body. The histochemical localization of the enzyme to the absorptive surfaces of the proximal convoluted tubule of the kidney,^{37,38} the small intestinal mucosa,³⁹ the syncytiotrophoblast of the placenta,⁴⁰ and the cell wall of *Escherichia coli*⁴¹ has suggested a connexion between these enzymes and transport, possibly of phosphate across cell walls, and there is some, albeit slender, evidence to support this contention,^{42,43,44,45} including the demonstration of the ability of alkaline phosphatase to bind inorganic phosphate.^{17,46}

Alkaline phosphatase may be connected with protein synthesis in the cell.^{47,48} The enzyme is thought to be concerned with nucleoprotein and nucleotide

metabolism.^{49,50,51} A functional relationship between alkaline phosphatase and RNA has been demonstrated,⁵² and AP may play a role in controlling DNA synthesis and growth;⁵³ the enzyme is capable of hydrolyzing both DNA⁵⁴ and RNA.⁵⁵ Alkaline phosphatases from many tissues possess both pyrophosphatase^{56,57} and phosphotransferase^{58,59} activities, and, in the case of intestinal, kidney, and bone phosphatases the two catalytic activities are the result of the action of the same enzyme. A wide range of compounds have been shown to act simultaneously as substrates of the hydrolase and the phosphotransferase activities of alkaline phosphatase,⁶⁰ and ATP is a good substrate for the enzyme.⁶¹

Clubb and his colleagues raised the serum AP of human volunteers to many times the normal activity by means of enzyme infusions and found no detectable effect on any parameter of calcium or phosphate metabolism, concluding that AP in the circulation appeared to be metabolically inert.⁶² Recent work suggests that intestinal AP is involved in calcium absorption⁶³ and that it may, in fact, be the same enzyme as calcium-activated adenosine triphosphatase.⁶⁴

Infusions of micellar solutions of oleic or octanoic acid into the cannulated rat duodenum result in increased synthesis of AP in the intestinal cell,⁶⁵ whilst similar infusion experiments in man suggest that intestinal alkaline phosphatase plays an active role in the absorption of oleic acid.⁶⁶ The enzyme may also be implicated in the hydrolysis of dietary sphingomyelin to ceramide and water-soluble compounds.⁶⁷ Although there is a good deal of information available about the relationship between fat absorption and intestinal alkaline phosphatase,⁶⁸⁻⁷³ the exact function of the enzyme in this context remains to be elucidated.

Alkaline phosphatases have their optimum reaction in an alkaline medium only at high substrate concentrations; at substrate concentrations approaching the physiological levels of phosphoric esters in the cell, the pH optimum of enzyme activity is close to the physiological pH values⁷⁴ and the enzyme possesses considerable activity even at a pH of 7.2. Hence the so-called 'alkaline' phosphatases can display almost their maximum enzyme activity under the conditions of pH and concentration of phosphate esters existing in the cell. It is clear that the function of the alkaline phosphatases *in vivo* remains unknown, a state of affairs more likely to be due to laboratory ineptitude than to enzymatic impotence.

Alkaline Phosphatase as an Isoenzyme

No definition is universally acceptable for the term isoenzyme; probably a broad definition such as 'different proteins with similar enzymatic activity' is at present the most suitable.⁵ The existence of serum alkaline phosphatase in more than one form was first indicated by paper electrophoresis when serum alkaline phosphatase was reported to migrate mainly in the α 2-globulin position, with a second fainter zone migrating in the position of β -globulin.⁷⁵ Following this, numerous attempts have been made to determine the organ source of serum alkaline phosphatase in health and disease by means of electrophoresis on a variety of media, including starch gel,^{76,77,78} cellulose acetate,⁷⁹ agar gel,⁸⁰ Pevikon C-870⁸¹ and, more recently, acrylamide gel.^{82,83} The results obtained have often been conflicting; in particular, the results obtained on one electrophoretic medium are difficult to relate to those

obtained on another. Even using the same medium, however, conflicting results have been obtained by different workers. Thus it has been claimed that using starch-gel electrophoresis the differentiation between bone and liver alkaline phosphatase can be made on the basis of the minor bands.⁸⁴ Chiandussi *et al* found that whilst the main band in neonates and in patients with bone and liver disease all gave an identical band in the β -globulin region, there were additional minor bands consistently present in the γ -globulin, β -lipoprotein and slow α 2-globulin regions in cases of biliary obstruction, whilst in patients with bone disease, additional minor bands were seen in the β -lipoprotein and slow α 2-globulin regions. In contrast, other workers have claimed to make the distinction between bone and liver enzymes by the position of the major bands, the liver band running slightly ahead of the bone band.⁸⁵ Finally, starch-gel electrophoresis has been found by some to be unhelpful in the differential diagnosis of disease.⁷⁸

This confusing and unsatisfactory state of affairs has now been largely resolved by three main factors. First, it is now realized that the different methods of extraction and preparation of the enzyme from tissue or blood will produce variations in electrophoretic pattern.⁸⁶ Secondly, it is now clear that attention should be focused not only on the position of the main band, but also on its shape; whilst the liver band on starch-gel tends to be slightly faster than bone, the difference is small and there is some overlap.⁸⁷ However, as Newton emphasized, the bone band is more diffuse than the compact liver band. Finally, the recent advent of polyacrylamide gel disc electrophoresis, which is known to give a superior resolution of proteins,⁸⁸ has demonstrated a clear and consistent difference in both mobility and shape between the slower, more diffuse bone band and the faster and more compact liver band.^{82, 83} On polyacrylamide gel slab electrophoresis⁸⁸ human serum alkaline phosphatase can be separated into four distinct isoenzymes. Apart from the bone and liver components which comprise the main band, a third isoenzyme, which is identical to intestinal alkaline phosphatase, is found in some individuals, whilst a fourth isoenzyme which remains at the origin is frequently found in patients with liver disease. Earlier claims that the presence of this origin band implied either biliary obstruction or hepatic metastases rather than hepatocellular disease⁸⁷ have not been substantiated. However, extrahepatic obstruction causing a raised alkaline phosphatase without jaundice is usually partial or intermittent and this may occur in chronic pancreatitis or carcinoma of the pancreas. The isoenzyme characteristics of both normal pancreatic alkaline phosphatase and a tumour variant have been recently described;⁸⁹ in chronic pancreatitis, on the other hand, hyperparathyroid bone disease or osteomalacia may coexist and isoenzyme studies will indicate that the raised alkaline phosphatase is not of hepatic, but of bone origin.

Other methods which have been used to study the isoenzyme characteristics of the alkaline phosphatases include immunological techniques, gel chromatography, and examination of heat stability and urea sensitivity. Immunologically, three antigenic classes of alkaline phosphatase exist. The first includes liver, bone, spleen, and kidney; intestinal alkaline phosphatase constitutes the second class and placental alkaline phosphatase the third.⁹⁰ The second and third classes partially cross-react with each other, and in turn cross-react with a minor kidney component. More recently, it has been shown that within the non-placental, non-intestinal group, an antiserum to liver phosphatase did not react with the phosphatases from bone or kidney.⁹¹ It appears that at

least three genetic loci operate in the production of human alkaline phosphatase isoenzymes.

A difference in relative thermostability between human bone and liver alkaline phosphatases was noted by Moss and King,⁹² and similar differences were later found when serum alkaline phosphatase from groups of subjects with skeletal and hepatobiliary disease was subjected to heat inactivation.⁹³ The clinical value of heat inactivation as a simple laboratory technique to differentiate between elevated serum alkaline phosphatase of bone or liver origin has been confirmed;^{94,95} the liver enzyme is relatively more heat stable than bone alkaline phosphatase, although both enzymes are vastly less heat stable than the placental isoenzyme.^{96,97}

Considerable differences have been found in the susceptibility of various human alkaline phosphatases to urea inhibition.^{98,99} The activation of hepatic alkaline phosphatase by low molarities of urea¹⁰⁰ has not proved to be of a sufficient magnitude to provide a clinically useful tool, but the greater urea stability of the liver enzyme compared to that of bone enables the distinction between the two to be readily made, and provides a technique of a similar order of precision and reliability to that obtained with heat inactivation.^{95,101}

It is also possible to differentiate between bone and liver alkaline phosphatase on Sephadex gel filtration; serum phosphatase from controls and patients with osteoblastic bone disease shows one main peak of enzyme activity moving in the 7S gamma-globulin region, whilst patients with raised alkaline phosphatase of hepatic origin show in addition another peak in the 19S macromolecular region (MW > 200 000), which comprises about 30% of the total activity.^{95,102} Techniques depending upon observations of varying molecular size are, however, too cumbersome for routine clinical use.

Intestinal Alkaline Phosphatase

In 1941 Gomori noted a positive reaction for AP on the surface of human intestinal epithelial cells;¹⁰³ this finding was soon confirmed and the reaction localized to the brush border and Golgi area.¹⁰⁴ The alkaline phosphatase of the small bowel brush border of the mouse is not a single entity¹⁰⁵ and the alkaline phosphatase activity of extracts of human small intestine is heterogeneous on DEAE-cellulose column chromatography¹⁰⁶ and on starch-gel electrophoresis.¹⁰⁷ Much information is available about the distribution and development of alkaline phosphatase in the small intestine.^{108,109,110} Intestinal alkaline phosphatase differs from non-intestinal phosphatases in substrate specificity^{111,112} and in electrophoretic mobility.⁸⁴ A significant advance came with the discovery by Fishman's group that intestinal alkaline phosphatase was strongly inhibited by the stereo-specific inhibitor L-phenylalanine, which inhibited the bone and liver isoenzymes to an insignificant extent,^{113,114} and shortly after this Robinson and Pierce demonstrated that human small intestinal AP differed from the other isoenzymes in being unaltered in electrophoretic mobility by neuraminidase,¹¹⁵ and this was quickly confirmed.²⁰ Up to 60% of normal human sera contain a slow-moving small-intestinal band on electrophoresis,¹¹⁶ and the presence of this isoenzyme in serum is known to be genetically controlled. Thus the small-intestinal band is seen more frequently in the serum of subjects who are blood group O or B than in subjects who are blood group A, and for any given blood group it is seen more fre-

quently in secretors than in non-secretors.^{117,118,119,120,121} The appearance of small-intestinal alkaline phosphatase in serum is, furthermore, known to be related to the ingestion of fat.^{71,116}

Intestinal Alkaline Phosphatase in Duodenal Juice

Duodenal juice contains high concentrations of alkaline phosphatase and isoenzyme studies have indicated that this is partly small intestinal in origin and partly derived from bile.¹²² Following the intravenous injection of both secretin and pancreozymin in man, there is a large increase in the output of alkaline phosphatase in duodenal juice, which is only partly due to increased bile flow since isoenzyme studies have shown large increases in concentration and output of intestinal AP after both secretin and pancreozymin.^{122,123} Both hormones have also been shown to release intestinal AP into duodenal juice in the cat.¹²⁴ Secretion of intestinal AP into the bowel lumen can also be produced by perfusion of long-chain fatty acids,^{125,126} and also by glucose.¹²⁷

Causes of an Elevated Serum Alkaline Phosphatase

The recognized causes of a high serum AP include skeletal disease, hepatic disorders, intestinal disease, tumours, pregnancy,^{3,128} thyrotoxicosis,¹²⁹ the ingestion of anticonvulsant drugs,¹³⁰ and rheumatoid arthritis.¹³¹

SKELETAL DISEASE

Robison first described alkaline phosphatase in the ossifying cartilage of rats and rabbits.^{30,31} Histochemically, the enzyme is found in association with osteoblasts,¹³² which are felt to be the source of the elevated levels of alkaline phosphatase found in growing children, as well as in a variety of bone disorders including rickets,¹²⁹ Paget's disease,¹²⁹ hyperparathyroidism,¹³³ familial osteoectasia,¹³⁴ and osteoblastic bone secondaries.¹³⁵ Estimations of serum alkaline phosphatase have been found more helpful in the diagnosis of osteomalacia following gastrectomy by some workers¹³⁶ than by others,¹³⁷ a disagreement probably based on the fact that neither group employed isoenzyme techniques, which should clearly indicate the presence of 'bone' type AP in the serum of patients with osteomalacia, even when the total serum AP level is only slightly elevated. Of the various techniques available for this purpose, heat inactivation studies are the simplest to employ but recent claims of the ability to quantitate roughly the amount of bone enzyme in serum by electrophoretic means are promising.¹³⁸

HEPATIC DISORDERS

The association between high levels of serum alkaline phosphatase and liver disease has been known for many years,¹³⁹ as has the classical concept that high serum levels of AP are found in 'obstructive' jaundice, whilst in 'toxic' or 'catarrhal' jaundice only slight elevations are found.¹⁴⁰ It is now realized that a clear separation of the various types of jaundice into 'obstructive' and 'non-obstructive' is not feasible.¹⁴¹ Thus, whilst it is true that more than 80% of patients with extrahepatic obstruction have a serum alkaline phosphatase of more than 30 KA units per 100 ml, up to 40% of patients with hepatitis also have enzyme levels exceeding this figure.¹⁴² There are two main theories to explain the elevations of serum alkaline phosphatase in liver disease. The

'retention' theory postulates that in biliary obstruction there is a failure of excretion through the biliary tract of alkaline phosphatase, derived from bone, whilst the 'regurgitation' theory considers the increase in serum AP in hepatic disorders to be due to the passage into the circulation of enzyme derived from bile by way of communications between the bile canaliculi and the sinusoids.¹⁴³ The conflicting experimental evidence in favour of each theory has been previously summarized^{1,3} but recent work has come down strongly on the side of the regurgitation theory. Thus, it has been shown that ligation of one hepatic duct in the experimental animal results in increased production of alkaline phosphatase by the liver rather than decreased excretion of the enzyme,¹⁴⁴ and experiments with isolated perfused liver preparations have shown that obstruction to biliary outflow leads to a rise in the AP activity of the perfusate, suggesting increased formation of the enzyme in the liver.¹⁴⁵

In man, experiments in which heat-stable human placental AP was infused into normal subjects and into patients with biliary obstruction suggest that it is unlikely that alkaline phosphatase is excreted via the biliary tract, since patients with biliary obstruction catabolize the infused enzyme at rates similar to those found in normal subjects and infused enzyme cannot be detected in the urine, faeces, or bile of recipients.⁶² Clubb *et al*, therefore, suggest that the reason for the elevation of serum alkaline phosphatase in patients with biliary obstruction is the increased delivery into the circulation of enzyme derived from the cholangiolitic epithelium. Further evidence against the excretion theory comes from isoenzyme studies since, as already mentioned, qualitative differences can be demonstrated between the alkaline phosphatase circulating in patients with hepatic disorders and that found in the serum of patients with osteoblastic skeletal disease. Finally, recent work has thrown light on the mechanism of the rise in serum AP following bile duct ligation.¹⁴⁶ Within twelve hours of bile duct ligation in rats, there is a sevenfold increase in the concentration of AP in the liver, whilst the enzyme concentration in serum increases by two and a half-fold due to an increase in an isoenzyme originating in liver. Since both rises were inhibited by cycloheximide, the rise in hepatic AP activity appeared to be due to protein synthesis *de novo*, in contrast to the rise in serum glutamic pyruvic transaminase which followed bile duct ligation which was due to simple leakage of preformed enzyme from damaged cells.

The poor correlation between serum AP and bilirubin levels in liver disorders is thus a reflection of the differing mechanisms responsible for the accumulation of bilirubin and alkaline phosphatase. In the diagnosis of liver disorders, AP determinations play an important part, particularly when performed in conjunction with isoenzyme studies; from the point of view of prognosis, however, they are of much less help.¹⁴⁷

INTESTINAL DISEASE

Observations of elevated levels of intestinal AP in disease have been few, and to a large extent uncontrolled. Unfortunately, until a healthy control population of differing age groups and of known blood groups and secretor status has been studied, both fasting and also after a standard fat meal, the normal range of intestinal alkaline phosphatase in serum will not be available.

Fishman *et al*¹⁴⁸ first reported that some cirrhotics had increased amounts of intestinal alkaline phosphatase in their serum; though the average value in cirrhosis did not differ from the normals, there was a wider range of values

amongst the cirrhotics (10-80% intestinal component). About one third of cirrhotics had high levels of intestinal AP and in one of these grossly elevated levels were seen. Most of this group with elevated intestinal AP levels had portal hypertension.

Other workers have assayed raised levels of intestinal AP in fasting blood electrophoretically in order to diagnose cirrhosis; it appears that the levels of intestinal AP reached in serum following a fatty meal are genetically controlled, being linked to the ABH system and the ABO blood groups in patients with cirrhosis, as in normals.¹⁴⁹

There is one report¹⁵⁰ of a case of steatorrhoea of unknown cause associated with a raised serum alkaline phosphatase of mainly intestinal origin. However, no definitive diagnosis was possible during life, and no necropsy carried out.

TUMOURS

Alkaline phosphatase has been reported in a variety of tumours and there is evidence that tumours may secrete variant forms of alkaline phosphatase into the circulation.¹⁵¹ Recently, a tumour isoenzyme, named the Regan isoenzyme after the patient in whom it was first found, has been isolated from the serum and tumour tissue of patients with a wide variety of tumours.^{152,153} This tumour isoenzyme can be recognized in serum since it has properties virtually identical to placental AP in being L-phenylalanine sensitive and very heat-stable. It appears to represent another example of the ectopic synthesis by tumour cells of an enzyme protein not normally manufactured in the tissue from which the tumour originated. The ectopic production of hormones such as ADH, and the synthesis by tumour cells of proteins normally found only in foetal tissue, is well recognized. The carcinoembryonic antigen,¹⁵⁴ and alpha-fetoglobulin¹⁵⁵ in the serum of certain patients with hepatoma may, like the Regan isoenzyme, result from the derepression of specific genomes within the tumour cell.¹⁵⁶ The Regan isoenzyme, however, probably represents a special case, since many tumours produce isoenzymes of alkaline phosphatase which are heat-sensitive and phenylalanine-resistant.^{89,157,158}

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