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REVIEW

ALKALINE PHOSPHATASE

LABORATORY AND CLINICAL IMPLICATIONS

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CONTENTS

List of abbreviations 420
1. Introduction 420
2. Laboratory implications 422
  2.1. Heterogeneity of ALP 422
  2.2. Methods of ALP isoenzyme differentiation 423
  2.3. Electrophoretic techniques 426
  2.4. Latest developments in isoelectric focusing 428
  2.5. Critical evaluation of ALP staining techniques 429
3. Clinical implications 431
  3.1. Alkaline phosphatase in daily routine 431
  3.2. Bone diseases 432
    3.2.1. Bone calcification 432
    3.2.2. Osteomalacia and rickets 433
    3.2.3. Osteoporosis 433
    3.2.4. Renal osteopathy 434
    3.2.5. Hyperparathyroidism 434
    3.2.6. Ostitis deformans (morbus Paget) 435
    3.2.7. Familial hyperphosphatasemia 435
    3.2.8. Skeletal disorders and fractures 435
  3.3. Hepatobiliary diseases 436
    3.3.1. Rheumatic diseases associated with abnormal ALP activities 436
  3.4. Hypophosphatasemia 437

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3.5. Placental alkaline phosphatase 437
3.6. ALP - a tumour-associated factor or "tumour marker?" 437
3.7. Neutrophil ALP 438
4. Summary 438
5. Acknowledgements 439
Note added in proof 439
References 439

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
AS	Anilinic acid
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilized pH gradients
LP-X	Lipoprotein X
PI-link	Phosphatidylinositol linkage of ALP to cell membranes
PLAP	Placental alkaline phosphatase
PTH	Parathyroid hormone

1. INTRODUCTION

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1., ALP) has, on the occasion of its 80th birthday, been described as a "tantalizing jigsaw puzzle" [1]. It may be somewhat provocatively stated that since its discovery in 1907 by Suzuki et al. [2], since the first descriptions of its presence in mammalian tissues [3,4] and its involvement in bone mineralization by Robison, and since the findings of Kay [5] and, independently, Roberts [6] of increased ALP activity in sera of patients with liver and bone disease, hardly anything has been revealed about the physiological substrate of this enzyme. Numerous researchers have, for years, been trying to fit the pieces of the jigsaw puzzle together [7–11] and we would like to dedicate this review to all the scientists who have worked and are working in the field of ALP research.

ALP acts on a number of synthetic substrates, the pH optimum of the catalysed reaction being ca. 10 in vitro. It is a metallo-enzyme with zinc as the constituent metal ion. The activity of the enzyme is increased in the presence of metal ions such as magnesium, cobalt and zinc, but for optimal activity a correct ratio of magnesium and zinc ions is required. The buffer systems used in measurements of ALP activity can be classified as inert (carbonate, barbital), inhibiting (glycine, propylamine) or activating (aminomethylpropanol, diethanolamine, Tris). The forms of ALP present in serum predominantly originate from the liver and bone, although forms from the placenta, the intestine and (rarely) the kidney may contribute toward the total ALP activity [12].

ALP is ubiquitous, being present in nearly every organ, although the highest activities are found in the intestinal epithelium, kidney tubules, osteoblasts, liver and placenta. It is closely associated with the cell membrane [13–15], and for a

long time it was believed to be integrated in the plasma cell membrane by its C-terminal end with hydrophobic interactions with the lipid bilayer, the major portion of the polypeptide chain being localized extracellularly. Studies with ALP from tissue sources thus involved solubilization of the enzyme, mainly after butanol extraction, although non-ionic and zwitterionic detergents or partial proteolysis with trypsin, pepsin or papain have also been used. Only recently has it become clear that ALP belongs to the class of proteins that are anchored to the plasma cell membrane via a phosphatidylinositol–glycan linkage (PI-link, Fig. 1), which has been characterized for human placental ALP [16–19]. Thus the solubilization of the enzyme is also possible with the use of phospholipase C with specificity for phosphatidylinositol [20–22]. This PI-link may represent an economical mechanism for the coordination of cell surface expression of ALP with the modification of intracellular metabolism by several independent second messenger pathways, such as 1,2-diacylglycerol (activator of protein kinase C) or glycosylinositol phosphate. ALP is a dimeric glycoprotein, and shows extensive intra- and inter-tissue charge heterogeneity and variations of the apparent molecular mass (M_r) of the purified enzyme. M_r values ranging between 130 and 220 kD have been reported after butanol extraction, 128 kD after solubilization with the detergent Emulgophen BC 720, 160 kD after solubilization with sulfobetaine and 122 kD after papain solubilization, depending on the tissue sources. Solubilization of liver ALP with PI-specific phospholipase C leads to forms similar to the serum-soluble forms, suggesting that the serum-soluble forms arise after enzymatic hydrolysis of phosphatidylinositol [23–29].

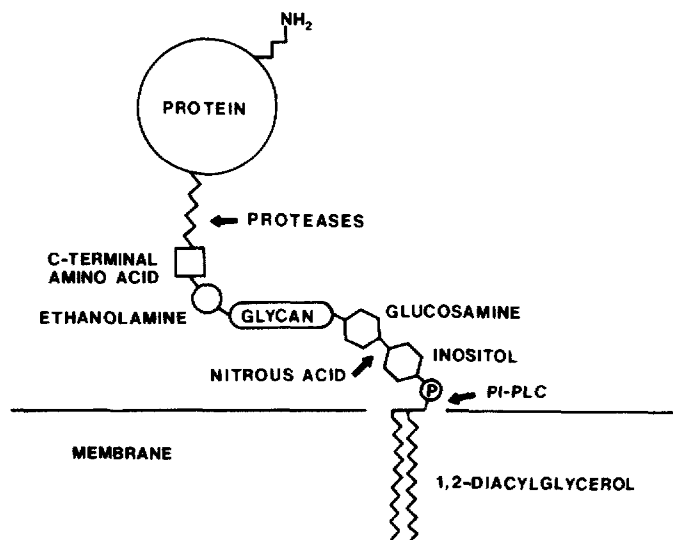

Fig. 1. Arrangement of the components involved in the PI-link with which ALP is anchored to the plasma cell membrane (according to Low, ref. 19, with permission).

2. LABORATORY IMPLICATIONS

2.1. Heterogeneity of ALP

The microheterogeneity of ALPs was demonstrated by the first attempts to purify the enzyme from different sources. The presence of multiple molecular forms was then confirmed by the use of peptide mapping of tryptic or cyanogen bromide digests, the use of substrates and inhibitors and immunological techniques using poly- or monoclonal antibodies directed against the enzyme purified from different sources. In general, the microheterogeneity can have a genetic basis or can be caused by post-translational modification of the enzyme after its synthesis [12].

Three distinct gene loci for the enzyme have already been established, namely the gene for placental ALP (PLAP), the gene for the adult intestinal ALP and a single gene locus, the so-called tissue-unspecific gene, for the ALP from liver, kidney and bone and perhaps other sources [30,31]. Extensive polymorphism is demonstrated only by the placental ALP gene, with the presence of three alleles (Pl^1, Pl^2, Pl^3), which give rise to six phenotypes (three heterozygous 2-1, 3-1, 3-2 and 1, 2 and 3 most likely homozygotes) of the heterodimeric enzyme. Furthermore, at least fifteen rare alleles giving rise to 38 phenotypes of placental ALP have been reported [32].

Although the fetal and adult intestinal ALP are similar in catalytic activity and seem also to be immunologically similar, they are coded by different genes [33]. Many forms of adult intestinal ALP have been reported, but these forms are probably due to post-translational glycosylation of the enzyme, although other mechanisms (e.g. phosphorylation, acylation, sulphation) cannot be excluded [34,35]. Thus together with the gene that codes for the liver, bone and kidney ALP, four distinct gene loci seem to code for ALPs. The forms of ALP derived from these four genes can be regarded as true isoenzymes [30] and those resulting from post-translational modifications as multiple or isoforms of the enzyme but not isoenzymes.

Recently published results on the structure and expression of ALP from rat osteosarcoma may be useful in reviewing the genetical basis of ALP heterogeneity [36,37]. The authors have purified ALP from the rat bone, kidney, placenta and osteosarcoma using monoclonal antibodies. The molecular mass of the enzyme varied from 68 to 82 kD, depending on the source, but converged to 52 kD after deglycosylation. The cDNA, which has 2415 bases, yielded in Northern blots a 2.5-kb band with RNA isolated from bone, liver, placenta, kidney and lung but not with the RNA from the intestine. Southern blot studies suggest the existence of a single gene for all sources of ALP mentioned above, except for the rat intestine. These results may express species specificity but at present they are inconsistent with the existence of a separate gene locus for PLAP.

Non-genetic causes for the microheterogeneity of ALP are post-translational modifications of the enzyme, mainly the presence of carbohydrate side-chains [38]. An association of ALP with other macromolecules can additionally lead to differences in charge and molecular mass [39–42]. Although the exact composi-

tion of the carbohydrate side-chain of ALP is unknown, work using neuraminidase has led to the clarification of the observed microheterogeneity. Except for the adult intestinal ALP, the charge-dependent differences in electrophoretic mobility of ALP from the same tissue are caused by the presence of terminal N-acetylneuraminic acid residues. These charge differences between the tissue enzymes can be abolished by extensive hydrolysis with neuraminidase [43]. Different glycosylation of the ALP from various tissues, e.g. liver, kidney and bone, may be responsible not only for the electrophoretic differences noticed but also for their thermolabilities [44]. It should, however, be remembered that owing to the vast numbers of purification techniques some of the reported multiple forms of ALP may be artefactual.

2.2. *Methods of ALP isoenzyme differentiation*

A wide variety of techniques have been used to study the isoenzymes of ALP. Generally, non-separation techniques can be differentiated from separation techniques. Table 1 summarizes the most important and commonly used methods.

The isoenzymes of ALP demonstrate different susceptibilities towards thermal denaturation. Treatment at 56°C for 15 min inactivates the isoforms in the following order: bone, liver, intestinal and placental. Treatment at higher temperature (65°C, 10 min) inactivates all but placental isoforms [10,12].

An alternative to heat inactivation studies are chemical inactivators: the most commonly used are urea or L-phenylalanine. Again, bone ALP is most susceptible to denaturation by urea at concentrations up to 3 *M*. Placental and intestinal ALP are most resistant, liver ALP having intermediate susceptibility. L-Phenylalanine, on the contrary, inhibits the placental and intestinal forms most and has very little effect on the bone and liver ALP forms. The inhibition of ALP isoforms by L-homoarginine is similar to that caused by urea. Certain forms of

TABLE 1

NON-SEPARATION AND SEPARATION TECHNIQUES OF ALP ANALYSIS

Non-separation techniques	Separation techniques
1. Heat inactivation	1. HPLC
2. Chemical inhibition	2. Chromatofocusing
Urea	3. Electrophoresis
L-Phenylalanine	(a) Zone electrophoresis
Homoarginine	Starch gels
Levamisole	Cellulose acetate
L-Leucine	Agarose gels
L-*p*-Bromotetramisole	Polyacrylamide gels
3. Immunological techniques	(b) Affinity electrophoresis
	Zone electrophoresis in the presence of lectins
	(c) Isoelectric focusing
	Carrier ampholytes
	Immobilized pH gradients

ALP (tumour-derived, e.g. placenta-like) can be inhibited by L-leucine [31]. Levamisole or its derivative L-*p*-bromoteramisole [45–48] inhibit most forms of ALP except the intestinal, and are effective at very low concentrations (0.1 m*M*). Thus a combination of heat and chemical inactivation, although not very specific, can be effectively used as a simple method for the determination of the origin of ALP isoforms in body fluids.

Amongst the separation techniques, the single most useful method has been electrophoresis of ALP isoforms in combination with specific enzyme staining. Before this is discussed in detail, it is worth mentioning a few methods that might gain importance in the future. The first of these is the analysis of ALP isoforms using high-performance liquid chromatography (HPLC) [49]. For example, serum or tissue homogenates can be applied to a strong anion-exchange (Mono Q) column and, after unbound material has been washed away, the bone forms of ALP (KI and KII) and the liver forms (LI and LII) can be eluted stepwise from the column with various amounts of lithium chloride (Fig. 2). [50]. The reproducibility of these results has been questioned in a recent article [51], which states that using the same column, elution profile and buffer systems, it was not possible to resolve the bone ALP (KII) from one liver isoform (LI).

A second relatively new separative technique is chromatofocusing, a liquid chromatographic procedure, which separates proteins according to their isoelectric points. Owing to different degrees of glycosylation, it is expected that the multiple forms of ALP should have different p*I* values. Chromatofocusing has been successfully applied for the fractionation of ALP forms from the brush border and the basolateral membranes isolated from the rat jejunum (Fig. 3). Numerous forms of ALP were fractionated when villous and crypt cells from the jejunal mucosa were solubilized and subjected to chromatofocusing. Solubilization and fractionation of brush border and basolateral membranes yielded single forms of ALP, with p*I* values of 5.6 and 4.7. Apparently the enrichment factor of

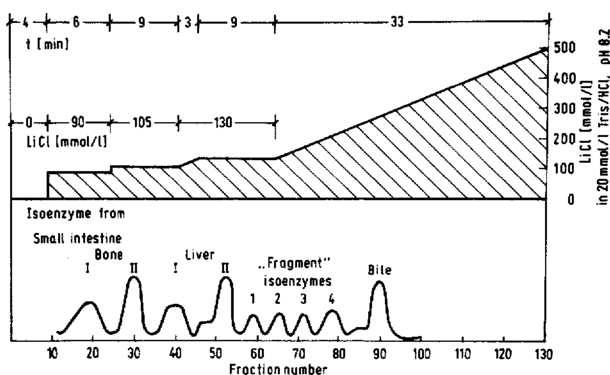

Fig. 2. Resolution of ALP isoforms in serum by HPLC. Chromatography of serum samples on a Mono Q HR 5/5 (50 mm ×5 mm I.D.) column. Schematic compilation of the methodology used. From top to bottom are the elution time of each lithium chloride concentration, the steps of lithium chloride concentrations and the respective fraction numbers. For illustration a fictional elution pattern is used. (Figure and legend from ref. 50, with permission.)

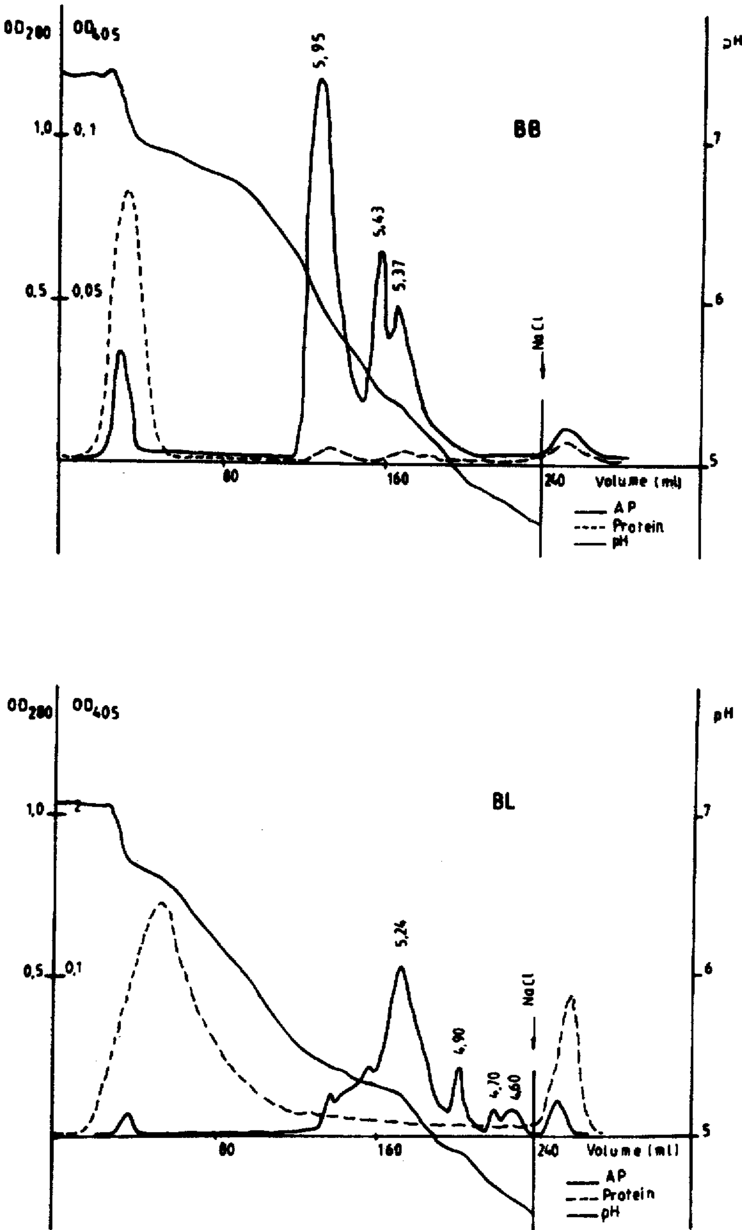

Fig. 3. Chromatofocusing of ALP released from brush border (BB) and basolateral (BL) membranes after 1 h incubation in the presence of 0.5% Triton X-100 at 25 °C. Polybuffer exchanger PBE94 equilibrated with 0.025 *M* imidazole buffer, pH 7.4. Elution with Polybuffer 74. (Figure and legend from ref. 52, with permission.)

purification is greater than 10 000, thus making chromatofocusing a potentially powerful tool for the isolation and analysis of ALP forms from different organs and body fluids [52].

2.3. Electrophoretic techniques

Electrophoretic analysis has been performed in starch, agar, agarose and polyacrylamide gels, as well as on cellulose acetate membranes. The use of starch gels has, since the introduction of polyacrylamide gels, practically become obsolete. Every other matrix used has its advantages and disadvantages. Non-sieving media, such as cellulose acetate and agarose, have the advantage that high-molecular-mass forms of ALP can easily enter the gel. The disadvantage of agarose electrophoresis, namely the electroendosmosis, has been reduced to a minimum by the introduction of relatively pure charge-neutral agaroses. The use of polyacrylamide gels is common in most laboratories. The greatest disadvantage of this sieving medium is that high-molecular-mass phosphatases that are present in serum of cholestatic patients do not enter the gel. This disadvantage, however, is more than balanced by the fact that the diffusion of protein zones is slow, thus enabling the staining times to be prolonged without loss of resolution.

At alkaline pH values (above pH 8.0) the ALP forms from the liver have the highest anodal mobility. The zones obtained from the bone are more diffuse and tend to overlap with the liver zones. Placental ALPs have more or less the same mobility as forms from the liver, and the separation depends on the phenotype. Intestinal ALP forms have lower mobility than those from the bone. In certain cases a "fast liver" form can be identified in cholestatic sera [12]. This simple technique can be combined with heat inactivation or chemical inactivation studies in order to increase the diagnostic specificity and sensitivity of ALP isoform analysis.

The main disadvantage of this method is the incomplete separation of the liver and bone ALP zones. Two simple ways of improving the separation have been reported. The first is the use of neuraminidase to hydrolyse the terminal N-acetylneuraminic acid residues. Treatment of serum samples with *Vibrio cholerae* neuraminidase (50 μl of serum plus 5 U of neuraminidase for 15 min at 37°C) changes the charge properties of both isoforms, enabling complete separation of the liver (most anodal) from the bone forms [53]. Extensive hydrolysis with neuraminidase abolishes the charge differences, leading to identical electrophoretic mobilities. Although it is still unclear why the terminal sialic acid residues of the bone ALP are more susceptible to neuraminidase treatment, the use of this limited hydrolysis technique is extremely useful for the separation of bone and liver ALP forms. The presence of tumour-derived or placental ALP may lead to confusing results due to their electrophoretic mobility, which is similar to those forms from the liver.

Improved resolution of the bone and liver ALP forms can be obtained using a simple and elegant technique described by Rosalki and Foo [54]. In this case, serum or plasma samples are treated with wheatgerm lectin, which specifically binds N-acetylglucosamine residues and preferentially precipitates ca. 80% of the bone isoforms, leaving the liver ALP in the supernatant. Some biliary ALP is also precipitated, but this can be avoided by inclusion of detergents, such as Triton X-100, in the precipitation mixture. Alternatively, an affinity electrophoresis procedure, which includes wheatgerm lectin in buffers used to soak the cellulose

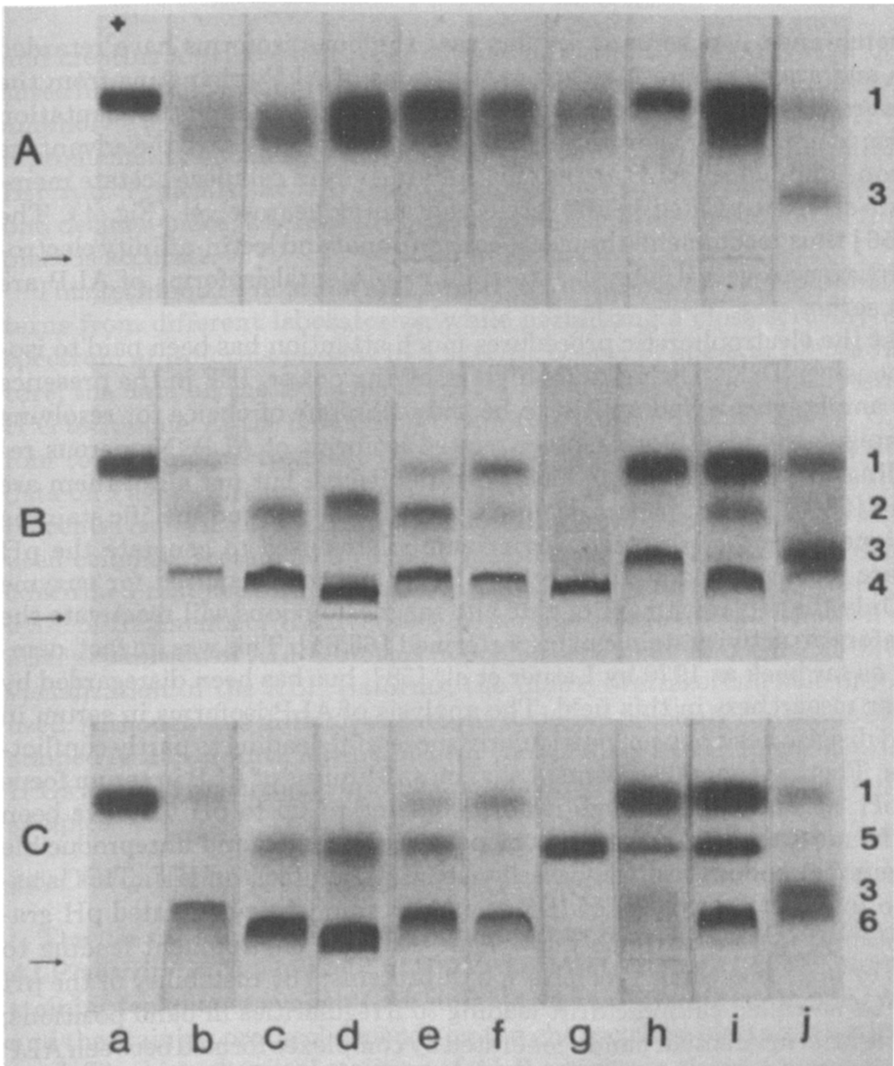

Fig. 4. Electrophoretic separation of ALP isoenzymes in agarose gel: (A) without lectin; (B) with lectin (0.05 mg/ml); (C) with lectin (0.05 mg/ml) and 0.1% Triton X-100. (a) Liver ALP isolated from serum by chromatography on DEAE-Sephacel; (b) healthy adult; (c) child (14 years old) with elevated bone and normal liver ALP; (d) patient with Paget's disease with high bone and low liver ALP; (e) and (f) patients with elevated bone and liver ALP; (g) biliary ALP isolated from serum by chromatography on DEAE-Sephacel; (h) patient with liver and biliary ALP; (i) patient with liver, bone and biliary ALP; (j) patient with liver, bone and intestinal ALP. Bands: 1 = liver ALP; 2 = non-precipitating bone ALP; 3 = intestinal ALP; 4 = precipitating bone ALP; 5 = non-precipitating bone ALP, non-precipitating biliary ALP or non-precipitating bone and biliary ALP; 6 = precipitating bone ALP. (Figure and legend from ref. 56, with permission.)

acetate membranes, can be used. In this case the bone isoforms have retarded mobilities and are clearly resolved from the forms of ALP originating from the liver. Numerous reports have been published, especially details on the adaptation of this basic technique for agarose gels [55–57]. Agarose gels have the advantage that the bone and biliary ALP are not just retarded as in cellulose acetate membranes, but are precipitated in the lectin-containing agarose gel (Fig. 4). The authors [56] thus recommend the use of conventional and lectin-affinity electrophoresis in agarose gels if biliary, intestinal or placental isoforms of ALP are present in serum.

Amongst the electrophoretic procedures much attention has been paid to isoelectric focusing (IEF). Owing to its high resolving power, IEF in the presence of carrier ampholytes would appear to be the technique of choice for resolving and unambiguously identifying closely related isoforms of ALP. Numerous reports dealing with this technique have been published, but not all of them are convincing [58–62]. Most papers dealing with IEF analysis and specific staining for ALP ignore the fact that the carrier ampholytes used to generate the pH gradient are strong chelators, which bind the metal ions required for enzyme activity. Only the replenishment of zinc and magnesium ions will reactivate the enzyme before an activity stain can be performed [63,64]. This was, in fact, demonstrated as far back as 1970 by Latner et al. [58], but has been disregarded by many other researchers in this field. The analysis of ALP isoforms in serum in health and disease has not been particularly successful, leading to partly conflicting results. There is general agreement that most isoforms of ALP in serum focus between pH 3.5 and 6.0, although isoforms having p*I* up to pH 7.0 have been reported. In most reports, however, poor patterns, smeared and unreproducible bands in number and intensities, have limited the usefulness of IEF. This is ascribable to inherent drawbacks of IEF in carrier ampholyte-generated pH gradients, notably: (a) the extremely low ionic strength environment leading to isoelectric or near-isoelectric precipitation of proteins; (b) instability of the pH gradient, the so-called cathodic drift leading to irregularities in band positions; and (c) potential artefactual bands generated by complexes formed between ALP and different carrier ampholyte species [65,66].

2.4. Latest developments in isoelectric focusing

These problems associated with conventional IEF in carrier ampholyte-generated pH gradients can be avoided with the use of a new fractionation technique, which has not yet reached the attention of many in the field of ALP research. This is IEF in immobilized pH gradients (IPGs). IPGs are a totally new concept in IEF [67]. They are based on the principle that a pH gradient, which exists prior to the IEF run itself, is copolymerized, and thus insolubilized, within the fibres of the polyacrylamide matrix. This is achieved by using, as buffers, a set of seven chemicals, weak acids and bases, called immobilines. The immobilines are derivatives of acrylamide, which at one end of the molecule contain the acrylic double bond, which is consumed during the polymerization reaction, thus immobilizing either a carboxyl or a tertiary amino group localized at the other end

and creating a pH gradient. The high ionic strength milieu typical of IPGs ensures full protein solubility and should disrupt any potential binding with carrier ampholyte species. Needless to say, cathodic drift does not occur in IPGs, so that reproducibility of band position is fully guaranteed, no matter how prolonged the IEF run. Typically the IPG technique generates p*I* values significant to the second decimal place, whereas in conventional IEF often not even the first decimal place is accurate.

This technique will allow much more reproducible and comparable ALP patterns from different laboratories, while permitting a close scrutiny of the fine spectrum of isoforms present in a specific tissue. While still preliminary in nature, the data on the isoforms of ALP in serum show the great potential of the IPG technique. At the moment two isoforms of ALP in bone disease (Paget) and four to six isoforms from the placenta have been unambiguously identified. The data on Paget's disease have been confirmed [64]. The presence of at least four placental isoforms of ALP has been confirmed recently by Cocco et al. [68], who used cellulose acetate as a support medium for IEF. Further IPG studies have confirmed that the forms of ALP in hepatobiliary disease are caused by the release of fragments of the liver cell membrane [69,70]. Fig. 5 shows a representative separation of ALP isoforms in pooled sera on an IPG (pH 3.5–6.0). For the visualization of the ALP isoforms, the indoxyl-tetrazolium salt procedure was used. Although it is difficult at present to define precisely the tissue origin of the stained ALP isoforms, it is quite clear that of all the techniques mentioned, only IPGs are able to demonstrate the microheterogenic complexity of ALP in serum samples.

2.5. *Critical evaluation of ALP staining techniques*

The use of specific staining techniques has been useful in the localization of ALP activity in tissue sections and after electrohoresis (zymograms). Not all staining techniques available are suitable for the differentiation of ALP isoforms, and the staining protocol depends on the choice of the electrophoretic technique used. The histochemical staining of ALP activity in tissue (bone) was reported as far back as 1923 by Robison [71], who incubated sections of bone from rachitic rats with calcium hexose monophosphate, followed by the treatment with silver nitrate. Intensive staining of those cartilage cells involved in provisional calcification was noticed, whereas the non-proliferative cartilage remained unstained. This was also the first report on the involvement of ALP in bone formation.

In 1939 Gomori [72] and Takamatsu [73] developed the classical stain for the light microscopical localization of ALP in tissue sections. The azo-coupling technique, introduced by Menten et al. [74] in 1944, was the real advance. The basis of the technique is the production of an insoluble azo dye at the sites of ALP activity, a method that is now used not only for ALP but also for a large number of other enzymes (e.g. proteases, glycosidases and esterases). In the 1960s Burstone [75] introduced naphthol-anilinic acid (AS) derivatives, which have the advantage that the released naphthol-AS is more insoluble in aqueous buffers than 1- and 2-naphthols. Using the technique described by McGadey in 1970 [76] (indoxyl phosphate in combination with tetrazolium salts), ALP activities can

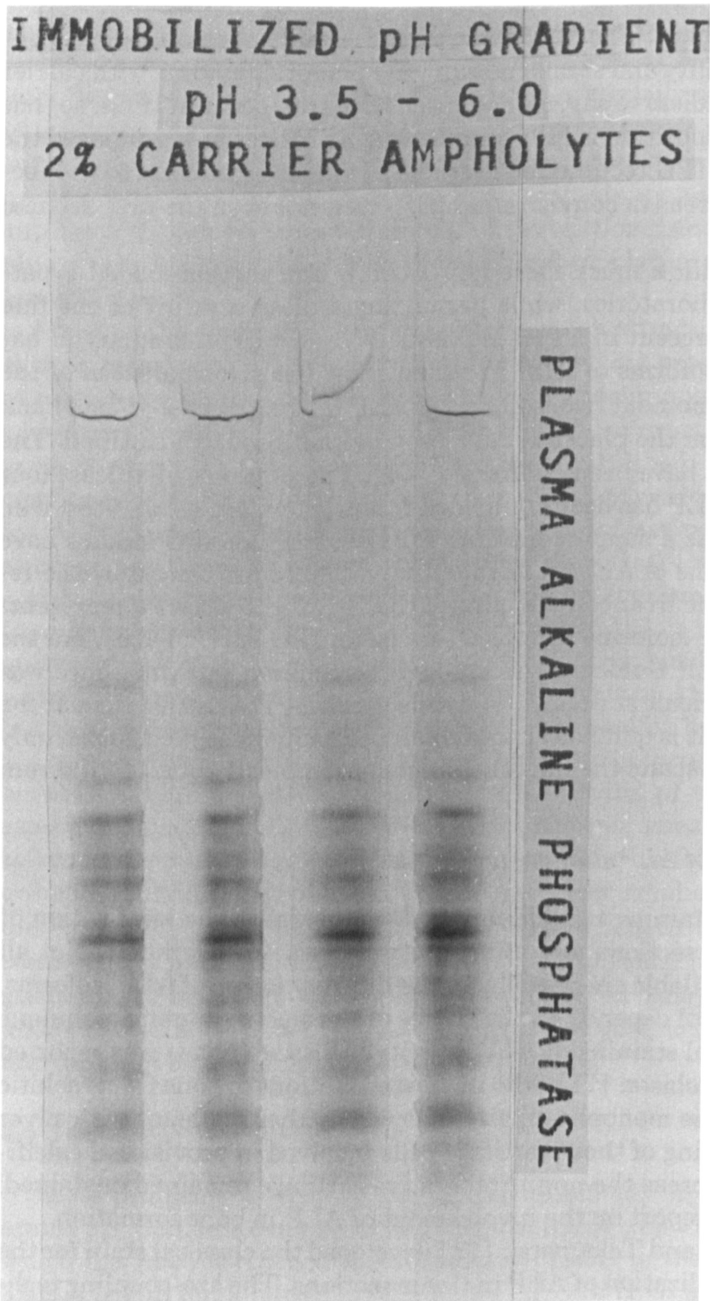

Fig. 5. Resolution of ALP isoforms in pooled ALP serum with a total activity of 520 U/l; 10 mU of ALP activity were applied to each lane for electrophoresis in an immobilized pH gradient (pH 3.5–6.0). After the electrophoretic run, the bands of ALP activity were visualized using the indoxyltetrazolium salt procedure mentioned in refs. 63 and 69. All stained bands represent isoforms of ALP in serum, thus showing the advantage of immobilized pH gradients for the demonstration of ALP microheterogeneity.

be visualized by the deposition of either insoluble formazan or indigo dyes, depending on the presence or absence of tetrazolium salts in the reaction mixture.

Although metal salt techniques have been adapted to gel electrophoretic systems [77], their use is cumbersome. Intense background staining of the matrix is observed and long destaining times are required. Metal salt techniques are fatal in IEF [48], since the carrier ampholytes, which are known chelators, bind lead and calcium ions, thus producing artefactual bands. The disadvantage of the azo-coupling technique lies in the instability of the diazonium salts required for the coupling procedure at pH values required for optimal ALP activity. The instability of diazonium salts above pH 8.2 is well known [75,78,79]. Additionally, the electrophilic diazonium ion $R\text{-}N^+$ exists in alkaline buffers in equilibrium with the unionized compounds and its salts. Hydroxides favour the conversion of the diazonium ion into compounds $R\text{-}N{=}N\text{-}OH$ and $R\text{-}N{=}N\text{-}O^-$, which do not couple with naphthols. Thus the stability of the diazonium salts appears to be rate-limiting, especially in view of the fact that longer staining times are required after electrophoresis than in tissue sections. All these problems are circumvented by the use of the McGadey technique [76], which has been adapted for the staining of ALP isoforms after IEF [63], using 5-bromo-4-chloro-3-indoxyl phosphate and a tetrazolium salt. After hydrolysis of the phosphate group, the resulting indoxyl molecules reduce tetrazolium salts to relatively water-insoluble formazans at the site of ALP activity. The first staining protocol included the use of a glycine buffer [63], but this was later replaced by an amino alcohol, diethanolamine [69]. The real advantage of this staining protocol is the stability of both the substrate and auxiliary reagent at high pH values. In a recent article, which directly compares the indoxyltetrazolium salt with the azo-coupling procedure, the former was found to be superior [64]. This technique can, in our experience, be generally recommended for the staining of ALP isoforms after electrophoresis.

3. CLINICAL IMPLICATIONS

3.1. Alkaline phosphatase in daily routine

In the routine clinical laboratory ALP is most commonly used for the diagnosis and therapeutic observation of bone and hepatobiliary diseases. In addition, as a tumour-associated marker, elevated serum ALP activity can be a helpful tool or an accidental hint for further differential diagnosis and investigations. Analysis of serum ALP isoforms are still in the hands of few specialized diagnostic enzymologists, whereas analysis of urine ALP, e.g. after renal transplantation [80] or in hypertension [81], is so far a domain of clinical researchers.

It has been well documented that normal children have a steadily increasing serum ALP. Pre-term infants delivered before the 30th week of gestation [82,83] may develop rickets, and for early diagnosis – even though there is a wide range – weekly measurements are recommended. In adolescence (due to bone growth) there is a peak of up to 500 ± 200 I.U./l, more in males than in females, and a constant concentration will be reached at the age of ca. 20 years [84]. In general,

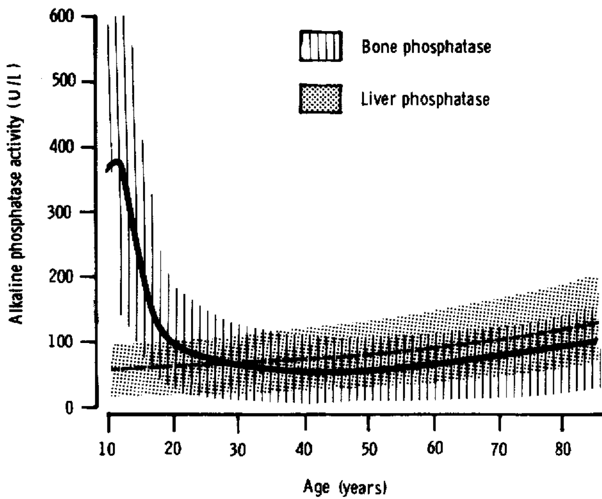

Fig. 6. Age-dependent changes in the activities of bone and liver ALP in serum (according to Moss and Whitby, ref. 82, with permission).

normal total serum ALP activity is slightly elevated in males compared to females [85]. The age-related increase (Fig. 6) has not been thoroughly investigated and may be due to metabolic changes induced by beginning hepatic or renal diseases.

3.2. Bone diseases

3.2.1. Bone calcification

ALP has long been recognized as an osteoblastic marker [71] – not a determinant – although its physiological role remains unsolved. The localization and its intracellular distribution have been discussed earlier. Serial sections through calcifying epiphysis of a number of species showed a progressive increase in ALP activity from unorganized cartilage through proliferating and hypertrophic cartilage to primitive spongiosa [86,87]. Osteoblasts and osteocytes are rich in ALP, but contain little acid phosphatase; this is in contrast to osteoclasts [88,89]. Robison's hypothesis, concerning a cause-and-effect relationship between ALP and tissue calcification, stated that circulating phosphate esters with a resulting increase in inorganic phosphate ions in the vicinity of phosphatase-producing cells cause a solubility product of various calcium phosphate compounds with a resulting deposition of hydroxyapatite and similar compounds. This was confirmed in detail by electron microscopy [90,91] and by cell tissue studies on the transformation of fibroblasts into osteogenic tissue accompanied by a local increase in ALP [92]. This theory has provoked criticisms ever since, e.g. calcification fails to occur in rickets bone and in bone implants of diphosphonate-treated animals [93], even though ALP is abundant. In addition, new theories were proposed, e.g. skeletal ALP may lead to the destruction of an inhibitor in the mineralizing process [94], and that pyrophosphates could serve as substrates for mammalian ALP [95]. Support for the theory was obtained from hypophosphatemic patients who excrete abnormally large amounts of pyrophosphate in their urine [96], allegedly

owing to their inability to hydrolyse this material enzymatically. ALP present in the plasma cell membrane [16,91] as an ectoenzyme can hydrolyse organic phosphates on the outer cell surface. During osteogenesis ALP is shed into the bloodstream and used as an indicator of bone formation and turnover [97]. ALP is abundant in cartilage cells prior to calcification. The time of its appearance in osteoblasts and cartilage cells, and the importance of phosphate in mineralization, have implicated the enzyme in this physiological pathway [98].

Owing to the lack of serological bone markers other than unspecific ALP, recent investigations have been carried out on different non-collageneous proteins from bone (osteocalcin, osteonectin) [99–104].

3.2.2. Osteomalacia and rickets

The definition of osteomalacia is difficult and often misinterpreted by clinicians. It is a histological rather than a serological finding. ALP activity might be normal, slightly above normal, or up to three times the upper reference range in severe osteomalacia. The skeleton is characterized by defective mineralization of newly formed preosseous matrix (osteoid) in mature lamellar bone. Static and dynamic measurements disclose the resulting lamellar osteoid accumulation and retarded appositional bone formation, documented by histomorphometric techniques, such as in vivo double tetracyclin labelling.

A heterogeneous group of hereditary and acquired metabolic and privational disorders may manifest themselves as osteomalacia or, prior to epiphyseal fusion, as impaired mineralization of the cartilaginous growth plate and primary spongiosa, namely rickets [105]. Histological, radiological and clinical symptomatic osteomalacia may even be present in the absence of elevated serum ALP [106]. Since histological investigations are not routinely available, radiological investigations including bone scans can be misleading, and the measurement of other serological parameters (including vitamin D metabolites) are only pieces of a mosaic. Different researchers have applied discriminant function analysis to the diagnosis of osteomalacia incorporating serum chemistry results of total calcium, phosphate and ALP [107]. Evaluating a diagnostic index, the limits of the range that indicates the need for bone biopsy can thus be determined by the cutoff points at maximum sensitivity and maximum specificity [108]. This approach has reduced iliac crest biopsies by ca. 50%.

3.2.3. Osteoporosis

Even in osteoporosis [109], the most common generalized skeletal disease, ALP and its isoforms are not a helpful parameter for clinical diagnosis or therapeutical control. Senile or post-menopausal osteoporosis is a skeletal disorder in which the absolute amount of bone is decreased relative to age-matched controls. The value of ALP measurements might be overestimated in the characterization of skeletal response to treatment, e.g. with fluoride [110]. Very few literature citations have evaluated this fact; in some observations ALP was inversely related to bone mineral content and total body calcium concentration, and there was a significant difference to age-matched controls [111]. In a cross-sectional longitudinal study [112] in normal post-menopausal women, total serum ALP was

positively correlated with body weight, plasma calcium and estrone, and with urine calcium and hydroxyproline. The most interesting of these correlations with ALP was negative for forearm mineral density. In addition, ALP levels were below normal or normal, and a significant difference was demonstrated with non-drinkers being slightly elevated compared with drinkers. Secondary osteoporosis due to specific endocrinopathies or from excessive and prolonged use of drugs that interfere with bone formation is not indicated by ALP. An increase may rather be due to bone fractures or aseptic necrosis. Corticosteroid osteoporosis induced by the depression of osteoblast function may be accompanied by bone healing after successful treatment of Cushing syndrome [113].

3.2.4. Renal osteopathy

Renal impairment and the association with secondary hyperparathyroidism and/or osteomalacia have been described and evaluated histologically [114,115]. An inverse correlation of serum ALP and highly significant bone ALP to the decrease of glomerular filtration rate was observed [116,117]. Accumulation of osteoid in bone correlated significantly with bone isoenzyme activity, but was rather loosely correlated with serum parathyroid hormone (PTH) concentrations. In addition, correlation of the bone isoenzyme and ionized serum calcium has been interpreted as an indicator for a hyperostoid state, because patients with normal bone ALP demonstrated exclusively histological parameters for resorption without increased bone apposition [118]. Elevated total ALP (up to two-fold) may rather demonstrate the activity of renal osteopathy than hyperparathyroidism from osteomalacia disorder in dialysis osteodystrophy [119]. Improved dialysis techniques may be one reason for discrepancies in the literature, e.g. atraumatic fractures in 1–37% of hemodialysis patients [120,121]. On the other hand, the prolongation of hemodialysis and the chronic administration of aluminium-containing phosphate binders may cause severe osteomalacia and encephalopathy in uremic patients, the so-called aluminium osteopathy [122], with an increase in ALP [123]. This disorder does not occur in patients on continuous ambulatory peritoneal dialysis [124]. Increased ALP in uremic patients may be an indication for bone biopsies and may help to decide further vitamin D metabolite treatment.

The recent introduction of cyclosporin into the management of transplant recipients has reduced steroid-related problems of bone metabolism, e.g. necrosis [125,126], but case reports of elevated ALP may be due to the liver rather than to bone tissue [127]. Since ALP activity decreased after renal transplantation, irrespective of graft function, it was suggested that it could be due to the primary toxic effects of high prednisolone therapy on osteoblasts [128,129].

3.2.5. Hyperparathyroidism

By 1929 the coincidence of increased serum ALP and hyperparathyroidism had been described [130], and this was later shown to be correlated with histological resorption parameters [131]. Patients with hyperparathyroidism may show normal ALP activities, whereas a significant increase may occur in advanced osteoporosis and may inversely correlate with radiological bone density [132].

3.2.6. Ostitis deformans (morbus Paget)

The etiology of Paget's disease [133] is still under debate, e.g. slow osteotropic virus with a mono- or polyostotic localized increase of osteoclastic activity. ALP is the most frequently used biochemical marker [134] and it is a good example for the use of total ALP and its isoforms, because cholestasis might be ruled out by routine hepatic enzyme analysis [65,69]. The turnover rate of bone-derived ALP is ca. two days in patients with Paget's disease [135].

The absolute levels of ALP, with a range from normal up to ten times the age-related normal mean, remain more or less constant, throughout life [136], but a progression rather than a spontaneous remission of biochemical and radiological observations may occur [137]. A significant correlation has been documented in the literature between total ALP activity and the extent of skeletal involvement measured radiologically [138] or using bone scans [139] and even with the use of calcium tracer kinetics [140]. Locally increased bone turnover is demonstrated by the correlation of ALP and urinary hydroxyproline excretion [140]. A skewed distribution of serum ALP has been demonstrated in patients with Paget's disease, and the analysis suggests an unimodal logarithmic normal distribution in all grades of severity of the disease. About 15% of our patients with this disease exhibit ALP values within the reference range, some of these patients being asymptomatic; osteosarcoma occurs in less than 1%. ALP is a helpful biochemical indicator of successful treatment with different agents, which today include calcitonin [141], diphosphonates [142] or a combination of the two [143], and may discriminate at an early stage between responders and non-responders.

3.2.7. Familial hyperphosphatasemia

A benign, transient, unexplained hyperphosphatasemia is a rarely recognized clinical event [144], and repetitive measurements are necessary to avoid the "Ulysses syndrome" [145]. This disorder is characterized by increased activity of serum ALP, typically up to five-fold the adult upper reference limit, in children below five years of age without evidence of liver or bone disease. The disease seems to be inherited via an autosomal recessive pathway and has been described as "juvenile Paget's disease" [146]. A viral etiology has been proposed, although a drug-induced etiology cannot be completely ruled out. Isoform analysis suggests a double tissue origin (bone and liver) of ALP in this disorder, whereby the most probable reason for the increase of ALP activity in serum is reported to be an impaired clearance from circulation due to viral infections, although little is known about this mechanism [147]. Unnecessary extensive investigations, e.g. bone biopsy, can be avoided by the examination of ALP isoforms since there is normalization of ALP activity within a few months. There are, however, reports in the literature of patients suffering from skeletal deformities, poor cortical bone formation and high serum ALP activities. In addition, familial hyperphosphatasemia unrelated to skeletal disorders has also been reported [148].

3.2.8. Skeletal disorders and fractures

A variety of skeletal dysplasias have been classified [149] but most, like achondroplasia, hypochondroplasia, spondyloepihyseal dysplasia, osteopetrosis or os-

teogenesis imperfecta and focal bone diseases of unknown origin such as osteopoikilose, are not accompanied by significant ALP elevations. Slight elevations might be due to sporadic atraumatic fracture callus, which occurs within days after the event and may last for weeks. Fractures in humans may be followed by a 50% increase of total ALP activity and may be absent in geriatric patients, owing to low bone turnover rate [150].

3.3. Hepatobiliary diseases

After the first report of elevated ALP in the presence of obstructive jaundice [6], many theories were proposed and extensively discussed until it was demonstrated that the high-molecular-mass ALP present in the plasma of patients with liver diseases, especially intra- or extrahepatic cholestasis, originates from the sinusoid domain of the plasma membrane of liver cells [151]. This could be caused by a disturbed transfer of newly synthesized proteins from the sinusoidal to the bile canalicular domain of the plasma membrane. This form of ALP has been alleged to possess a considerable number of attributes, all of which have irritated clinicians more than clarifying the issue.

There is a known association of ALP with other membrane-bound enzymes, such as 5-nucleotidase, γ-glutamyltransferase and leucine aminopeptidase in cholestasis [152,153]. Lipoprotein X (LP-X), a complex consisting of phospholipids, free cholesterol and protein, mainly apolipoprotein C and albumin, formed in serum after bile regurgitation, is supposed to have diagnostic value, but does not exceed that of ALP and γ-glutamyltransferase [154–158]. There might be an association of ALP with LP-X, and a proposal has been made to call the cholestatic complex "ALP–LP-X complex" in order to reserve the term "membrane particle ALP" for the high-molecular-mass ALP without LP-X seen in hepatic malignancies [159].

ALP in the human liver is located in the endothelial cells around the portal and central veins, in the sinusoids as well as in the bile canaliculi [160]. From experimental bile duct ligation data [161,162] it has been documented that, in addition to mechanical obstruction leading to reduced excretion, there is an increased de novo synthesis of ALP stimulated by bile salts [163]. In a series of elegant experiments it has been shown that this de novo synthesis is not due to an increase in the transcription but due to enhanced translation of mRNA [164].

ALP and cholestasis have recently been extensively reviewed [1,165]. Many drugs may cause cholestasis and thus elevate ALP, from one to ten times normal values. In patients with chronic diphenylhydantoin treatment it was suggested that serum ALP elevation derives from bone disease; despite conflicting results elevation is generally attributable to hepatic enzyme induction.

3.3.1. Rheumatic diseases associated with abnormal ALP activities

Elevated total ALP activities are difficult to interpret in rheumatic diseases but, with a few exceptions (bone involvement in morbus Bechterew), are a sign of hepatic manifestation of systemic disease processes. Some 26% of rheumatoid arthritics exhibit mild to moderate ALP elevations [166], and increases of ALP

in vasculites [167], Wegener's granulomatosis and polyarthritis, Sjögren syndrome [168] and Felty syndrome are probably due to hepatobiliary involvement. Even therapeutic doses of anti-inflammatory drugs (e.g. salicylates) produce raised serum transaminases and ALP activities [169]. The response to corticosteroids may be judged by the decreasing levels of ALP.

3.4. Hypophosphatasemia

Hypophosphatasemia in infants is present in a perinatal lethal form [170] and in a more mild childhood form, which may result from defective regulation of an intact structural gene for the tissue non-specific isoenzyme of ALP [171]. Clinically it is characterized by dental abnormalities and rickets/osteomalacia and biochemically by subnormal circulating serum ALP.

Low or even undetectable levels of ALP have been described in Wilson's disease. The mechanism responsible for this decrease is still uncertain, but it is possible that copper may compete with zinc and be incorporated in the metalloenzyme, which should then have reduced activity. Large amounts of bilirubin present in the plasma of patients with Wilson's disease may cause problems in the determination of enzymes including ALP, whereas the addition of copper and zinc does not influence the enzymic activity. Treatment with D-penicillamine has, in one case, led to an increase of serum ALP activity [172,173]. Decreased activities of ALP were also reported under exposure to drugs, e.g. clofibrate [194].

3.5. Placental alkaline phosphatase

Since the first description of ALP during pregnancy [172], conflicting results have been reported about PLAP. It is generally accepted that it is normally released from microvilli of the syncytiotrophoblast membrane [175]. During the first two trimesters of gestation, PLAP's gradual rise is followed by a very steep increase reaching maximum at term [176]. Levels of PLAP, unlike those of other placental proteins, do not correlate with fetal or placental weight, which indicates the possibility of using PLAP clinically as a monitor of placental function. It should be noted, however, that PLAP can be further differentiated into two forms, the early or fetal placental ALP, with properties similar to unspecific tissue phosphatase, and the term placental ALP [31].

3.6. ALP – a tumour-associated factor or a "tumour marker"?

Since the first description of ectopic production of ALP by tumour tissue, much attention has been paid to the characterization of the nature of the enzyme. Up till now three different isoenzymes from independent gene loci have been demonstrated in cancer patients: (a) Regan isoenzyme or term placental ALP; (b) Nagao, testicular or PLAP-like; and (c) Kasahara or fetal intestinal ALP [31,174].

Possibilities hitherto unknown for the examination of these enzyme variants have been opened after the introduction of hybridoma technology. Numerous

assays have been developed for quantification, and this has led to conflicting results concerning the reference ranges in serum or plasma of the ectopically produced enzyme, an essential for the specificity as a potential tumour marker [175,176].

Including all routine laboratory parameters, total plasma ALP has been described to be a "most useful" indicator in breast cancer with a sensitivity for metastases of 30–40% [177]. For survival time of prostatic cancer, elevated total ALP has been evaluated as a "significant" prognostic factor [178]. Using various statistical methods, sensitivity, specificity and accuracy for liver metastases in lung cancer were 71, 89 and 86%, respectively [179]. Plasma bone ALP was twice as sensitive as total enzyme activity in the diagnosis of the presence of bone metastases [178]. Overall, 45% of metastatic deposits were recognized by isoform analysis, but significantly less (24%) by total ALP, and worse than radiological imaging [180]. Serum PLAP has been described as a potential marker in seminoma patients [181,182], but it has been recognized that environmental influences affect PLAP levels significantly, e.g. smoking leads to a re-expression of PLAP [183,184]. Despite these environmental influences, PLAP has been used successfully in the monitoring of testicular and ovarian carcinoma [185–187].

At present it can be said that, owing to (a) the use of diverse immunological techniques that are not standardized internationally, (b) the relatively low sensitivity and specificity of the tests used, (c) small patient collectives, (d) unknown (or unreported in the publication) tumour prevalence, the potential use of ALP isoenzymes as a tumour marker is limited since the predictive value is either too low or uncalculable because of missing statements in the publications. The use of ALP isoenzymes for diagnosis and therapeutic control is therefore still overestimated and uncritically used in daily routine by clinicians. It still remains a field of interest for basic research, which might find its application in the future.

3.7. Neutrophil ALP

Neutrophil granulocytes contain ALP, and the nature of this isoform is unclear. Neutrophils have been reported to contain a unique ALP [188], or largely placental ALP [189] or even liver, kidney or bone ALP [190]. Furthermore, a distinct ALP has been described in patients with lymphatic leukemia and infectious mononucleosis, which is related to the clinical state of the patients with leukemia and disappears from circulation after recovery from infectious mononucleosis [190]. Neutrophil ALP has been reported to be decreased in myeloid leukemias [192,193], increased activity may be observed in polycytemia vera and osteomyelo fibrosis (= myeloproliferative syndrome) with no change in myelodysplastic syndrome.

4. SUMMARY

Alkaline phosphatase (ALP), which was discovered in 1907, is extensively reviewed. The first section deals with biochemical aspects of ALP, e.g. the anchorage of ALP to cell membranes via a phosphatidylinositol linkage, the charge and molecular mass heterogeneities and their causes, and methods for the separation and analysis of ALP isoforms using the newest electrophoresis techniques, such

as affinity electrophoresis with wheatgerm lectin and isoelectric focusing in immobilized pH gradients. The second section deals exclusively with the clinical implications of ALP analysis in diseased states, e.g. cholestasis, hyper- and hypophosphatasemias and neutrophil ALP. Extensively discussed is the involvement of ALP in numerous bone diseases. The role of ALP and its isoforms in tumours and their applicability as potential tumour markers is critically evaluated.

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NOTE ADDED IN PROOF

The never-ending stream of articles on ALP has continued since our article was submitted to the journal. Since we still have one last chance, we would like to add three new articles that have appeared in the past few months in order to extend the literature list. Two of these deal with the fetal intestinal ALP in serum and amniotic fluid [195] and the significance of PLAP as a tumor marker [196]. The meticulous work of Endo et al. [197] has clarified the structure of the carbohydrate moiety of PLAP. Purified PLAP contains one asparagine-linked sugar chain, which after hydrolysis, can be separated by paper electrophoresis in one neutral and two acidic fractions. The structure of the neutral fraction has been elucidated. The acidic oligosaccharide fractions seem to be mixtures of mono- and disialyl derivatives of the neutral fraction.

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