

## Structural evidence that human liver and placental alkaline phosphatase isoenzymes are coded by different genes

(gene products/NH<sub>2</sub>-terminal sequence/peptide map/molecular weight)

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**ABSTRACT** Human liver alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] was purified, and some of its physical and chemical properties were examined and compared to those of human placental alkaline phosphatase. The results indicated a different peptide structure for each, based upon NH<sub>2</sub>-terminal residue sequence, two-dimensional tryptic peptide maps, and different amino acid compositions. These data are interpreted to indicate that the enzymes are synthesized by different structural genes. Other molecular properties differentiating the two enzymes were a higher apparent molecular weight for the liver enzyme from sodium dodecyl sulfate gel electrophoresis, a higher  $s_{20,w}$  value, different carbohydrate content, and a different isoelectric point. The immunochemical specificity of each enzyme was not affected by removal of sialic acid groups. Both enzymes are similar in that they are dimers of equal molecular weight subunits, and are probably homodimers.

Different molecular forms of human alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] exist and the general subject of these isoenzymes has been well reviewed (1, 2). Alkaline phosphatase isoenzymes have been identified by their electrophoretic and immunochemical properties, thermostability, and differential response to substrates and effectors (1, 2). However, classification of the alkaline phosphatase of individual tissues by these properties has not been satisfactory because these properties often overlap, and because the molecular differences between these isoenzymes has not been ascertained (1, 2). Although it has been suggested that the major classes of human alkaline phosphatase isoenzymes are coded by different structural genes (3), there is no rigorous evidence of molecular structure in support of this suggestion (2). The most definitive way to evaluate this suggestion is to compare the peptide structure of alkaline phosphatases from different tissue sources. Characterization of partial peptide structure has been used to show identity between human placental alkaline phosphatase and an enzyme synthesized by a nontrophoblastic neoplasm (4).

The purpose of the present paper was to investigate whether the tissue-specific alkaline phosphatases are indeed coded by different genes and have different peptide structures, or whether the molecular differences depend upon post-translational factors, such as variation in glycosylation, e.g., sialic acid residues (2, 5). In this paper we describe the purification to homogeneity of the human liver alkaline phosphatase and compare its peptide structure and other molecular properties with those of the placental enzyme. Previous studies have suggested that these isoenzymes may be different molecular species; they are immunochemically distinct (3, 6), and pre-

liminary data indicated different NH<sub>2</sub>-terminal residue sequences (4).

### MATERIALS AND METHODS

**Purification of Liver Alkaline Phosphatase.** Livers were obtained from patients who had died of trauma or of myocardial infarction. Alkaline phosphatase was extracted and purified by a modification of the methods previously described (6). In brief, the tissue was homogenized in saline containing 0.5 mM MgCl<sub>2</sub> and 1-butanol (20% vol/vol), and the homogenate was stirred for 3 hr and centrifuged at 9000 × *g* for 10 min. The pellet was rehomogenized with saline and 1-butanol, extracted overnight, and the aqueous phase from each homogenization was combined. Fractional acetone precipitation was used to concentrate the enzyme in the 30–60% vol/vol acetone fraction.

The acetone precipitates were resolubilized, pooled, and dialyzed against 0.01 M Tris-HCl, 0.5 mM MgCl<sub>2</sub> buffer, pH 7.4 (at 20°), containing 0.03 M NaCl and loaded on a 5 × 83 cm column packed with DEAE-cellulose (Whatman DE52). The loaded column was washed with the same buffer (3140 ml) followed with buffer containing 0.05 M NaCl (3460 ml). The column was developed using a linear gradient from 0.05 to 0.09 M NaCl in the Tris-Mg<sup>++</sup> buffer with 5 liters in each reservoir. The fractions with highest specific activity were pooled and concentrated by acetone precipitation (60% vol/vol). This precipitate was dissolved in the Tris-Mg<sup>++</sup> buffer containing 0.1 M NaCl, dialyzed against the same buffer, concentrated with dry sucrose, and applied to a 5 × 85 cm gel filtration column (Sephadex G-200, Pharmacia).

The fractions of highest specific activity were pooled and isoelectric focusing was performed with Ampholine solution to the range pH 3–6 in an LKB no. 8101 column with a working volume of 110 ml (7). The sample was added to the dense solution after two-thirds of the gradient had been pumped in. Previous experiments had shown that most of the enzyme activity would be lost below pH 3.7 if the run was continued until a constant current was reached (4). By using an initial voltage of 300 V gradually stepped up to 500 V within 6 hr, and staying at this voltage for 18 hr, we recovered an enzyme peak between pH 3.8 and 4.4. The enzyme-containing fractions were immediately pooled, dialyzed against Tris-Mg<sup>++</sup> with 0.1 M NaCl, concentrated against dry sucrose, and applied to the same Sephadex G-200 column for removal of ampholytes.

**Placental Alkaline Phosphatase.** The placental alkaline phosphatase used was extracted and purified from human placentas as previously described (4).

**Enzyme Assay and Immunoassay.** Enzyme activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (Sigma) at 405 nm (8) at 37°, pH 11.5.

Abbreviations: NaDodSO<sub>4</sub>, sodium dodecyl sulfate; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

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Table 1. Purification of liver alkaline phosphatase

Purification procedure	Total protein (mg)	Total enzyme (units*)	Specific activity (units/mg)	Recovery of enzyme (%)	Relative purity
Crude extracts	250,000	23,000	0.09		1
I acetone precipitate	45,300	20,400	0.45	89	5
DEAE, fractions 740-920	632	9,568	15	42	167
II acetone precipitation	370	9,086	24	40	278
IG-200, fractions 55-67	89	6,140	69	27	767
Electrofocusing <sup>†</sup>					
II G-200, fractions 54-94	2.0	1,848	924	8	10,266

\* See text.

<sup>†</sup> The presence of Ampholine prevents quantitative measurements at this purification step.

The substrate solution contained 0.54 mM *p*-nitrophenyl phosphate, 0.05 M monoethanolamine, and 0.5 mM MgCl<sub>2</sub>. A unit of activity was defined as the amount of enzyme capable of releasing 1 μmol of *p*-nitrophenol per min. Immunochemical assays were carried out using a double precipitation reaction as described by Sussman *et al.* (6). Protein concentrations were expressed as the absorbance at 280 nm.

**Polyacrylamide Gel Electrophoresis.** Disc electrophoresis was performed in a slab gel apparatus (9) using 7½% polyacrylamide with the buffer system of Davis (10) with stacking pH 8.3 and running pH 9.5 (gel reagents from Canalco). Bromophenol blue was used as tracking dye. Enzyme activity was located by incubating the slab briefly in substrate solution and marking the yellow bands indicating release of *p*-nitrophenol. The gels were then fixed with 5% acetic acid in 50% methanol and stained with Coomassie blue. For estimation of the molecular weight of the subunits, a sodium dodecyl sulfate (NaDodSO<sub>4</sub>) system was employed (11). The molecular weight standards used were bovine albumin (68,000), chymotrypsinogen (27,000), trypsin (23,300), and myoglobin (17,000). The logarithms of the relative mobilities of the markers were plotted against the molecular weights and the molecular weight of the samples were estimated from this standard curve.

**Ultracentrifugation.** The sedimentation constant  $s_{20,w}$  was determined by centrifugation in a 12.0 ml, linear sucrose gradient, 5–20% wt/wt sucrose in Tris, using a Beckman L2-65B ultracentrifuge with an SW41 rotor (12). The sample was layered on top in a volume of 0.1 ml. Bovine serum albumin (Sigma)  $s_{20,w} = 4.3$  S and bovine immunoglobulin G (Mann)  $s_{20,w} = 7.0$  S were used as markers. The gradients were centrifuged at 39,000 rpm for 20 hr at 4°. Fifty-two fractions were collected per tube and the gradients were checked by refractometry. The enzymes were detected by enzyme assay and, when both were contained in the same tube, distinguished by immunoprecipitation. The marker proteins were located by reading the absorbance at 280 nm. The  $s_{20,w}$  values for the enzymes were calculated by setting the ratio of  $s_{20,w}$  enzyme to  $s_{20,w}$  marker equal to the ratio of the distances traveled from the meniscus by the two proteins.

**Amino Acid Analysis.** For the amino acid analysis the enzyme proteins were dialyzed against saline for removal of Tris, lyophilized, and hydrolyzed in 6 M HCl for 24 hr at 110° under anaerobic conditions. The amino acid composition was determined on a Beckman-Spinco automatic amino acid analyzer model 120C.

**Amino-Terminal Sequence Analysis.** Amino-terminal sequence analysis was carried out according to Weiner *et al.* (13). 1-Dimethylaminonaphthalene-5-sulfonyl (dansyl) derivatives were used to identify the NH<sub>2</sub>-terminal amino acids by means

of thin-layer chromatography on two-sided polyamide sheets. Identification of the NH<sub>2</sub>-terminal residues was made by spotting the sample alone on one side of the sheet and the sample plus a selected dansylated standard on the other side and cochromatographing them (14). The dansyl residues were made visible and traced under ultraviolet light.

**Peptide Mapping.** Reduction and 5-carboxymethylation was done according to Sawyer *et al.* (15), and 273–300 μg of protein were used to make two maps. For digestion the carboxymethylated protein was dissolved in 1 ml 0.1 M ammonium bicarbonate buffer, pH 8.2, at 37°. Trypsin [Worthington, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated] was added in 2 ml aliquots initially, and after 1 hr from a solution containing 2 μg/ml. The pH was checked several times during the reaction and, when necessary, adjusted with tiny flecks of dry ice. Total incubation time was 4 hr. The lyophilized peptide mixture was spotted on two 20 × 20 cm plates of thin-layer silica gel on plastic (Eastman no. 6061). The plates were developed simultaneously in 1-butanol/pyridine/acetic acid/water (75:45:15:30). The pyridine had been distilled in the presence of ninhydrin to reduce background. The plates were left to dry overnight. Electrophoresis then took place in a Desaga chamber cooled to 4° in 8.8% formic acid at 300 V for 2 hr 10 min. The plates were dried and sprayed either with ninhydrin-cadmium or with fluorescamine (Roche Diagnostics), 2 mg in 30 ml acetone per plate. On the fluorescamine plates the peptide spots were made visible with ultraviolet light and traced directly on the plate.

**Treatment with Neuraminidase.** Both liver and placental enzymes were treated with neuraminidase to remove sialic acid residues (16). The phosphatase enzymes were dialyzed against 0.01 M sodium acetate buffer at pH 5.0. A working solution of neuraminidase was made by dissolving the commercial enzyme (Worthington NEUP, 1.5 unit/mg) in the same acetate buffer to a final concentration of 1.5 unit/ml. From the working solution 0.02 units of neuraminidase were added per mg of phosphatase protein initially, and again after 4 hr. The reaction took place at room temperature for 24 hr, during which time the outside buffer was changed twice. The dialysis bags were then transferred to 4° and dialyzed against three changes of the Tris-Mg<sup>++</sup> buffer.

## RESULTS

**Purification of Liver Alkaline Phosphatase.** A summary of the purification steps is found in Table 1. The enzyme was eluted from the DEAE column as a broad peak between 0.06 and 0.08 M NaCl. The first gel filtration through Sephadex G-200 yielded a symmetrical peak of activity on the shoulder of a broad protein peak. Electrofocusing gave a single enzyme

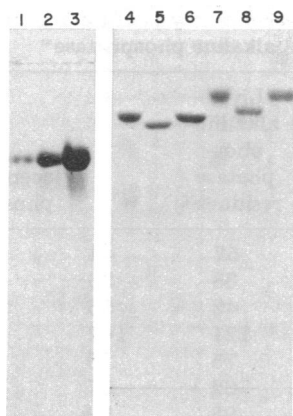


FIG. 1. Polyacrylamide gel electrophoresis of purified liver alkaline phosphatase, stained with Coomassie blue. Positions 1, 2, and 3 contain 2, 8.5, and 25  $\mu$ g of protein. Positions 2 and 3 were overloaded to detect the presence of minor impurities. Enzyme activity was coincident with the protein bands. Positions 4–9: NaDodSO<sub>4</sub> polyacrylamide gel electrophoresis. Positions 4–6 are placental alkaline phosphatase and positions 7–9 are liver alkaline phosphatase. Positions 4 and 7 show the native enzymes, and 5 and 8 show the enzymes after treatment with neuraminidase; 6 and 9 are enzyme controls that have been processed as 5 and 8 but without the addition of neuraminidase. For details, see text.

peak, and the Ampholines were removed by gel filtration on Sephadex G-200. The enzyme eluted from this column yielded a single symmetrical protein peak coinciding with the enzyme activity.

Acrylamide gel electrophoresis was performed with various concentrations of the purified enzyme. In each case a single protein band coinciding with the band of enzyme activity was found (Fig. 1). The  $R_F$  value of the liver enzyme was found to be 0.28. In the immunochemical assay 94–98% of the enzyme activity was removed by the antiserum specific for liver alkaline phosphatase.

**Placental Alkaline Phosphatase.** The placental alkaline phosphatase used in this study was a single protein band on acrylamide gel with an  $R_F$  value of 0.32. It had a specific activity of 1000 units/mg and was precipitated 96–99% with antiserum specific for placental alkaline phosphatase.

**Molecular Weight Estimation.** From the NaDodSO<sub>4</sub> gel electrophoresis a subunit molecular weight of 69,000 was found for the liver enzyme and 64,000 for the placental enzyme.

In the ultracentrifugation studies the liver enzyme sedimented slightly further in the gradient than the placental enzyme. This was independent of whether the enzymes were centrifuged together or separately (Fig. 2). The  $s_{20,w}$  value found for liver alkaline phosphatase was 6.7 S and a value of 6.5 S was found for the placental enzyme.

The molecular weight was estimated by using a standard curve of  $s_{20,w}$  versus  $\log M_r$ , values for known spherical proteins. By using data from Edsall (17) for the standard curve, we found molecular weights of 135,000 and 127,000 for the liver and the placental enzyme, respectively.

**Amino-Terminal Sequence Analysis.** The first two amino acid residues of the subunit of the liver alkaline phosphatase have been reported to be Leu-Val (4). This was confirmed in the present study, and the third residue was determined as Phe. The first four residues of the placental enzyme's subunit confirmed the sequence previously reported: Ile-Ile-Pro-Val (4).

**Peptide Mapping.** The peptide maps produced by the two proteins showed totally different patterns (Fig. 3). A total of 45 peptides was found for the liver enzyme and 46 for the placental. Based on the subunit molecular weights, the known quantities of glucosamine and sialic acid groups, and the amino acid compositions, one should expect no more than 54 peptides for either enzyme if the two subunits of each are identical. If other carbohydrates are present in the molecules, there will be fewer peptides. The maps in Fig. 3 are representative of the four maps made using the technique of spotting in 1% formic acid and performing chromatography before electrophoresis. Only a few minor differences were observed, and these are no greater between maps spotted with material from different digests than between maps spotted with the same material. Fluorescamine revealed more spots than ninhydrin. However, there were two peptides in the placental digest that did not show with fluorescamine and that did stain with ninhydrin. These two peptides could be detected on the fluorescamine plates by spraying with ninhydrin following the tracing of the fluorescent spots.

**Amino Acid Composition.** The major difference in amino acid composition between liver alkaline phosphatase and pla-

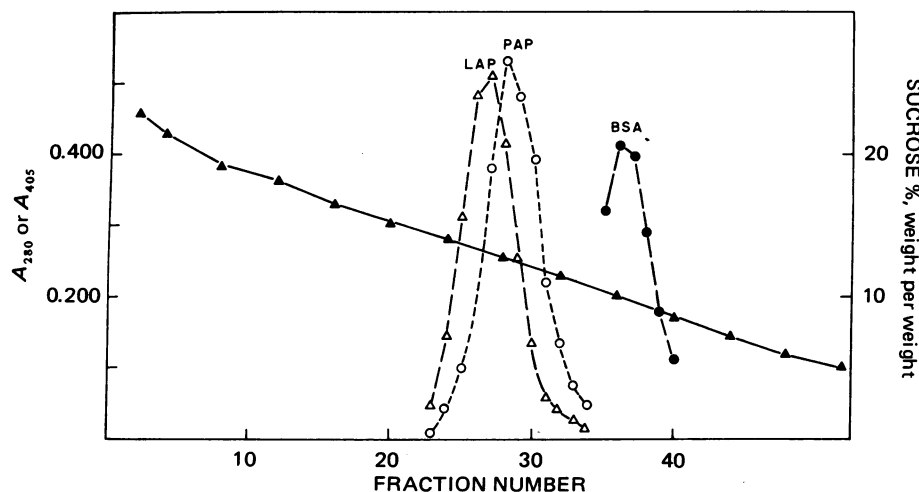


FIG. 2. Sedimentation patterns for liver alkaline phosphatase (LAP) and placental alkaline phosphatase (PAP) in a sucrose gradient. Bovine serum albumin (BSA) and bovine immunoglobulin G (not shown) were used as markers. Protein concentration of bovine serum albumin (●) is expressed as  $A_{280}$ , and enzyme activity (○, △) as  $A_{405}/\mu$ l per 5 min. Sucrose concentration (▲) is expressed as percent wt/wt. Fractions were collected from the bottom, and each tube yielded 52 fractions.

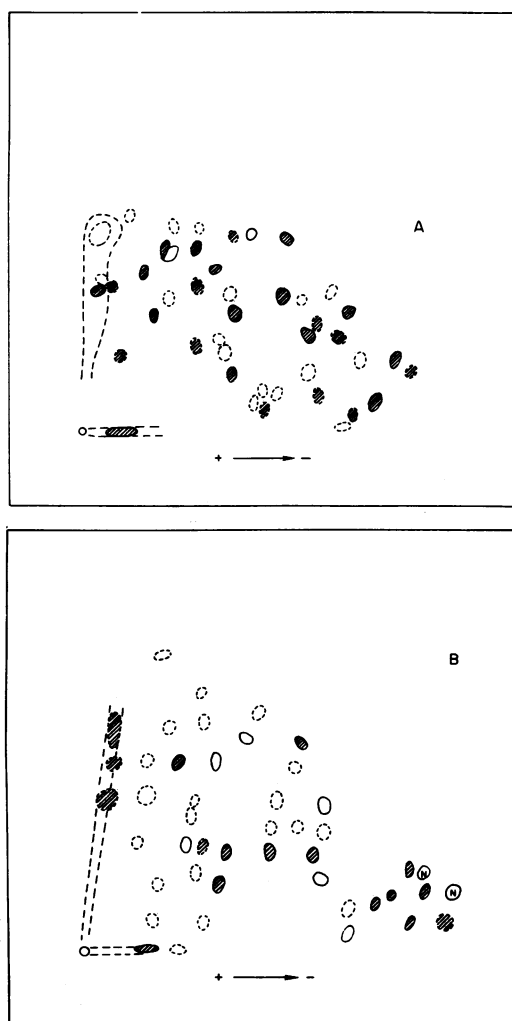


FIG. 3. Tryptic peptide maps. (A) Liver alkaline phosphatase, and (B) placental alkaline phosphatase. Spots drawn with a solid line were strongly fluorescent with fluorescamine. Spots drawn with a broken line were weakly fluorescent. Shaded spots were also stained with ninhydrin. The two spots marked with an N were seen only with ninhydrin. For details, see text.

cental alkaline phosphatase is that Lys, His, and Asp are higher for liver than for placental alkaline phosphatase and that Arg and Ala are lower (Table 2). Glucosamine was higher for liver than for placental alkaline phosphatase. Comparison of the amino acid composition of placental alkaline phosphatase in this and previous studies (18, 19) showed the greatest variation in measurement of individual amino acids was for Ser, Gly, and Ala.

**Functional Properties.** Enzyme activity was measured as a function of pH in the range 8.5–11.5 using 0.05 M glycine buffer in the assay mixture. With this glycine buffer the liver enzyme was found to have a pH optimum at 10.3 to 10.5. Above pH 11.0 the enzyme became so unstable that good duplicates could not be obtained. The placental enzyme had its optimum at pH 11.0–11.3 and became unstable at 11.5. The enzyme preparations were negative for acid phosphatase activity.

Heat stability of the enzymes was measured at 56° and 65° in two different buffer systems: Tris buffer at pH 7.5 and monoethanolamine at pH 11.5, protein concentration 0.2 μg/ml. Under these conditions the liver enzyme was more labile.

The long-term stability of the enzymes in Tris buffer was

Table 2. Amino acids analysis of human liver and placental alkaline phosphatase\*

Amino acids	Liver alkaline phosphatase residues †		Placental alkaline phosphatase ‡	
Lys	57	44	50	46
His	38	26	27	29
Arg	42	56	57	61
Asp	121	102	96	103
Thr	75	70	65	64
Ser	62	57	61	49
Glu	98	99	104	106
Pro	49	56	57	53
Gly	92	107	96	94
Ala	91	115	100	116
Cys	—	—	—	14 §
Val	72	64	77	66
Met	24	22	23	23
Ile	31	29	34	36
Leu	82	83	84	82
Tyr	35	36	34	35
Phe	31	35	35	37
Glucose-NH <sub>2</sub>	62	18		

\* Number of residues is expressed as moles per 1000 moles of amino acids.

† The values are average values of two separate double determinations.

‡ The first column shows data from one double determination done in series with one of the liver enzyme determinations, the second column shows data from Sussman and Gottlieb (18), and the third column shows data from Harkness (19).

§ Not included in summation to 1000. Measured as cysteic acid; not determined in the other studies.

followed at room temperature and at 4°. The enzymes were not stable at room temperature and after 27 days each enzyme had less than 10% of its original activity. At 4° after 160 days the liver enzyme retained 60–90% of the original activity, whereas the placental enzyme showed only 20–40% of the original activity.

**Treatment with Neuraminidase.** After neuraminidase treatment, the  $R_F$  values in the standard acrylamide gel were 0.15 and 0.25, respectively, for the liver and the placental enzyme versus 0.28 and 0.35 for the native enzymes (Fig. 1). In the NaDodSO<sub>4</sub> gel system both proteins migrated faster after incubation with neuraminidase, an indication of a reduction in molecular weight equivalent to about 4000 for the liver enzyme and 3000 for the placental enzyme, which brought the apparent subunit molecular weights to 65,000 and 61,000, respectively. The removal of sialic acid by neuraminidase did not affect the antigenicity of either enzyme.

## DISCUSSION

It is important to have a more definitive classification of human tissue-specific alkaline phosphatase isoenzymes, since these enzymes are frequently used as genetic markers in cell biology and as parameters of tissue or organ disease in medicine. The present study addressed this question by comparing the subunit and structural properties of the alkaline phosphatases from two different human tissues, liver and placenta.

Our study provides evidence that the liver alkaline phosphatase has a different peptide structure than the placental

enzyme, and so represents a different gene product. The evidence for this is the data indicating different amino acid sequences, different two-dimensional peptide maps following tryptic digestion, and different amino acid compositions. Although differences in peptide structure were demonstrated, without sequence information the data from this study are not sufficient for evaluating the degree of homology between these enzymes.

Other molecular differences between the two enzymes were demonstrated. The liver enzyme had a higher apparent molecular weight than the placental enzyme by sedimentation velocity in a sucrose gradient for the native enzyme, and by NaDodSO<sub>4</sub> gel electrophoresis for the subunit. None of these techniques define whether the difference in molecular weight between the liver and placental enzymes was due to the polypeptide or the carbohydrate component of the molecule. It was shown, however, that liver alkaline phosphatase had a higher content of glucosamine. Neuraminidase treatment indicated that the liver enzyme also had a higher content of sialic acid groups than the placental enzyme on the basis of a greater reduction in apparent molecular weight and in electrophoretic mobility. The demonstration that the antigenic specificity of each enzyme was not affected by treatment with neuraminidase provided evidence that sialic acid groups do not determine the antigenic differences between the enzymes. The  $R_F$  values for the liver enzyme were less than for the placental enzyme in standard polyacrylamide gel electrophoresis, and its isoelectric point was lower, confirming results previously reported (4). Differences in the functional properties of the two enzymes were also demonstrated.

The native liver enzyme, like the placental enzyme (18, 19), was shown to be a dimer of equal molecular weight subunits. This was demonstrated by the single protein band observed with both enzymes by NaDodSO<sub>4</sub> polyacrylamide gel electrophoresis. Our data also suggest that each enzyme may be a homodimer, based upon the demonstration of a single set of amino acid residues in the NH<sub>2</sub>-terminal sequence for each enzyme, and the fact that for each enzyme the number of spots found on the peptide maps was of the right order of magnitude based on molecular weight and amino acid composition. Calf intestine alkaline phosphatase is a dimer of equal molecular weight subunits and was postulated to be a homodimer (20). The *Escherichia coli* alkaline phosphatase is a dimer coded by a single cistron (21), and the possibility exists that alkaline phosphatase is a general family of enzymes throughout evolutionary development. The mammalian enzymes are glycoproteins; the *E. coli* enzyme has no carbohydrate (22).

The importance of characterizing the molecular structure of alkaline phosphatase was recently emphasized from the purification and characterization of the alkaline phosphatase of KB cells, an aneuploid cell line (23). The KB alkaline phosphatase is immunochemically indistinguishable from placental

alkaline phosphatase, although its properties during purification are different. However, two catalytically active peptide subunits were identified from purified KB enzyme preparations; these subunits differed in apparent molecular weight, the initial NH<sub>2</sub>-terminal amino acid, and in carbohydrate content. It was not ascertained whether the native KB enzyme is a heterodimer, or whether two homodimers were present and both were carried through the purification.

We hope that these studies will encourage the purification and structural characterization of alkaline phosphatases from other tissues so that a more extensive series of these isoenzymes can be classified on the basis of their peptide structures.

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