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HUMAN ALKALINE PHOSPHATASES

EVIDENCE OF THREE ISOENZYMES (PLACENTAL, INTESTINAL AND LIVER-BONE-KIDNEY-TYPE) BY LECTIN-BINDING AFFINITY AND IMMUNOLOGICAL SPECIFICITY

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Summary

The structural relationship between human alkaline phosphatase isoenzymes from placenta, intestine, liver, bone and kidney was investigated by lectin-binding affinity chromatography. In addition, antibody-binding sites of the enzymes were studied using monospecific antisera against each of the isoenzymes. Evidence is offered for the existence of three classes of alkaline phosphatases: the placental isoenzyme, the intestinal isoenzyme and the liver-bone-kidney-type-isoenzyme: 1. A high affinity to bind to concanavalin A and lentil lectin characterizes the placental isoenzyme in contrast to the other isoenzymes. The intestinal isoenzyme remains totally unbound. The liver-bone-kidney-isoenzyme demonstrates a microheterogeneity with bound and unbound parts. A small unbound fraction can be detected in the placental isoenzyme, also when lentil lectin is used. 2. The placental isoenzyme and the isoenzymes purified from liver, bone and kidney are bound by wheat germ lectin-Sepharose, but not the intestinal isoenzyme. All isoenzymes are eluted as a homogeneous peak. 3. Using *Helix pomatia* lectin-Sepharose, all isoenzymes are unreactive except a minor fraction of kidney alkaline phosphatase. 4. Antibodies to the placental isoenzyme show a partial cross-reaction with the intestinal isoenzyme, but can be obtained monospecific after absorption. 5. Antibodies to the intestinal isoenzyme show a partial cross-reaction to the placental isoenzyme, respectively, but are monospecific again after absorption. 6. Antibodies to liver, bone or kidney isoenzymes show a complete cross-reaction,

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Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; Temed, *N,N,N',N'*-tetramethylenediamine.

but are unreactive with the placental and intestinal isoenzyme; after absorption with a heterologous isoenzyme of this group, no further reaction can be demonstrated with any of the three isoenzymes. Thus, lectin-binding affinity identifies the same isoenzyme classes by their carbohydrate parts, as antibodies presumably do by the protein parts of the isoenzymes. Furthermore, lectin-binding affinity demonstrates a microheterogeneity of the placental isoenzyme with lentil lectin-Sepharose and of the liver-bone-kidney-type-isoenzyme with different lectins.

Human serum alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC. 3.1.3.1) is one of the most important parameters in clinical chemistry. Human alkaline phosphatases occur in multiple forms as demonstrated by an enormous microheterogeneity in polyacrylamide gel electrophoresis. Clinical chemical methods, specific for one isoenzyme deriving from a definite tissue, could not be developed because of the unresolved structural relationships among human alkaline phosphatase(s). In recent years, the existence of three different structural genes has been suggested by four different experimental approaches: these genes code three different isoenzymes: the placental isoenzyme (P), the intestinal isoenzyme (I) and the liver-bone-kidney-type isoenzyme (L, B and K). The following methods are involved: (a) The catalytical properties of alkaline phosphatase(s) isolated from different human organs was shown to vary when different activators or inhibitors were used [1—9], (b) The stability of the molecule, as measured by the relative heat resistance, was higher for P- alkaline phosphatase than for other alkaline phosphatase(s) [10—12]. (c) The antigenic determinants of P- and I-alkaline phosphatase showed a close immunological relationship (Refs. 13—15, divergent results, Refs. 5, 16—18), but differed from those of L-, B- and K-alkaline phosphatase. Antibodies to these isoenzymes seem to suggest a close immunological relationship between L-, B- and K-alkaline phosphatase [3,5,14,16,19—21]. (d) A different primary structure of different isoenzymes was demonstrated by partial proteolytic-peptide 'mapping' [22,23]. However, activation and inhibition studies as well as thermostability experiments only provide an indirect support for the structural relationship of alkaline phosphatase(s). The immunological studies are still incomplete and partially controversial, and little is known about the carbohydrate part of different alkaline phosphatase(s). In this paper, we present a complete immunological study of human alkaline phosphatase(s); and we demonstrate that lectin-binding affinity is able to (a) discriminate the same isoenzymes as antibodies do, and (b) can demonstrate a not yet described heterogeneity of the liver-bone-kidney-isoenzyme.

Materials and Methods

Purification of human alkaline phosphatases

All isolation steps were performed at 4° C in 0.01 M Tris buffer with 0.002 M $MgCl_2$, pH 7.5 (for B-alkaline phosphatase, 0.005 M $ZnCl_2$ was added). 7 P-, 6

TABLE I

Purification protocol of alkaline phosphatase(s) (AP) used for immunisation (P-alkaline phosphatase$_1$, I-alkaline phosphatase$_1$, L-alkaline phosphatase$_1$, B-alkaline phosphatase$_F$, B-alkaline phosphatase$_1$; K-alkaline phosphatase$_1$ was a gift of Professor G. Pfleiderer, Stuttgart) and lectin-binding affinity chromatography (P-$_2$, I-$_2$, L-alkaline phosphatase$_2$, B-alkaline phosphatase$_2$ and K-alkaline phosphatase$_2$). P-AP is activated, B-AP partially denatured during the isolation procedure. Crystalline preparations: P-alkaline phosphatase$_1$, I-alkaline phosphatase$_1$, L-alkaline phosphatase$_1$ and B-alkaline phosphatase$_1$. The other 5 P-, 4 I-, 14 L-, 6-B and 1 K-alkaline phosphatase preparations were isolated in analogy, except for some L-alkaline phosphatase preparations, additionally purified by preparative polyacrylamide gel electrophoresis (PAGE). Data represented in units AP.

	P-AP$_1$	P-AP$_2$	I-AP$_1$	I-AP$_2$	L-AP$_1$	L-AP$_2$	B-AP$_F$	B-AP$_1$	B-AP$_2$	K-AP$_2$
n-Butanol extract	17980	14 743	2639	855	1560	2956	210	234	1251	605
Acetone precipitation	23 100	16 367	2236	757	1215	2725	—	—	—	—
Heat step	24 600	15 163	—	—	—	—	—	—	—	—
$(NH_4)_2SO_4$ precipitation	23 600	17 950	2164	496	1092	2183	77.7	—	—	518
DEAE-A 50 Sephadex	22 950	15 709	1634	145	1050	1624	64.9	160	1012	213
Sephadex G-200 or Ultrogel	12 760	11 372	1437	127	710	1123	42.2	128	997	182
$(NH_4)_2SO_4$ or ultrafiltration	9 380	11 339	1141	112	616	661	37.8	113	653	158
Preparative PAGE	—	—	—	—	—	—	—	79.9	602	—
Specific activity	196.2	145.4	736.0	747.0	41.34	49.88	3.22	3.51	4.68	4.61
Purification factor	204.2	177.3	566.2	238.7	188.0	43.0	38.3	255.1	42.3	16.4
Yield (%)	52.2	76.9	43.2	13.1	20.8	22.3	18.0	34.2	48.1	26.1

I-, 16 L-, 9 B- and 2 K-alkaline phosphatase preparations were isolated and stored in $(NH_4)_2SO_4$ at −20° C. Table I shows a brief isolated protocol of all alkaline phsophatase(s) used for immunization (P-alkaline phosphatase$_1$, I-alkaline phosphatase$_1$, L-alkaline phosphatase$_1$, B-alkaline phosphatase$_F$, and B-alkaline phosphatase$_1$; N-alkaline phosphatase$_1$ was a gift of Professor G. Pfeiderer, Dept. of Organic Chemistry and Biochemistry, University of Stuttgart, F.R.G.) and for lectin-binding affinity chromatography (P-alkaline phosphatase$_2$, I-alkaline phosphatase$_2$, L-alkaline phosphatase$_2$, B-alkaline phosphatase$_2$ and N-alkaline phosphatase$_2$). The other preparations in the study were isolated in analogy, except L-alkaline phosphatase which was further purified by preparative polyacrylamide gel electrophoresis (see below). All alkaline phosphatase(s) with the exception of B-alkaline phosphatase were stable during the purification procedure. The specific activities, cited in Table I, were not always the highest attainable. However, contamination by other phosphatase(s) was excluded and antibody preparations were completely absorbed if necessary.

Extraction. Human term placentae were collected immediately after birth and washed through the cord vessels with heparin containing saline. The blood-free placental tissue was homogenized with 0.25 M sucrose (1 : 5, w/v) and extracted with *n*-butanol (1 : 2, w/v). After remaining at 37° C for 60 min the homogenate was stored overnight at 4° C and the layer containing the enzyme was passed through a cheese cloth. Small intestinal mucosa was obtained from autopsy material less than 12 h after death by scraping the jejunum and ileum mucosa off with a glass microscope slide. Livers and kidneys were collected from autopsy material. Bones (sternum, humeri, femurs and vertebrae) were obtained either from aborted fetuses (4—9 months old) for preparation of B-alkaline phosphatase$_F$, or from adults (for all other preparations), autolyzed in double distilled water, pH 8.5, for 48 h and then extracted as described for placentae. All tissues were macro- and microscopically normal.

Acetone precipitation. The enzyme solution was saturated with acetone at −7° C up to 30%, stirred for 30 min and centrifuged at −7° C at 12 000 × *g*. The supernatant was saturated up to 55%, stirred for 30 min and centrifuged. The sediment was suspended in buffer (see above) and dialyzed free of butanol and acetone against buffer for at least 48 h.

Heat step. This was used only for P-alkaline phosphatase; the dialyzed material was heated at 65° C for 10 min, cooled in an ice bath and centrifuged at 12 000 × *g*.

$(NH_4)_2SO_4$ precipitation. The supernatant was saturated up to 45% by addition of solid $(NH_4)_2SO_4$, stirred for at least 4 h and centrifuged at 20 000 × *g*. Further saturation was performed by addition of $(NH_4)_2SO_4$ up to 62—75% (until less than 8% of the enzymatic activity could be detected in the supernatant of a centrifuged specimen). The solution was stirred overnight and centrifuged at 20 000 × *g*. The sediment was suspended in buffer and dialyzed free of $(NH_4)_2SO_4$ against the equilibration buffer of the anion exchange chromatography.

Anion exchange chromatography. DEAE-Sephadex A-50 chromatography was performed with a 1 : 10 diluted buffer for equilibration and with a discontinuous NaCl gradient system [14] for elution using a LKB-Ultrograd gradi-

ent mixer with a level sensor system. The alkaline phosphatase activity-containing fractions were pooled and concentrated by precipitation with $(NH_4)_2SO_4$ or ultrafiltration using a PM-30 or XM-50 membrane in an Amicon ultrafiltration cell (Oosterhout, The Netherlands).

Gel filtration. The material was passed through a Sephadex G-200 column, with buffer, for equilibration and elution. The enzymatic activity-containing fraction was again pooled and concentrated by addition of $(NH_4)_2SO_4$ or ultrafiltration. In some preparations Ultrogel AcA 34 was used instead of Sephadex G-200.

Crystallization. Some preparations (like P-alkaline phosphatase$_1$, L-alkaline phosphatase$_1$, I-alkaline phosphatase$_1$ and B-alkaline phosphatase$_1$) were crystallized and, if necessary, recrystallized in buffer at pH 7.3—7.4 by slow addition of $(NH_4)_2SO_4$ over weeks.

Preparative polyacrylamide gel electrophoresis. Some L-alkaline phosphatase and B-alkaline phosphatase preparations were further purified by preparative polyacrylamide gel electrophoresis (see below). Only one peak with enzymatic activity could be detected in all experiments in the elution profile.

Antibodies

Antisera were obtained in rabbits immunized with purified alkaline phosphatase(s) in complete Freund's adjuvant according to the methods and the scheme described [24]. Anti-P-alkaline phosphatase-IgG was absorbed with soluble pure I-alkaline phosphatase and anti-I-alkaline phosphatase-IgG by affinity chromatography on CNBr-activated Sepharose 4B coupled to pure P-alkaline phosphatase as reported earlier [14]. IgG fractions were obtained by two consecutive precipitations with $(NH_4)_2SO_4$ (40 and 35% saturation) and chromatography on DEAE-cellulose or DEAE-Sephacel.

Anti-L-alkaline phosphatase-γ-globulin was absorbed either with soluble B-alkaline phosphatase (step-wise absorption) or with affinity chromatography on CNBr-activated Sepharose 4B coupled to pure B-alkaline phosphatase. Anti-B-alkaline phosphatase serum was similarly absorbed with pure L-alkaline phosphatase coupled to CNBr-activated Sepharose 4B as absorbent. Antibodies to minor contaminants, detectable with high titer antisera against tissue concentrates, could be removed by consecutive affinity chromatography according to the scheme described for anti-I-alkaline phosphatase-IgG [25]. All antisera were controlled against tissue extracts (from which the antigen has been purified), against the pure antigen and other pure alkaline phosphatase(s) as well as standard human serum (Behring, Marburg, F.R.G.), both in the double-diffusion technique according to Ouchterlony [26] and Piazzi [27], in immunoelectrophoresis according to Grabar and Williams [28], in Scheidegger's modification [29] with staining for proteins (Coomassie brillant blue) and for enzymatic activity (see below). Purity of IgG preparations were assured with anti-IgA, anti-IgG and anti-IgM sera from Miles-Seravac (Rehovot, Israel) in immunoelectrophoresis.

Polyacrylamide gel electrophoresis

Analytical vertical slab polyacrylamide gel electrophoresis was carried out in a discontinuous voltage and pH gradient system according to Ornstein [30] and

Davis [31]. A 2.5% spacer gel (pH 8.3, 2 mm thick, length 3 cm) and a 7.5% separation gel (pH 9.5, 2 mm thick, length 10 cm) were used. 0.47 M Tris buffer, containing Temed (pH 6.9) was the spacer gel buffer; 3.0 M Tris buffer, containing Temed (pH 8.9) the separation gel buffer and 0.039 M Tris/0.39 M glycine buffer (pH 8.3) the electrode buffer solution as described by Davis [32] and Maurer [33] (Ultraphor apparatus, Colora, Heidelberg, F.R.G., 4°C). Bromphenol blue was used as marker. Gels were stained for proteins with Coomassie brillant blue. Alkaline phosphatase activity was detected by staining with α-naphthylphosphate and fast blue B salt according to Khattab and Pfleiderer [34] and fixed with 5% acetic acid.

Preparative polyacrylamide gel electrophoresis was performed in analogy to the analytical procedure with a 5 mm thick gel (2 cm spacer gel, 5 cm separation gel, elution buffer: 0.124 Tris buffer, pH 8.1). The eluted fractions were recorded by an ultraviolet densitometer at 280 nm, collected in a Ultrorac collector (both from LKB, Bromma, Sweden), and assayed for enzymatic activity. Peak fractions were concentrated by $(NH_4)_2SO_4$ precipitation or ultrafiltration.

Enzymatic activity

Alkaline phosphatase was measured in all experiments by the method of Bessey et al. [35] in 0.05 M glycine buffer, containing 0.0005 M $MgCl_2$ at pH 10.5 with *p*-nitrophenylphosphate as substrate.

Protein was determined with the biuret reagent and $(NH_4)_2SO_4$ molarity with Nesslers reagent by the methods of Beisenherz et al. [36]. Staining for alkaline phosphatase activity was carried out as described under 'Polyacrylamide gel electrophoresis'.

Quantitative precipitation

Quantitative titration of pure alkaline phosphatase(s) was performed by incubation of antigen with increasing amounts of antibodies in a final volume of 0.5 ml of 0.01 M Tris buffer, containing 0.002 M $MgCl_2$ (for B-alkaline phosphatase, 0.005 M $ZnCl_2$ was also added), 5% pure human albumin and (in most of the experiments) 5% poly(ethylene glycol) 6000 (pH 7.5) at 25°C for 60 min. Centrifugation was carried out at 4°C for 30 min at 20 000 × *g*, the supernatant was reincubated at 25°C and alkaline phosphatase activity was determined.

Lectin-binding affinity chromatography

Pure alkaline phosphatase(s) were dialyzed against the corresponding equilibration buffer of the lectin column, applied to a K 9/15 column (Pharmacia, Uppsala, Sweden) containing a 5 cm high-lectin gel layer (ϕ, 0.9 cm; l = 15 cm; maximal protein load, 0.5 mg pure alkaline phosphatase = large excess of lectin) and eluted at a flow rate of 6.8 ml/h after an incubation period of 10 min. The eluate was recorded by an ultraviolet densitometer at 280 nm, collected in 1.4-ml fractions and measured for alkaline phosphatase activity. Rechromatography of single peaks was performed in analogy following dialysis against the equilibration buffer. Peaks containing enzymatic activity were pooled and concentrated by ultrafiltration on a XM 50 membrane in an

Amicon UF cell 12 or 8 MC cell. The activity of each peak was calculated on the basis of the activity after dialysis and before the start of the chromatography. Conditions: Con A-Sepharose 4B; 0.1 M sodium acetate buffer/0.001 M $MgCl_2$/0.001 M $CaCl_2$/0.5 NaCl (pH 6.5) for equilibration and first elution, with 0.05 α-methyl-D-mannoside (Sigma Chemical Co., St. Louis, MO) for elution of the bound material. Lentil lectin-Sepharose 4B; 0.05 M di-sodiumhydrogenphosphate buffer with 0.2 M NaCl (pH 7.0) for equilibration and first elution and with 0.15 M α-methyl-D-mannoside for elution of the bound material. Wheat germ lectin-Sepharose 6MB; same buffer as lentil lectin-Sepharose, elution with 10% *N*-acetyl-D-glucosamine (Fluka, Buchs, Switzerland). *Helix pomatia* lectin-Sepharose 6MB; same buffer as lentil lectin-Sepharose, elution with 1% *N*-acetyl-D-galactosamine (Sigma, St. Louis, MO).

Chemicals

N,N,N',N'-tetramethylethylendiamine, acrylamide, *N,N'*-methylenebisacrylamide, tetrabromphenolsulphataleine, riboflavine and all other chemicals for polyacrylamide gel electrophoresis, DEAE-cellulose and Coomassie brillant blue R-250 were purchased from Serva, Heidelberg, F.R.G. α-Naphthylphosphate was prepared by Boehringer, Mannheim, F.R.G. DEAE-Sephadex, DEAE-Sephacel, Con A-Sepharose, lentil lectin-Sepharose 4B, wheat germ lectin-Sepharose 6MB, *Helix pomatia* lectin-Sepharose 6MB and CNBr-activated Sepharose 4B were reagents of Pharmacia, Uppsala, Sweden. Human albumin and complete Freund's adjuvant were reagents from Behring, Marburg, F.R.G.; agar Noble from Difco Lab., Detroit, MI; Ultrogen AcA 34 from LKB, Bromma, Sweden, and poly(ethylene glycol) 6000, fast blue B salt and all other chemicals from Merck, Darmstadt, F.R.G.

Results

Lectin-binding affinity of human alkaline phosphatases

Con A-Sepharose. The placental isoenzyme is completely bound to concanavalin A, the intestinal isoenzyme remains completely unbound and emerges in the void volume. The liver, bone and kidney isoenzymes are separated in two peaks: the first peak appears in the void volume, the second peak is bound to concanavalin A, but easily eluted with α-methyl-D-mannoside (Fig. 1). The recovery rates and the percentage activity within different peaks are contained in Fig. 1; the data for P- and K-alkaline phosphatase are the mean of two determinations. All first unbound peaks of L-, B- and K-alkaline phosphatase were dialyzed against the equilibration buffer of this column, rechromatographed and recovered in the same position (void volume) as before. Thus, an overload of the column during the first experiment is excluded. The recovery rates of the rechromatographs were 90.8, 85.7 and 93.4%.

Lentil lectin-Sepharose. P-Alkaline phosphatase demonstrates a small, loosely bound fraction (4.42 and 4.71% in the first and second experiments, respectively) eluted after the void volume by washing with equilibration buffer. If this peak is rechromatographed, it appears in the void volume. The main fraction is bound to lentil lectin and eluted with α-methyl-D-mannoside. I-Alkaline

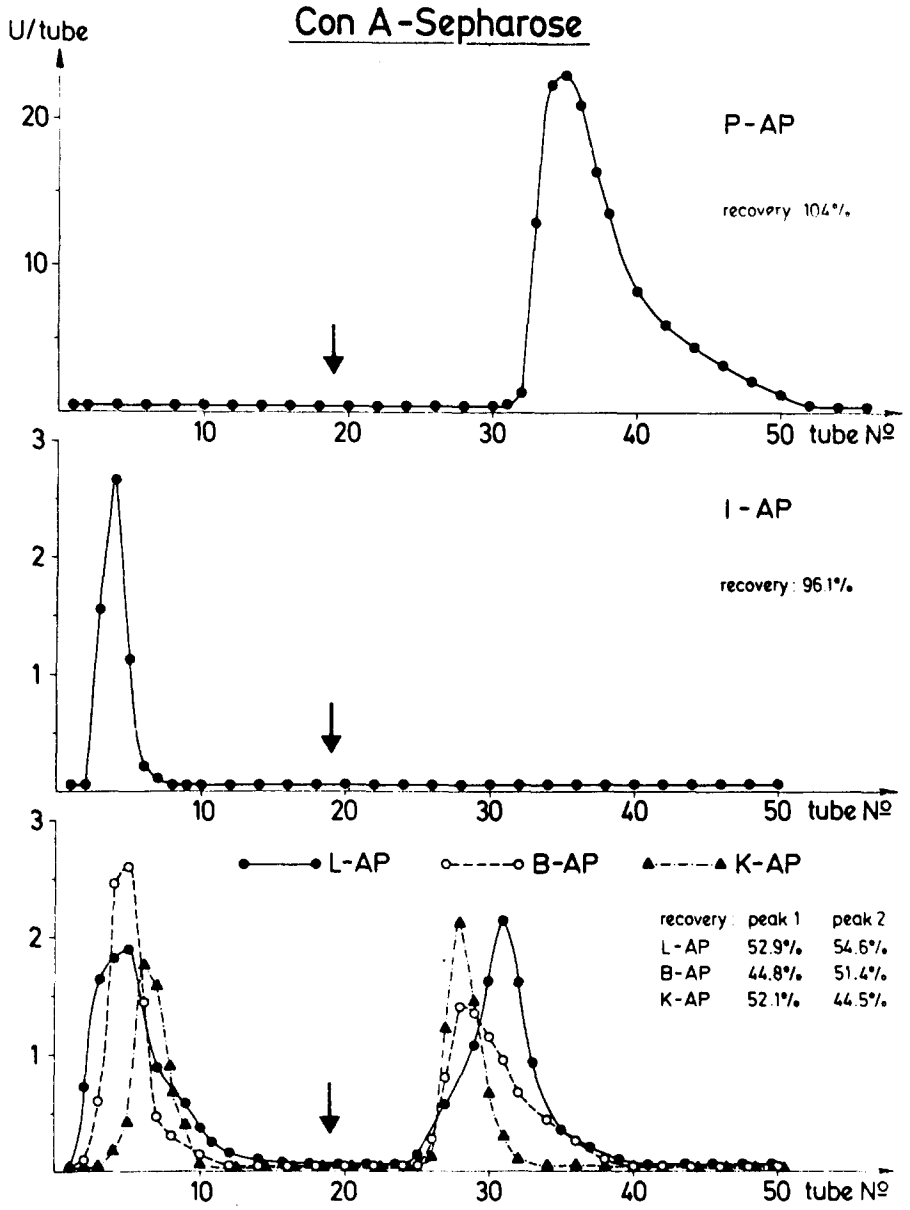

Fig. 1. Lectin-binding affinity of human alkaline phosphatase(s) on Con A-Sepharose.

phosphatase remains completely unbound. L-, B- and K-alkaline phosphatase yield two peaks in the concanavalin A experiment: the first peak appears in the void volume, the second peak is bound to the column (Fig. 2). The recovery rates as given in Fig. 2 are the mean of two experiments for P-alkaline phosphatase, and the mean of three experiments for L- and B-alkaline phosphatase. The first peaks each of the P-, L- and B-alkaline phosphatase elution profiles were dialyzed against the equilibration buffer of the column and appeared in the same position on rechromatography (except P-alkaline phosphatase, see above).

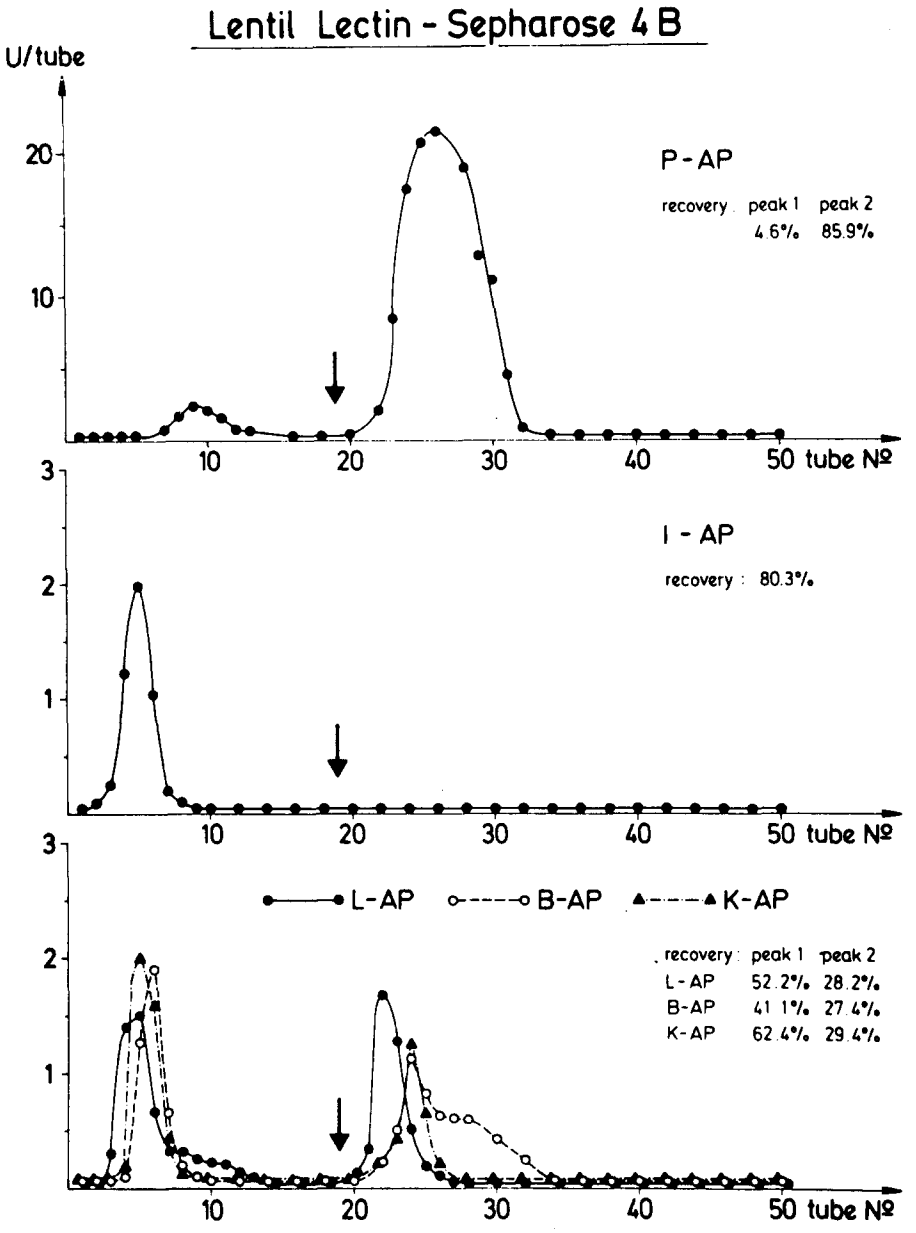

Fig. 2. Lectin-binding affinity of human alkaline phosphatase(s) to lentil lectin-Sepharose 4B.

Wheat germ lectin-Sepharose. P-, L-, B- and K-alkaline phosphatase are completely bound to the wheat germ lectin, whereas I-alkaline phosphatase is eluted with the void volume (Fig. 3).

Helix pomatia lectin-Sepharose. All alkaline phosphatase isoenzymes appear in the void volume and remain unbound except a small second peak of K-alkaline phosphatase of 19.4% in the first and 14.8% in the second experiment (Fig. 4). The recovery rates are indicated in Fig. 4.

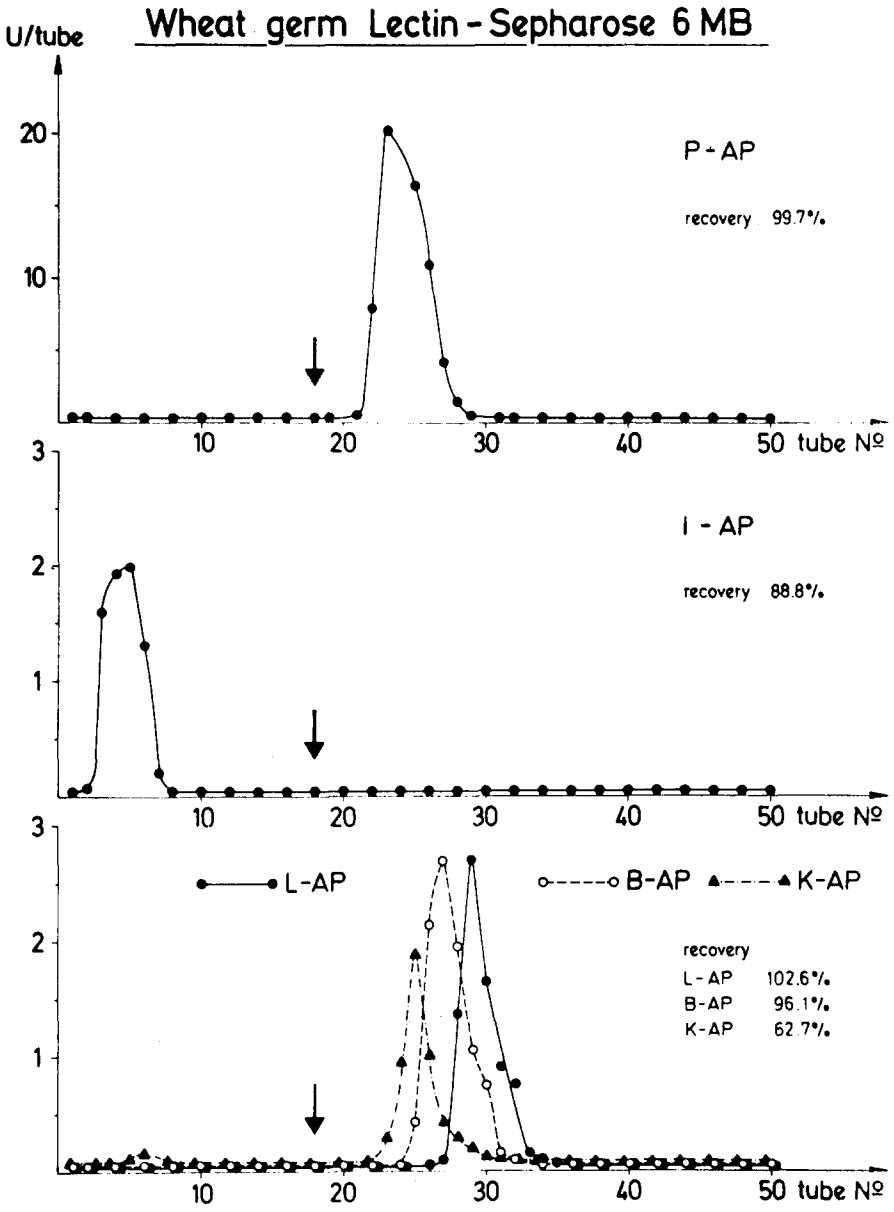

Fig. 3. Lectin-binding affinity of human alkaline phosphatase(s) to wheat germ lectin-Sepharose 4B.

In analytical polyacrylamide gel electrophoresis, the electrophoretic mobility of the two L-alkaline phosphatase fractions separated by concanavalin A was retarded towards the cathode, and that of peaks I and II separated by lentil lectin-Sepharose was increased, as compared to unfractionated L-alkaline phosphatase (Fig. 5A). However, the electrophoretic mobility of peaks I and II neither differed in the concanavalin A nor in the lentil lectin-binding experiment. On the other hand, all fractions of B- and K-alkaline phosphatase bound

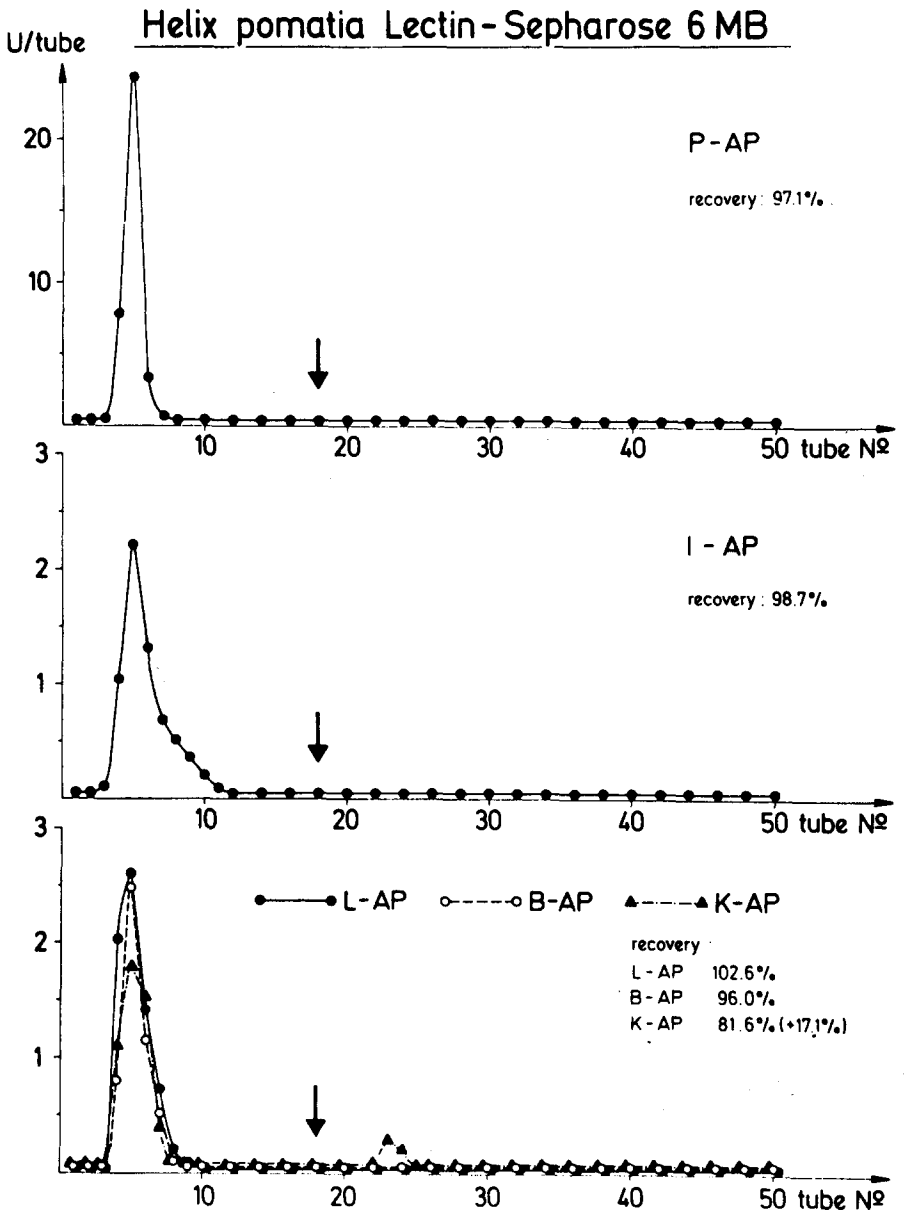

Fig. 4. Lectin-binding affinity of human alkaline phosphatase(s) to *Helix pomatia* lectin-Sepharose 6MB.

to concanavalin A, lentil lectin, or *Helix pomatia* lectin (only K-alkaline phosphatase) showed more anodic migration than the unbound fractions of B- and K-alkaline phosphatase as eluted with the void volume (Fig. 5B and C). P-alkaline phosphatase, Con A-Sepharose-bound P-alkaline phosphatase, the first P-alkaline phosphatase peak loosely bound to lentil lectin-Sepharose and the lentil lectin-Sepharose-bound P-alkaline phosphatase peak have a similar electrophoretic mobility (Fig. 5B). The second peak of lentil lectin-Sepharose,

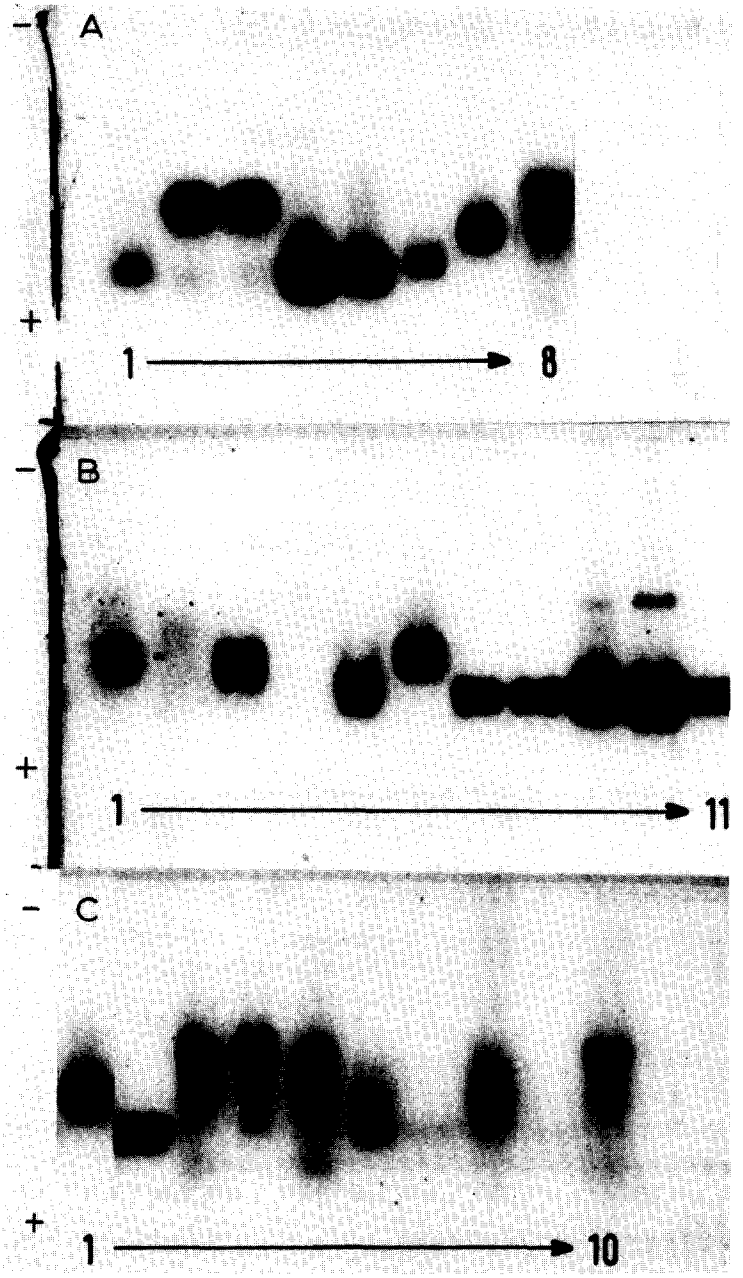

Fig. 5. Analytical polyacrylamide gel electrophoresis of human alkaline phosphatase(s) (AP) before and after affinity chromatography through lectin gel columns. (A) 1. L-AP; 2. L-AP, concanavalin A, 1. peak; 3. L-AP, concanavalin A, 2. peak; 4. L-AP, lentil lectin, 1. peak; 5. L-AP, lentil lectin, 2. peak; 6. L-AP; 7. B-AP; 8. K-AP. (B) 1. B-AP; 2. B-AP, concanavalin A, 1. peak; 3. B-AP, concanavalin A, 2. peak; 4. B-AP, lentil lectin, 1. peak; 5. B-AP, lentil lectin, 2. peak; 6. B-AP; 7. P-AP; 8. P-AP, concanavalin A, peak; 9. P-AP, lentil lectin, 1. peak; 10. P-AP, lentil lectin, 2. peak; 11. P-AP. (C) 1. B-AP; 2. P-AP; 3, K-AP; 4. K-AP, concanavalin A, 1. peak; 5. K-AP, concanavalin A, 2. peak; 6. K-AP, lentil lectin, 1. peak; 7. K-AP, lentil lectin, 2. peak; 8. K-AP, *Helix pomatia* lectin, 1. peak; 9. K-AP, *Helix pomatia* lectin, 2. peak; 10. K-AP.

however, migrates a little bit more towards the anode than the first peak. Following chromatography on lentil lectin-Sepharose both of the peaks contain a faint second band located toward the cathode as compared with the enzyme prior to chromatography (Fig. 5B).

Immunological relationship of human alkaline phosphatases

Placental isoenzyme. P-alkaline phosphatase is precipitated by antibodies to P- and I-alkaline phosphatase, but does not react with antibodies to B-, L- or K-alkaline phosphatase (Fig. 6). Ouchterlony double-diffusion demonstrates a partial cross-reaction with the intestinal isoenzyme (Fig. 9). After absorption of anti-P-alkaline phosphatase-IgG with purified I-alkaline phosphatase, the antiserum is monospecific for P-alkaline phosphatase (Fig. 6), whereas anti-I-alkaline phosphatase-IgG, absorbed with P-alkaline phosphatase, no longer reacts with P-alkaline phosphatase (Fig. 6). Thus, P- and I-alkaline phosphatase share antigenic determinants. A monospecific antiserum can be produced by absorption of anti-P-alkaline phosphatase serum with I-alkaline phosphatase.

Intestinal isoenzyme. I-Alkaline phosphatase is precipitated by its own antibodies as well as by anti-P-alkaline phosphatase serum, but it does not react with anti-L-, anti-B-, and anti-K-alkaline phosphatase-antibodies (Fig. 7). Again, a partial immunological cross-reaction is demonstrated in double-diffusion (Fig. 9). After absorption of anti-I-alkaline phosphatase-IgG with purified P-alkaline phosphatase, the antiserum is monospecific for I-alkaline phosphatase and remains unreactive with all other human alkaline phosphatases

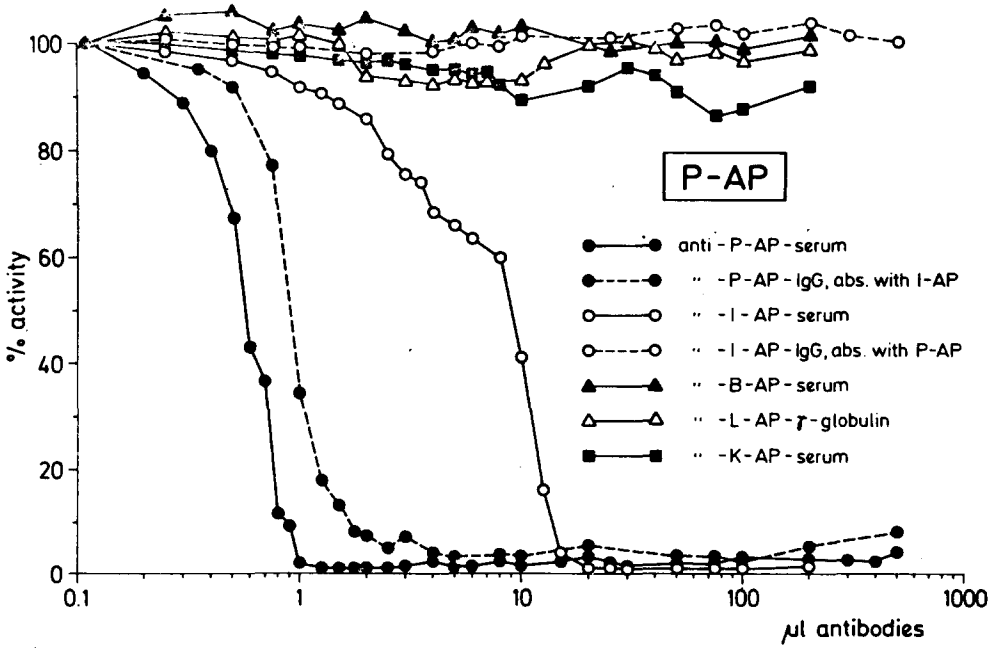

Fig. 6. Precipitation of P-alkaline phosphatase by antisera to human alkaline phosphatase(s). The anti-P-alkaline phosphatase serum, absorbed with I-alkaline phosphatase, still reacts with P-alkaline phosphatase, but is unreactive to I-alkaline phosphatase.

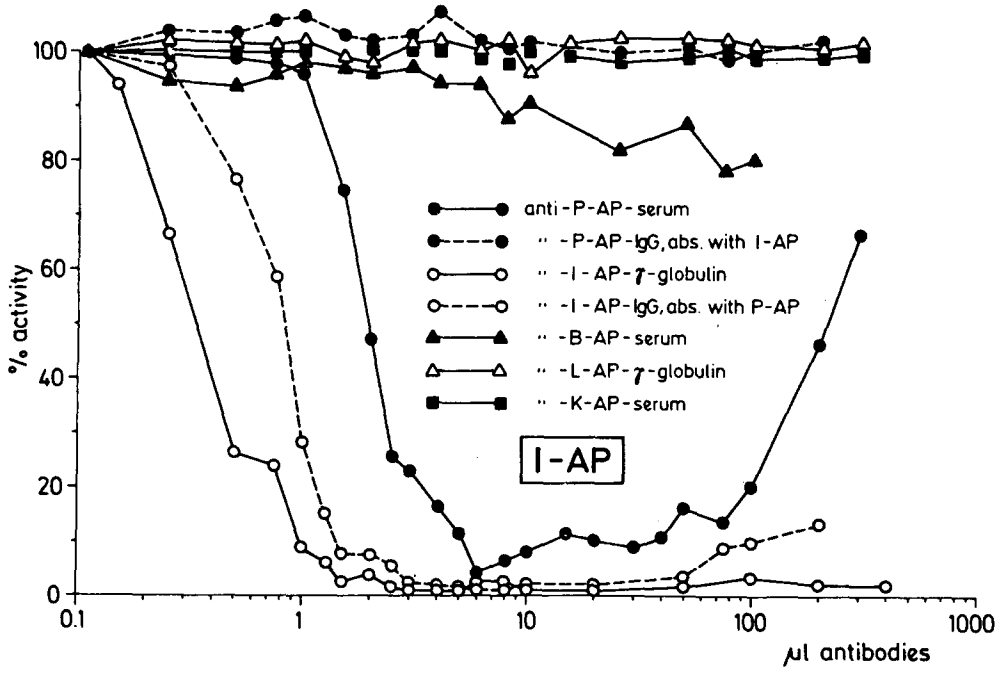

Fig. 7. Precipitation of I-alkaline phosphatase by antisera to human alkaline phosphatase(s). The anti-I-alkaline phosphatase serum, absorbed with P-alkaline phosphatase still reacts with I-alkaline phosphatase, but is unreactive to P-alkaline phosphatase.

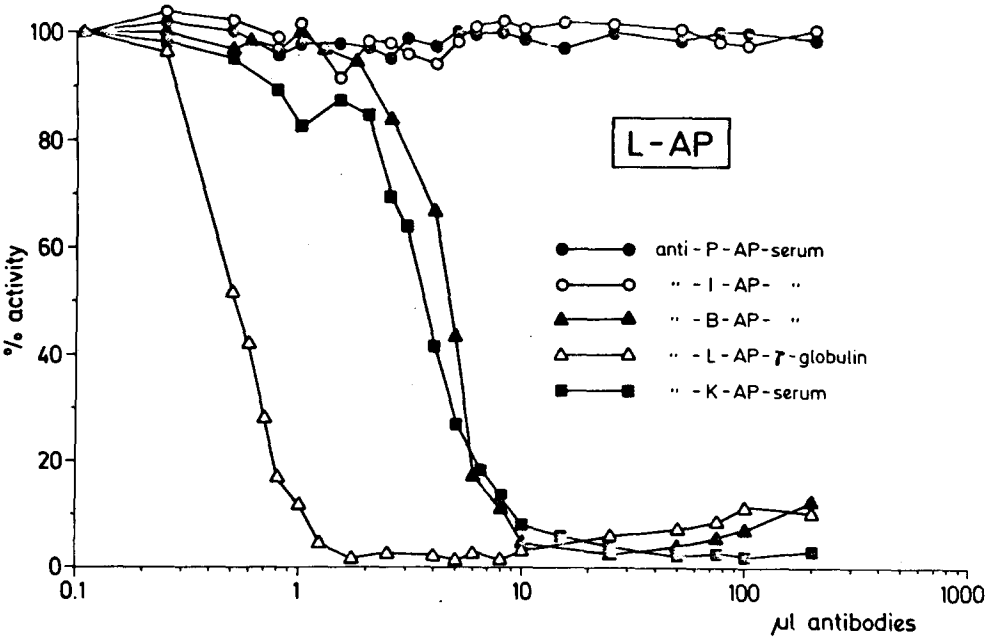

Fig. 8. Precipitation of L-alkaline phosphatase by antisera to human alkaline phosphatase(s). The use of B- or K-alkaline phosphatase instead of L-alkaline phosphatase demonstrates similar slopes (data not shown).

(Fig. 7), whereas anti-P-alkaline phosphatase-IgG, absorbed with I-alkaline phosphatase, no longer reacts with I-alkaline phosphatase (Fig. 7). Thus, a monospecific antiserum to I-alkaline phosphatase can be prepared by absorption of anti-I-alkaline phosphatase serum with P-alkaline phosphatase.

L-, B- and K-alkaline phosphatase isoenzyme. L-Alkaline phosphatase is equally precipitated by anti-L-, anti-B- and anti-K-alkaline phosphatase-antibodies, whereas anti-P- or anti-I-alkaline phosphatase serum do not react (Fig. 8). The same results can be obtained with B- and K-alkaline phosphatase instead of L-alkaline phosphatase: again, the antibodies to L-, B- and K-alkaline phosphatase easily precipitate B- and K-alkaline phosphatase, whereas B- and K-alkaline phosphatase are not precipitated by anti-P- and anti-I-alkaline serum. In the double-diffusion technique, a line of complete identity between L- and B-alkaline phosphatase can be demonstrated with antibodies to L- and B-alkaline phosphatase (Fig. 9).

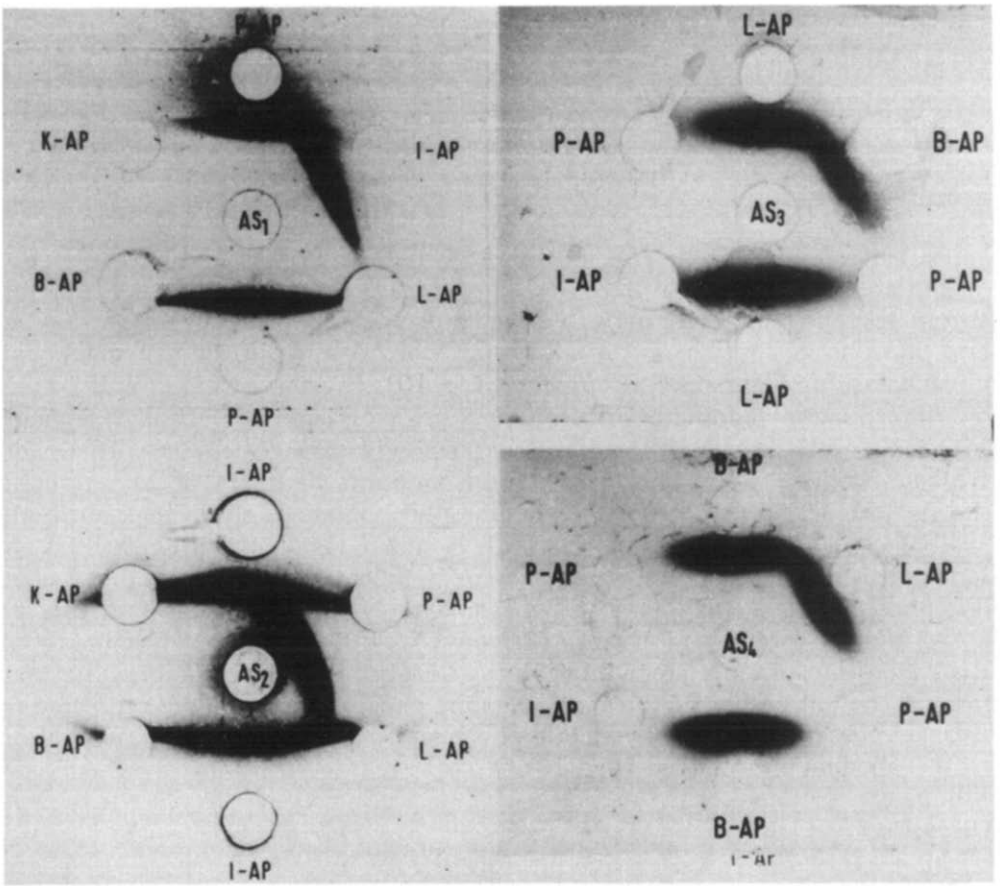

Fig. 9. Ouchterlony double-diffusion demonstrates a partial cross-reaction between P- and I-alkaline phosphatase and complete identity between L- and B-alkaline phosphatase. AS_1 = anti-P-alkaline phosphatase serum; AS_2 = anti-I-alkaline phosphatase serum; AS_3 = anti-L-alkaline phosphatase-γ-globulin; AS_4 = anti-B-alkaline phosphatase serum. The gel plates were stained for enzymatic activity.

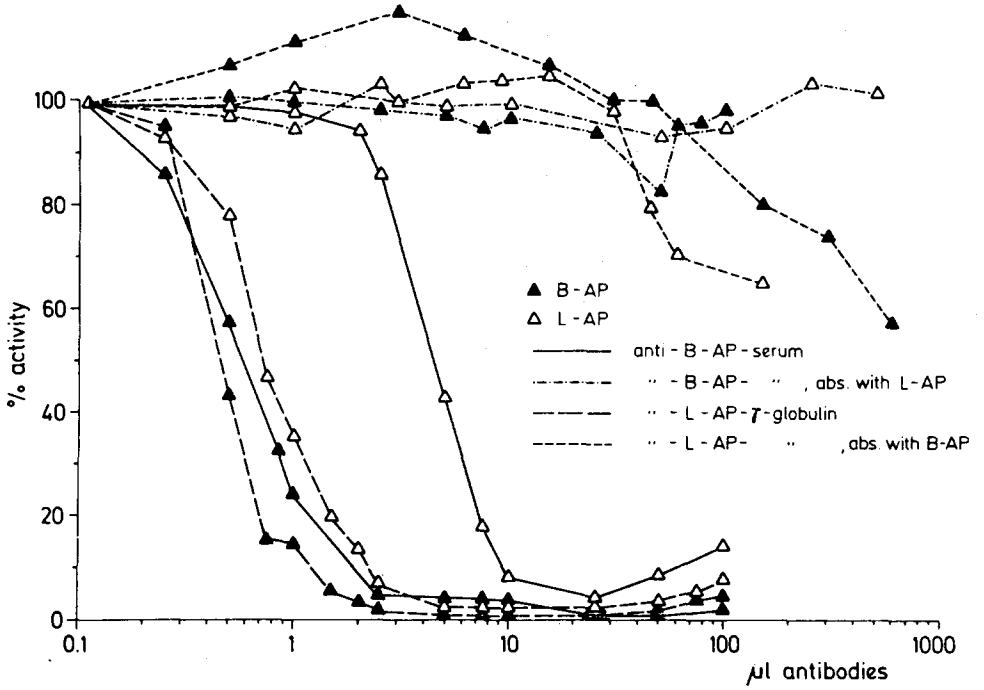

Fig. 10. Precipitation of L- and B-alkaline phosphatase by unabsorbed and absorbed anti-L- and anti-B-alkaline phosphatase sera.

The absorption of anti-B-alkaline phosphatase serum by means of affinity chromatography on CNBr-activated Sepharose 4B, coupled to purified L-alkaline phosphatase, results in an antiserum that does not react with B-alkaline phosphatase or L-alkaline phosphatase (Fig. 10). The absorption of anti-L-alkaline phosphatase-γ-globulin with soluble, purified B-alkaline phosphatase yields an equal result: before absorption, the antibody strongly reacts with L- and B-alkaline phosphatase. After absorption, amounts of up to 30 µl γ-globulin remain unreactive with B- and L-alkaline phosphatase. Only in high antibody excess, can a minimal precipitation with B- and L-alkaline phosphatase be observed, this occurs due to minor absorption deficiencies with the soluble B-alkaline phosphatase isoenzyme. Thus, it seems likely that all the antibodies in the anti-B-alkaline phosphatase serum directed to the antigenic determinants of B-alkaline phosphatase are removable by absorption with L-alkaline phosphatase, as the antibodies of the anti-L-alkaline phosphatase-γ-globulin directed to antigenic determinants of L-alkaline phosphatase are removable by absorption with B-alkaline phosphatase (Fig. 10).

Discussion

The same three alkaline phosphatase isoenzyme classes can be discriminated by lectin-binding affinity as well as with immunological methods. Similar results are obtained with lentil lectin-Sepharose and with Con A-Sepharose. Chromatography on concanavalin A or lentil lectin columns clearly separates

the immunologically related isoenzymes from placenta and intestine, and demonstrates that both isoenzymes are different from the liver-bone-kidney-type. The latter is apparently microheterogeneous. The interpretation of the first loosely bound P-alkaline phosphatase peak of lentil lectin-Sepharose chromatography is difficult, since several placentas have been used in each P-alkaline phosphatase preparation procedure; thus, genetically-determined phenotypes (F, S, I, FS, FI, SI) or other variants (like the D-variant) may contribute to this phenomenon. Wheat germ lectin is unable to bind I-alkaline phosphatase, but binds all other investigated alkaline phosphatase isoenzymes. The intestinal isoenzymes can therefore be separated from all other isoenzymes studied in this paper using one purification step on chromatography on wheat germ lectin-Sepharose. Finally, our studies on *Helix pomatia* lectin-Sepharose demonstrates unreactivity towards all isoenzymes investigated in this study. A small peak of K-alkaline phosphatase, however, was bound to the matrix and eluted with *N*-acetyl-D-galactosamine, but this K-alkaline phosphatase preparation was not completely purified (see Table I). Further studies should exclude an involuntary contamination of the preparation.

The structural basis of the microheterogeneity of L-, B- and K-alkaline phosphatase indicates two types of isoenzymes with different terminal glucosylation. Analytical polyacrylamide gel electrophoresis proved that the unbound fraction and the fraction bound to concanavalin A or lentil lectin differ in their electrophoretic mobility. It has been suggested that L-, K- and B-alkaline phosphatase are coded by the same structural gene [23]. But their carbohydrate moieties are non-identical because of varying contents of sialic acid, and they express post-translational glucosylation of membrane-bound and secreted proteins [23]. We are at present studying the influence of neuraminidase digestion of alkaline phosphatase isoenzymes on their ability to bind to lectins. Using an alternative approach, we immunized rabbits with fractions of L-, B- and K-alkaline phosphatase able to bind or not to bind to lectins, in order to study their immunological relationship.

The aspect of the purification of alkaline phosphatase(s) and of the characterization of normal and variant alkaline phosphatase(s) is associated with another aspect: our lectin-binding affinity studies suggest that this method may also be applied to the separation of alkaline phosphatases occurring in the serum of patients. Provided that the lectin-binding affinity of circulating alkaline phosphatases is identical with that of purified alkaline phosphatase(s), wheat germ lectin may be suitable for the isolated determination of the intestinal isoenzyme, and concanavalin A or lentil lectin for the differentiation between the three isoenzyme classes. Further studies, using other lectins with different carbohydrate specificity, as well as investigations with neuraminidase digestion should elucidate whether or not the most interesting clinical problem, the separation of the circulating L-alkaline phosphatase from the circulating B-alkaline phosphatase can be performed by a simple chromatographic step.

Our immunological investigations enlarge previous incomplete studies [3,5, 13—34]. They clearly demonstrate three immunologically distinct isoenzymes, the placental, the intestinal and the liver-bone-kidney-type. Absorption experiments demonstrate that monospecific antisera and thus specific immunoassays can be obtained for: 1, P-alkaline phosphatase [37]; 2, I-alkaline phosphatase

[25] and 3, the L-, B- and K-alkaline phosphatase isoenzyme. The P-alkaline phosphatase assay is suitable for monitoring of pregnancy and for the detection of the Regan-isoenzyme in cancer patients [37]. The I-alkaline phosphatase assay in feces is a quantitative, non-invasive parameter for the damage of the brush border of the intestine under experimental [38] or clinical [39,40] conditions. The absorption of antisera to adult L-, B- or K-alkaline phosphatase preparations demonstrated a complete immunological identity of these three isoenzymes. Immunization with fractions binding or not binding to lectins, as well as immunization with L-, B- and K-alkaline phosphatase preparations following partial digestion of carbohydrate chains should prove whether or not an immunological differentiation of this 'universal' type [21] of alkaline phosphatase will be possible.

In summary, our studies by lectin-binding affinity and immunoprecipitation provide direct experimental support for the existence of at least three alkaline phosphatase genes coding P- and I-alkaline phosphatases and the L-, B- and K-alkaline phosphatase isoenzymes [22,23]. Furthermore, microheterogeneity due to a difference of the terminal carbohydrates of different isoenzymes has been demonstrated.

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