Human Alkaline Phosphatase

IMMUNOCHEMICAL IDENTIFICATION OF ORGAN-SPECIFIC ISOENZYMES

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SUMMARY

The antigenic distinctiveness of individual organ-specific isoenzymes of human alkaline phosphatase was shown by immunochromical means. A two-step method involving two separate antigen-antibody reactions was utilized to identify and measure the isoenzymes. The first reaction consisted of reaction of the isoenzyme with its antiserum; the second reaction consisted of precipitating the enzyme-antibody complex with anti-y-globulin. The second reaction was necessary because the enzyme was neither inhibited nor precipitated by specific antibody. By this method, individual isoenzymes could be identified from mixtures of isoenzymes.

The results clearly show that antibody to liver phosphatase and antibody to placental phosphatase were specific for their respective homologous antigens, and did not react with alkaline phosphatase from bone, neutrophils, kidney, or intestine. Thus, there are at least three antigenic types of alkaline phosphatases: one derived from liver, one from placenta, and one or more from other organs.

In the present study, an immunochromical method capable of specific and quantitative measurement of individual molecular forms was used to examine alkaline phosphatases isolated from individual organs.1 Evidence derived from these immunochromical studies is presented for the existence of antigenically distinct, organ-specific alkaline phosphatase isoenzymes.

MATERIALS AND METHODS

The chromatographic materials used were DEAE-cellulose (Bio-Rad), Sephadex G-200 (Pharmacia), and hydroxyapatite (Hyapatite, Lot 6281, Clarkson Chemical Company). Acrylamide gel reagents were obtained from Canalco, and p-nitrophenyl phosphate was a Sigma product.

Enzyme Assay—Enzyme activity was assayed at 37° with p-nitrophenyl phosphate as the substrate, and the release of p-nitrophenol was measured at 410 nm (23). Enzyme activity is expressed as the change in absorbance per unit of time at that wavelength. Specific activities are expressed as micromoles of p-nitrophenol released per min per mg of protein with a measured molar absorbance of 1.84 × 104 for p-nitrophenol in 0.02 N NaOH.

Protein was measured as absorbance at 280 nm, with the use of an extinction coefficient of 1.0 for a 1.0 mg per ml aqueous solution read in a 1-cm light path.

Polyacrylamide Gel Disc Electrophoresis—Electrophoresis was conducted at room temperature. A 5% upper gel and a 7.5% lower gel were used with 0.065 M Tris-HCl-0.04 M glycine buffer, pH 8.3, in both upper and lower electrode compartments; 2 ma per disc were applied (24). Enzyme protein was detected by incubating the gel cylinder in p-nitrophenyl phosphate for 10 to 15 sec at room temperature and observing the development of yellow color, indicating release of p-nitrophenol in the region of enzyme activity. The boundaries of enzyme activity were marked with two razor cuts immediately after color development. The gel was then counterstained with Amido black to delineate the relationship between enzyme activity and protein bands.

Purification of Enzymes—Alkaline phosphatase was extracted and purified from the following: liver, placenta, kidney, bone, intestine, and neutrophils. Although the initial preparation of each tissue was different, subsequent extraction with n-butyl

1 A preliminary report on this subject has been presented (22).
alcohol and initial processing during purification were similar for all.

Liver and Kidneys—Livers and kidneys were obtained within 24 hours after death from cadavers of patients who had died of trauma or of myocardial infarction. Nonparenchymal tissues were removed, and excess blood was washed out.

Placenta—Placentas were initially processed by N. Kontaxis (Stanford Research Institute, Menlo Park, California), who kindly provided an acetone powder of the extracted enzyme. Prior to extraction, the placentas had been washed and then perfused with cold 0.25 M sucrose and heparin to remove blood.

Bone—Human bone and cartilage were collected at autopsy from the costochondral junction, patella, tibial and femoral epiphyses at the knee, and the pelvis of adults and children who had died of trauma or heart disease. The bones were stored at −20°C, and 300 g of tissue were processed.

Neutrophils—Neutrophils were obtained from the buffy coat of multiple units of centrifuged venous blood from normal subjects. Two hundred milliliters of buffy coat were used for enzyme preparation. The erythrocytes present were lysed by osmotic shock (25), and the neutrophils were washed with 0.01 M Tris-HCl buffer, pH 7.4.

Intestine—A small intestine was obtained at autopsy from a 20-year-old white man who had died of trauma 8 hours previously. The luminal surface was washed with running tap water, and the mucosal cells were scraped off and frozen. Two days later the cells were thawed and washed three times with 20 volumes of cold 0.14 M NaCl solution. Microsomes were prepared by differential centrifugation after the cells had been homogenized in 0.01 M Tris-HCl, pH 7.4, with a motor-driven Teflon pestle with a ground glass homogenizer. The initial, 600 x g supernatant was centrifuged at 20,000 x g for 20 min to remove mitochondria. The supernatant from this step was centrifuged at 105,000 x g for 2 hours to obtain the microsomal pellet.

Enzyme Extraction and Purification—Alkaline phosphatase was extracted from each tissue by a modification of Morton's procedure (26). Each tissue and the intestinal microsomes were homogenized in 0.25 M sucrose and 20% (v/v) n-butyl alcohol at 4°C. The resulting homogenate was incubated at 37°C for 1 hour, kept overnight at 4°C, and centrifuged at 1000 x g for 2 hours. The enzyme activity was recovered in the supernatant. The sediment was re-extracted with butyl alcohol and homogenized as before, and the aqueous phase from this was pooled with that from initial extraction.

Bone enzyme was extracted by a modification of the method of Volkin (27). Fragments were allowed to autolyze in Tris-HCl buffer at room temperature for 2 weeks prior to homogenization with butyl alcohol.

Fractional acetone precipitation of the tissue extracts was conducted at 4°C. Enzyme was precipitated in the 30 to 50% (v/v) n-butyl alcohol fraction. In the case of placental phosphatase, an acetone powder was prepared from a single 60% precipitation. The precipitates were then suspended in 0.01 M Tris-HCl buffer, pH 7.4, and dialyzed free of butyl alcohol and acetone.

Chromatography and Gel Filtration—The liver, placental, and kidney enzymes were further purified following acetone precipitation by chromatography and gel filtration; neutrophil, bone, and intestinal phosphatase preparations were not. All chromatographic procedures were conducted at 4°C.

DEAE-cellulose was equilibrated with 0.01 M Tris-HCl, pH 7.4. Liver and placental enzymes were purified on columns (5.5 x 20 cm) of the equilibrated DEAE-cellulose; a linear gradient of 0.0 to 0.14 M NaCl in the Tris buffer was used, with 1000 ml in each reservoir. Kidney phosphatase was purified on a DEAE cellulos column (2.5 x 25 cm) by means of a linear gradient (0.0 to 0.3 M NaCl in the Tris buffer), with 500 ml in each reservoir. A single peak of enzyme activity was obtained in each case. Tubes of highest specific activity were pooled, concentrated by pressure dialysis at 4°C, and purified further by gel filtration on a column (2.5 x 90 cm) of Sephadex G-200 equilibrated with 0.01 M Tris-HCl buffer, pH 7.4, containing 0.14 M NaCl. A single peak of enzyme activity was again obtained for each preparation.

An aliquot of the liver phosphatase from the Sephadex column was further purified by passage through a column (12 x 0.5 cm) of hydroxylapatite in 0.001 M phosphate buffer, pH 6.8. The column was developed at a constant pH by stepwise elution with phosphate solutions of increasing concentration, 0.001 to 0.040 M, all at pH 6.9 (C). ———%, protein concentration; A280; -----, enzyme activity; ---, gradient concentration.

**Fig. 1.** Chromatographic patterns of human liver alkaline phosphatase separated on DEAE-cellulose with a 0.0 to 0.14 M NaCl gradient in 0.01 M Tris-HCl, pH 7.4, for elution (A); Sephadex G-200 with 0.14 M NaCl in 0.01 M Tris-HCl, pH 7.4, as eluant (B), and hydroxylapatite with stepwise elution with phosphate solutions of increasing concentration, 0.001 to 0.040 M, all at pH 6.9 (C). ———%, protein concentration; A280; -----, enzyme activity; ---, gradient concentration.
3.500
96
72
24
1600 3600 5400 7200

EFFLUENT VOLUME (ml)

FIG. 2. Chromatographic patterns of human placental alkaline phosphatase separated on DEAE-cellulose with a 0.0 to 0.09 M NaCl gradient in 0.01 M Tris-HCl, pH 7.4, used for elution (A) and Sephadex G-200 with 0.14 M NaCl in 0.01 M Tris-HCl, pH 7.4, as eluent (B). - - - -, protein concentration, A_280; ---, enzyme activity; - - - - - -, gradient concentration.

TABLE I
Purification of phosphatase from human liver and placenta

<table>
<thead>
<tr>
<th>Purification procedure</th>
<th>Total protein, mg</th>
<th>Total enzyme units</th>
<th>Specific activity</th>
<th>Relative purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone precipitate, 30-50%</td>
<td>7,000</td>
<td>455</td>
<td>0.065</td>
<td>1.0</td>
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<tr>
<td>DEAE-cellulose chromato</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>graphy</td>
<td>77</td>
<td>430</td>
<td>5.5</td>
<td>85</td>
</tr>
<tr>
<td>Sephadex G-200 gel filtration</td>
<td>26</td>
<td>183</td>
<td>7.0</td>
<td>108</td>
</tr>
<tr>
<td>Hydroxylapatite chromatography</td>
<td>-a</td>
<td>-a</td>
<td>14.7</td>
<td>226</td>
</tr>
<tr>
<td>Human placenta enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone precipitate, 60%</td>
<td>8,800</td>
<td>26,920</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>240</td>
<td>13,200</td>
<td>55</td>
<td>18</td>
</tr>
<tr>
<td>Sephadex G-200 gel filtration</td>
<td>27</td>
<td>8,856</td>
<td>328</td>
<td>109</td>
</tr>
</tbody>
</table>

*a An aliquot rather than the total sample was purified by this procedure.

quot containing 120 μg of enzyme protein (specific activity, 7 μmoles per min per mg) was placed on each analytical gel cylinder, and after electrophoresis the site of enzyme activity in the gel cylinder was determined as previously described. The enzyme-containing bands were immediately cut out from the gels and stored at -20°C until used. The remainder of the cylinder was stained for protein to ascertain that the removed section included any non-enzyme protein (Fig. 3). Only sections free of non-enzyme protein were used for immunization. Twenty gel sections were used for primary and booster immunizations. The sections were placed in a tuberculin syringe and fragmented by slowly squeezing them through a No. 25 needle. The fragments were suspended in 0.5 ml of complete Freund's adjuvant prior to inoculation. Approximately 0.2 mg of protein was used for each immunization.

Antiserum 234 was made in sheep in response to liver phosphatase which had been purified with DEAE-cellulose and Sephadex G-200 (specific activity, 7 μmoles per min per mg). Both primary and booster immunizations contained 2.0 mg of protein in 0.5 ml of NaCl emulsified with 0.5 ml of complete Freund's adjuvant.

Antiserum 333 to the purified placental phosphatase (specific activity, 328 μmoles per min per mg) was made in sheep. This material had a single, coinciding enzyme protein band on disc electrophoresis. The primary immunization contained 0.8 mg of protein in 0.5 ml of NaCl emulsified with 0.5 ml of complete Freund's adjuvant. An initial booster containing 0.3 mg of protein emulsified with the same amount of Freund's adjuvant did not initiate an adequate antienzyme response. A satisfactory response was obtained with a second booster containing 2.7 mg of protein.

The antigen emulsions were injected intramuscularly at multiple sites. Antibody activity was evaluated in 6 weeks by techniques to be described. The animals were bled between 10 and 16 days after the booster injections, when antibody was present in a satisfactory titer.

Preparation of Burro Anti-sheep γ-Globulin—γ-Globulin was prepared from sheep serum by sodium sulfate precipitation (28). Three milligrams of the sheep γ-globulin in 0.5 ml of NaCl were...
Comparison of $R_f$ values of alkaline phosphatase isoenzymes

The $R_f$ values of alkaline phosphatases from different organs were determined after polyacrylamide gel disc electrophoresis. The conditions of electrophoresis were 5% upper gel, 7.5% lower gel, 0.005 M Tris-0.04 M glycine buffer, pH 8.3; run at 25° at 2 ma per cylinder for 180 min. The front was defined as 1.0 and was determined by a riboflavin marker.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.27</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.27</td>
</tr>
<tr>
<td>Bone</td>
<td>0.28</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>0.28</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.28</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.34</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.48</td>
</tr>
<tr>
<td>Liver and intestine</td>
<td>0.28</td>
</tr>
<tr>
<td>Liver and placenta</td>
<td>0.27; 0.33</td>
</tr>
<tr>
<td>Intestine and placenta</td>
<td>0.27; 0.33</td>
</tr>
</tbody>
</table>

Electrophoretic Properties of Enzyme Preparations—A single band of enzyme activity was observed for each enzyme preparation after separation by disc electrophoresis on polyacrylamide gel. The $R_f$ values were the same for all human isoenzyme preparations, except for the placental isoenzyme, in which it was greater (Table II). Escherichia coli phosphatase, studied for comparison, had a greater $R_f$ value than any of the human preparations. Electrophoresis of isoenzyme mixtures confirmed these observations.

Immunochemical Isoenzyme Assay—The alkaline phosphatase isoenzymes were neither directly inhibited nor precipitated by the respective antibodies (Fig. 4). Because of this, a two-step method for measurement was devised. The first step consisted of allowing the isoenzyme to react with sheep antienzyme antiserum specific to it. The second step consisted of precipitating the resultant enzyme-antienzyme complex from solution with antibody to sheep $\gamma$-globulin. In the first reaction, an excess of antienzyme antiserum was used to ensure combination with all of the specific isoenzyme present. The second reaction, however, was conducted in the equivalence zone to ensure complete precipitation of the enzyme-antienzyme complex. Residual enzyme activity in the supernatant then represents an isoenzyme (or isoenzymes) that did not react with the particular antienzyme antiserum.

The equivalence zones for anti-sheep $\gamma$-globulin with respect to each antienzyme antiserum and SGG\(^2\) were determined from quantitative precipitin curves and after reaction of the initial supernatant in each tube with SGG and anti-SGG in agar diffusion plates. From such studies the amounts of SGG in each antiserum was estimated and the ratio of antiserum or SGG to anti-SGG at equivalence was calculated (Fig. 5). This ratio was used to determine the amounts of each component for the precipitation reaction of the enzyme assay. Each new batch of anti-SGG was standardized in the same manner.

The amount of antienzyme antiserum necessary to combine with the respective enzyme was determined independently of the precipitation reaction. Graded volumes of antiserum were added to a constant amount of purified enzyme of measured high activity. The quantity of $\gamma$-globulin in the reaction vessel was emulsified with 0.5 ml of complete Freund's adjuvant and injected at multiple sites intramuscularly into a burro. The animal was bled 6 weeks later, and antibody activity was measured by observing its ability to form precipitin lines against sheep $\gamma$-globulin in an agar gel diffusion plate (29). A booster injection of 3.0 mg was given, and 6 days later another sample was tested in the same manner, following which the animal was bled. Burro anti-sheep $\gamma$-globulin was prepared from the burro serum by sodium sulfate precipitation.

![Fig. 4. Failure of antienzyme to inhibit enzyme activity.](image)

**Fig. 4. Failure of antienzyme to inhibit enzyme activity.** □, control reaction mixtures contained 2.5 ml of $p$-nitrophenyl phosphate (2.0 mg per ml) and 0.005 $\mu$g of liver phosphatase; ●, test reaction mixtures contained, in addition, 30 $\mu$l of antiliver phosphatase (Phase) Antiserum 234. The temperature was 25°, and the reaction was followed directly at 410 nm in a Cary model 14 spectrophotometer. This amount of antiserum (30 $\mu$l) was capable of reacting with all of the phosphatase used when the enzyme-antienzyme complex was precipitated with anti-SGG.

**Fig. 5. Quantitative precipitin curves to determine the equivalence zone for precipitation of sheep $\gamma$-globulin with burro anti-sheep $\gamma$-globulin.** △, total precipitate with SGG; ○, anti-SGG (DASGG) precipitated with SGG, □, total precipitate with Antiserum 233; ▲, total precipitate with Antiserum 234. $\mu$Gm, micrograms; $R_x$, reaction.

\(^2\)The abbreviations used are: SGG, sheep $\gamma$-globulin; anti-SGG, burro anti-sheep $\gamma$-globulin.
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Fig. 6. Determination of the amount of antienzyme Antiserum 233 necessary to combine with all of a constant amount of enzyme of high activity. The equivalence zone was maintained by the addition of appropriate amounts of nonantibody SGG to each reaction vessel to ensure complete precipitation of the enzyme-antienzyme complex. Residual activity in the supernatant represents the excess of enzyme for each amount of antienzyme added.

Fig. 7. The range of activity of liver phosphatase isoenzymes measurable in the final assay system following precipitation reaction with anti-SGG. ○, enzyme activity without added antienzyme antiserum; ‡, enzyme activity with added antienzyme antiserum. Results are shown for Antiserum 233; similar relations were observed for Antisera 234 and 333. The units expressed as milliOD.₄₀₀/MIN represent (change in absorbance at 410 µm per min) × 10⁻⁴.

kept constant by the addition of reciprocal, graded amounts of nonspecific sheep γ-globulin. This procedure maintained the SGG:anti-SGG ratio within the equivalence zone for the precipitation reaction. The entire enzyme-antienzyme complex is precipitated within this equivalence zone, and any residual activity can be interpreted as isoenzyme present in excess of that which reacted with the amount of antienzyme used. Fig. 6 displays the results of such an experiment, in which Antiserum 233 was evaluated. Antisera 234 and 333 were evaluated similarly. These studies also show the independence of the two reactions, antienzyme complex formation and precipitation.

The range of isoenzyme activity which would react with the volume of antienzyme antiserum used in the finally selected assay system was also determined (Fig. 7). In this assay system, in a total volume of 400 µl, 10 µl of sheep antiserum and 5 mg of anti-SGG were used with Antisera 234 and 333; with Antiserum 233 the amounts were 25 µl and 12.5 mg. The concentration of γ-globulin in each antiserum was similar, as determined by quantitative precipitin reactions, and so the ratio of antiserum to anti-SGG in the reaction was the same in all instances.

The assay procedure consisted first of allowing the enzyme to react with antienzyme antiserum for 30 min at room temperature. Next, anti-SGG was added in amounts to give maximum precipitation, and 0.9% NaCl solution was added to bring the mixture to a constant volume. The reaction mixture was then incubated at 37° for 30 to 60 min and kept at 4° overnight. The next day the precipitate was separated by centrifugation at 800 × g for 20 min at 4°, and an aliquot of the supernatant was assayed for alkaline phosphatase activity. The background phosphatase activities of the antienzyme antiserum and of the anti-SGG were measured in control tubes which had been taken through all steps in the assay, and the values obtained were subtracted from the appropriate specimens. In most instances the background activity was small (5 to 10%) compared to the sample enzyme activity. With specimens of lowest enzyme activity, the background was as high as 40%.

Specificity of Antisera—The specificity of each antienzyme antiserum was determined by allowing it to react with the purified phosphatase preparations made from the various organs. The subsequent removal of activity from the supernatant depended entirely upon the specificity of the enzyme-antienzyme reaction, because the precipitation reaction was conducted in the equivalence zone and was complete. The precipitation reaction was independent of the enzyme-antienzyme reaction (Fig. 6). The data are presented in Table III. Antiserum 233 was specific for liver phosphatase, and Antiserum 333 was specific for placental phosphatase. Antiserum 234 was not specific; it reacted com-
**Table IV**

Discrimination experiments: ability of antisera to identify specific organ isoenzymes from mixtures

Data are mean values from duplicate analyses performed on separate days. Incubation times: 30 and 33 min; temperature, 37°C. Assay vessels contained 0.10 ml of supernatant from precipitation reaction; 0.50 ml of p-nitrophenyl phosphate, 6.0 mM; and 0.05 ml of MgCl₂, 8.0 mM.

<table>
<thead>
<tr>
<th>Individual values</th>
<th>Control</th>
<th>After reaction with antiliver phosphatase antibodies</th>
<th>No. 234</th>
<th>No. 233</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>4.22</td>
<td>4.22</td>
<td>4.22</td>
<td>4.22</td>
</tr>
<tr>
<td>Liver</td>
<td>10.45</td>
<td>0.80</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5.85</td>
<td>5.85</td>
<td>5.85</td>
<td>5.85</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.90</td>
<td>8.00</td>
<td>8.00</td>
<td>8.00</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
</tr>
</tbody>
</table>

| Sum of individually measured values | 29.77 | 12.47 | 19.27 |
| Measured value of a mixture of the listed isoenzymes | 34.40 | 13.80 | 11.20 |

**Discussion**

In these studies, antigenically distinct, organ-specific isoenzymes of human alkaline phosphatase have been found. Individual isoenzymes were identified on the basis of antigenic differences by a two-step immunochemical method. The first step, reaction of antienzyme antiserum with isoenzyme, was shown to be specific. The second step, precipitation of the resulting enzyme-antienzyme antibody complex with anti-y-globulin, was necessary because the antienzyme antibody did not inhibit or precipitate the enzyme. The second step was independent of the first, and did not interfere with its specificity.

Organ-specific forms of a number of mammalian enzymes have been found. Although human alkaline phosphatase has been assumed to be heterogeneous, evidence for specific organ-derived forms has been contradictory. With the exception of placental phosphatase, electrophoretic and chromatographic characteristics of human alkaline phosphatase isoenzymes are similar, so that these techniques have not been entirely satisfactory for distinguishing individual forms (8-14, 19, 30, 31). Possible explanations for this difficulty are that the properties measured by these techniques are unaltered by molecular substitutions which do not affect size or charge, but can be altered by molecular binding to other components in biological fluids as well as by buffer effects. Although differential inhibition and differential substrate activity have been reported for some phosphatase isoenzymes (16-18), the results are inconclusive, possibly because alkaline phosphatase is a relatively nonspecific enzyme capable of monophosphate hydrolysis activity with a variety of substrates. Kinetic measurements have not been satisfactory for distinguishing phosphatase organ isoenzymes because of similar $K_m$ values (10, 19, 20). The low $K_m$ values and the multiple steps in the over-all reaction reduce the discriminatory ability of this type of study. Furthermore, for comparison of $K_m$ data to be of value, uniform conditions of enzyme measurement must be used (32, 33).

Immunoechemical methods can be specific and precise for distinguishing individual forms. The material was purified with osmotic shock by a method which has not been published.

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1. E. coli enzyme was the gift of Dr. R. W. Brockman, National Institute of Arthritis and Metabolic Diseases. The material was purified with osmotic shock by a method which has not been published.
tistinguishing structural differences. If molecules are antigenically distinct, they have different structures; if they are antigenically indistinct, the structures may or may not be the same. In previous studies, antigenic differences of organ-specific mammalian enzymes, including human alkaline phosphatases, have been shown by various immunochemical methods (1-7). In the studies of human alkaline phosphatase, however, cross-reactions prevented conclusive distinction of organ isoenzymes. It was not possible to determine whether these cross-reactions were due to antigenic similarity of isoenzymes, to contaminants, or to non-specific antisera. The present study was designed to circumvent this difficulty by making specific antisera and by testing these against purified isoenzyme preparations. This required purification of isoenzyme from the designated organs, both for immunization and for subsequent determination of specificity.

The efficacy of using purified enzyme to make specific antisera was established when no cross-reactivity was observed with anti-liver phosphatase Antiserum 233 and antiplaclental phosphatase Antiserum 333. In contrast, Antiserum 234, prepared from a less pure liver phosphatase preparation than was 233, completely cross-reacted with neutrophil alkaline phosphatase and partially cross-reacted with kidney phosphatase. These observations suggest that the cross-reactivity found in previous studies may have been due to nonspecificity of antisera or to mixtures of isoenzymes in the assay standards, rather than to common antigenicity.

The present data, based on immunochemical identification of isoenzymes, establish that alkaline phosphatases from human liver and placenta can be distinguished from each other and also from alkaline phosphatase isolated from human bone, intestine, neutrophils, or kidney. The antigenic specificity of liver and placental enzymes suggests that these are separate molecular species. Bone, intestinal, and kidney phosphatases were not isolated in sufficient quantities to make specific antisera. Bone and intestinal phosphatases did not react with any of the antisera, whether specific (for liver, No. 233; or placenta, No. 333) or nonspecific (No. 234). Kidney phosphatase reacted partially with Antiserum 234, but not with the other two. These findings suggest that phosphatases of bone, intestine, and kidney might constitute additional distinct molecular species. The partial reaction of kidney phosphatase with antiliiver phosphatase Antiserum 234 may have been due to heterogeneity of the kidney enzyme preparation as well as the antiserum. The cross-reaction of Antiserum 234 with neutrophil phosphatase probably was due to heterogeneity of the liver enzyme preparation used for immunization to make that antiserum. The heterogeneity could have resulted from phosphatase of neutrophils if present in the liver in sufficient quantity, or from common antigenicity between neutrophil phosphatase and phosphatase of fixed or free hepatic macrophages. Either case suggests the existence of another phosphatase isoenzyme. None of the antisera reacted with E. coli phosphatase, which was to be expected in view of the phylogenetic difference.

From these data it is concluded that there are at least three antigenically distinct, organ-specific molecular forms of alkaline phosphatase, i.e. liver, placental, and one or more others. Indirect evidence suggests the existence of more than three antigenically distinct isoenzymes, since Antiserum 234 reacted fully with neutrophil phosphatase and partially with kidney phosphatase, and phosphatase of bone and intestine reacted with none of the antisera.

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