Chem. Pharm. Bull. 23(10)2369—2375(1975)

UDC 547.466.1.05 : 577.15.072

# Purification and Properties of Human Liver Alkaline Phosphatase[1]

Mamoru Sugiura, Kazuyuki Hirano,[2a] Shiro Iino, Hiroshi Suzuki, and Toshitsugu Oda[2b]

*Department of Pharmacy, Tokyo College of Pharmacy[2a] and 1st Department of Medicine, Faculty of Medicine, University of Tokyo[2b]*

(Received March 24, 1975)

Human liver alkaline phosphatase (E.C. 3.1.3.1) has been purified by butanol extraction, acetone precipitation, and a combination of diethylaminoethyl (DEAE)-cellulose, carboxymethyl(CM)-cellulose, Sephadex G-200 gel chromatography. The homogeneity of purified enzyme was demonstrated by disc electrophoresis and micro-Ouchterlony.

The heat activation for hydrolysis of *p*-nitrophenyl phosphate was calculated as 21000 cal/mole and liver alkaline phosphatase was inhibited by homoarginine.

A molecular weight of 180000 was obtained by gel filtration and isoelectric point of pI 4.7 was determined by isoelectric focusing. The optimum pH for the hydrolysis of *p*-nitrophenyl phosphate was 10.6 and optimum temperature was 40° in the standard assay system and other enzyme properties were also examined.

Elevation of alkaline phosphatase (orthophosphoric monoester phosphohydrolase E.C. 3.1.3.1) activity in serum is known to occur in variety of hephatic, extrahepatic and bone diseases.[3] For example, hepatobiliary diseases cause an elevation of that alkaline phosphatase which originates from the liver. It seems, therefore, to be of diagnostic value to detect the origin of the elevated serum alkaline phosphatase in the various diseases. The diagnostic value of alkaline phosphatase determinations has long been recognized.

There is evidence that human alkaline phosphatase exists in multiple forms[4] and that the forms found in different organs possess distinct properties[5] by which they may be recognized. However, interpretations have not always been in agreement on the existence or definition of specific organ forms. Some of the difficulties may have been due to insufficiently purified enzyme preparations and to the limitations of using physical properties or differential substrate activity to difine molecular differences.

In the previous papers, the authors had reported that the purification of human placental,[6] intestinal,[7] and biliary[8] alkaline phosphatase and their properties.

In this paper, the purification of alkaline phosphatase from human liver was performed and its properties were studied.

## Materials and Methods

**Crude Enzyme**——Frozen human liver from metastatic liver cancer (tumor tissue was removed) 300 g was homogenized with water and butanol (liver: water: butanol=1: 3: 1). The supernatant was treated

1) A part of the work was published as preliminary communication: M. Sugiura, K. Hirano, S. Iino, H. Suzuki, and T. Oda, *Chem. Pharm. Bull.* (Tokyo), **23**, 686 (1975); This paper forms Part CVIII of "Studies on Enzymes" by M. Sugiura.
2) Location: *a*) *Ueno-sakuragi, 1-Chome, Taito-ku, Tokyo, 110, Japan*; *b*) *Bunkyo-ku, Tokyo, 113, Japan.*
3) H.A. Fritsche, Jr. and H.R. Adams-park, *Clin. Chim. Acta*, **52**, 81 (1974); P.G. Dingjam, T. Postma, and J.A.P. Stroes, *Z. Klin. Chem. Klin. Biochem*, **11**, 167 (1973).
4) H.H. Sussman, P.A.Jr. Small, and E. Cotlove, *J. Biol. Chem.*, **243**, 160 (1968).
5) W.H. Fishman and N.K. Ghosh, *Adv. Clin. Chem.*, **19**, 255 (1967).
6) S. Iino, K. Abe, T. Oda, H. Suzuki, and M. Sugiura, *Clin. Chim. Acta*, **42**, 161 (1972).
7) M. Sugiura, M. Isobe, K. Hirano, S. Iino, H. Suzuki, and T. Oda, *Chem. Pharm. Bull.* (Tokyo), **23**, 1537 (1975).
8) M. Sugiura, K. Hirano, S. Iino, H. Suzuki, and T. Oda, *Chem. Pharm. Bull.* (Tokyo), **23**, 2019 (1975).

with 60% acetone in the cold to precipitate the protein. Distilled water was added to the precipitate and its dissolved part was used as the crude enzyme solution.

**Assay of the Alkaline Phosphatase Activity**——Method A: Disodium phenylphosphate was used as a substrate.[9] Two ml of 0.1M carbonate buffer (10.5) containing 10 mM substrate and 2 mM 4-aminoantipyrine was preincubated at 37°. One tenth ml of enzyme solution was added and the enzyme reaction was carried out at 37° for 15 min. The reaction was stopped by adding 2 ml of 0.2 M borate solution containing 6 mM potassium ferricyanate and absorbancy was determined at 500 nm. One unit of alkaline phosphatase activity was defined as the amount which produces 1 μmole of phenol per min.

Method B: *p*-Nitrophenyl phosphate was used as a substrate.[10] One ml of 10 mM substrate solution and 3 ml of glycine–KCl–KOH buffer (pH 10.5) was preincubated at 37°. One ml of the enzyme solution was added and the enzyme reaction was carried out at 37° for 30 min. The reaction was stopped by adding 2 ml of 0.1N NaOH and the absorbancy was determined at 430 nm. Method B was used for study of liver alkaline phosphatase properties.

**Assay of the Protein**——The absorbancy was determined at 280 nm by Hitachi electric photometer Model 101 and the protein was also measured by Lowry's method[11] with bovine serum albumin as a standard.

**Disc Electrophoresis**——Disc electrophoresis was carried out with the 7.5% polyacrylamide gel. For pH 8.4 glycine–Tris buffer was used and electrophoresed at the constant current of 4 mA/tube for 70 min. Staining was made with Amido Black 10B.

**Isoelectric Focusing**——Isoelectric focusing was carried out as decribed by Vesterberg and Svensson[12] using 1% carrier ampholyte (pH 3.5—5.0) at the constant voltage of 800 V for 48 hr.

**Preparation of Antiserum of Human Liver Alkaline Phosphatase**——Antiserum of liver alkaline phosphatase was prepared as previously reported.[6,7] Rabbit foodpad was injected with 1 ml containing 200 μg of the purified human liver alkaline phosphatase and an equal volume of Freund's complete adjuvant. Booster immunization was made by subcutaneous injection twice every two weeks.

## Results

### Purification of Alkaline Phosphatase from Human Liver

The fractional precipitation by ammonium sulfate was applied on the crude enzyme solution according to Fig. 1.

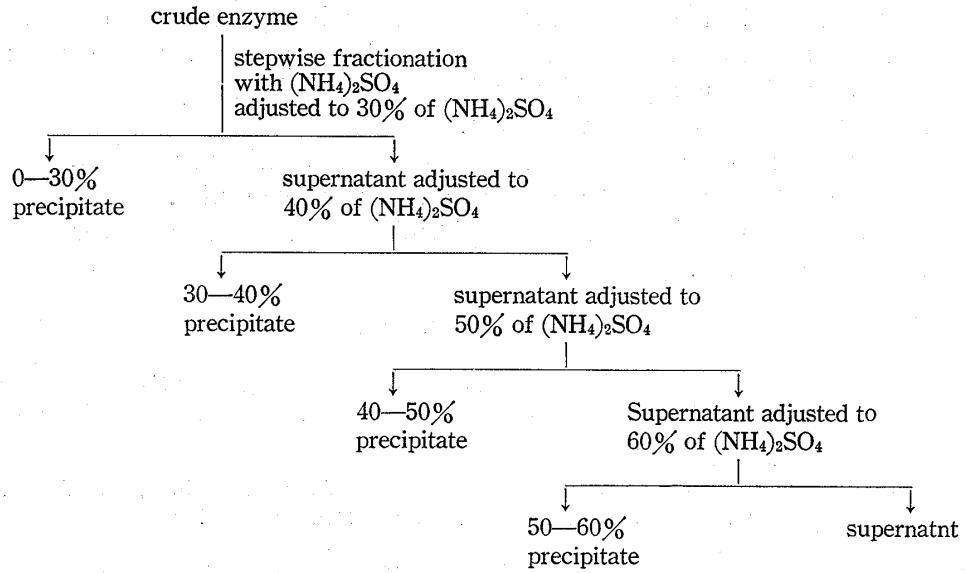


Fig. 1. Scheme for Ammonium Sulfate Fractionation of Alkaline Phosphatase from Human Liver

9) P.R.N. Kind and E.J.J. King, *J. Clin. Pathol.*, **7**, 322 (1954).
10) O.A. Bessy, O.H. Lowry, and M.J. Brock, *J. Biol. Chem.*, **164**, 321 (1946).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) O. Vesterberg and H. Svensson, *Acta. Chem. Scand.*, **20**, 820 (1966).

Table I. $(NH_4)_2SO_4$ Fractionation of Alkaline Phosphatase from Human Liver

| Concentration of $(NH_4)_2SO_4$ | Protein (OD 280 nm) | Activity (U) |
|---|---|---|
| 0 — 30% | 396 | 25.7 |
| 30 — 40% | 570 | 25.7 |
| 40 — 50% | 666 | 95.1 |
| 50 — 60% | 255 | 1400 |
| 60% | 279 | 10.7 |

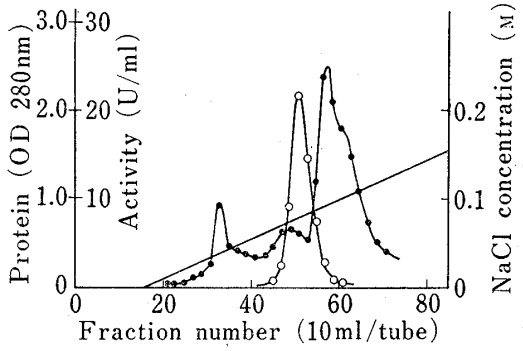


Fig. 2. Column Chromatogram of Alkaline Phosphatase from Human Liver on DEAE-cellulose

DEAE-cellulose had been equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 10 μM of $ZnCl_2$ and $MgCl_2$. Elution was carried out by changing the concentration of NaCl, linearly to 0.15M. Alkaline phosphatase activity (○) of each fraction was measured as described in Materials and Methods and expressed in OD units per ml of fraction. The concentration of protein (●) was measured in terms of the absorbance at 280 nm. column size: 3.0×10 cm, flow rate: 60 ml/hr.

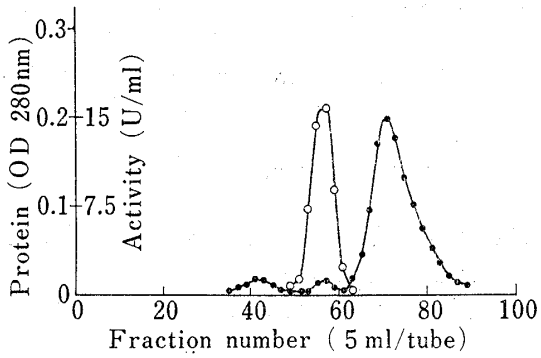


Fig. 3. Column Chromatogram of Alkaline Phosphatase from Human Liver on Sephadex G-200

The column had been equilibrated with 10 mM Tris-HCl buffer (pH 9.0) containing 0.1M of NaCl and 10 μM of $ZnCl_2$ and $MgCl_2$. Elution was carried out with the same buffer. The measurement of activity (○) and protein (●) were performed in the same manner as described in Fig. 2. column size : 2.5 ×10 cm, flow rate: 20 ml/hr

The precipitate obtained at the concentration of 0.5—0.6 saturation of ammonium sulfate, which had the highest activity, as shown in Table I, was collected and dialyzed against 4 liters of 10 mM Tris-HCl buffer (pH 7.4) containing 10 μM of $ZnCl_2$ and $MgCl_2$ for 24 hr.

The dialyzed enzyme solution was purified by column chromatography with diethylaminoethyl (DEAE)-cellulose. The enzyme solution was applied on the column which had been equilibrated with the same buffer as used for dialysis. After the column washed, alkaline phosphatase was eluted by changing concentration of NaCl gradiently from 0 to 0.15M. This result is shown in Fig. 2. The active fractions was collected and concentrated by the membrane filter.

The enzyme solution was dialyzed against 10 mM citrate buffer (pH 5.0) containing 10 μM of $ZnCl_2$ and $MgCl_2$ for 5 hr. The solution was passed through a column (2.0×30 cm) of CM-cellulose which had been equilibrated with the same buffer as used for dialysis and the effluent was collected. After concentration, the solution was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 0.1M of NaCl and 10 μM of $ZnCl_2$ and $MgCl_2$.

The dialyzed enzyme solution was purified on a column of Sephadex G-200 which had been equilibrated with the same buffer as used for dialysis. The result is shown in Fig. 3 and the active fraction was concentrated and dialyzed against Tris-HCl buffer (pH 8.0).

Above purification procedures are summarized in Table II.

The alkaline phosphatase from human liver was purified up to about 8900-fold from the crude enzyme level with a recovery of 10%. The resulting product was used as the specimen

Table II. Purification Procedures of Alkaline Phosphatase from Human Liver

| Procedure | Total activity (U) | Total protein (mg) | Specific activity (U/mg) |
|---|---|---|---|
| Crude enzyme | 2000 | 12450 | 0.16 |
| Fractional precipitation by ammonium sulfate | 1400 | 383 | 3.66 |
| DEAE-cellulose column chromatography | 1070 | 43.8 | 24.4 |
| CM-cellulose column chromatography | 470 | 5.73 | 82.0 |
| Sephadex G-200 gel filtration | 200 | 0.14 | 1430 |

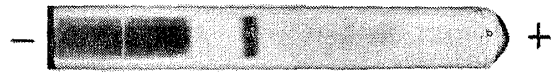


Fig. 4. Disc Electrophoretic Pattern of Purified Alkaline Phosphatase from Human Liver

For details see the text.

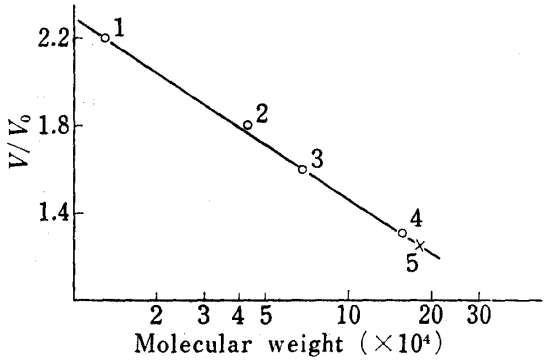


Fig. 5. Determination of the Molecular Weight of Alkaline Phosphatase from Human Liver by Gel Filtration of Sephadex G-200

column size: 2.8×65 cm,
buffer: 10 mM, Tris–HCl, pH 7.5
1 : cytochrome c, 2 : egg albumin, 3 : bovine serum albumin, 4 : γ-globulin, 5 : human liver alkaline phosphatase

of the purified enzyme. As shown in Fig. 4, the purified enzyme gave a single band of protein by disc electrophoresis at pH 9.4.

**Molecular Weight**

The molecular weight of alkaline phosphatase from human liver was determined using the method of Whitaker[13] by gel filtration on Sephadex G-200. From the result, as shown in Fig. 5, the molecular weight was calculated to be 180000 for human liver alkaline phosphatae.

**Isoelectric Point**

The isoelectric point was determined by isoelectric focusing using a carrier ampholyte (pH 3.5—5.0). As the result, the alkaline phosphatase activity was obtained as a single peak at pH 4.7.

**Optimum pH and pH Stability**

As shown in Fig. 6, liver alkaline phosphatase had optimum pH at 10.6 in the standard assay system. The effect of pH on the stability of the enzyme was examined. It was found that under the condition of 0° for 1 hr, more than 80% of the original activity was retained at the pH range of 4—11.

**Immunological Properties**

Immunodiffusion pattern of purified liver alkaline phosphatase is presented in Fig. 7. By this method only one precipitin line was seen when as much as 10 μg of the purified enzyme was tested. From this result, human liver alkaline phosphatase antibody was found to be specific for human liver alkaline phosphatase, which was purified according to our method, and homogeneity of purified enzyme was also supported by the result of disc electrophoresis.

**Effect of Temperature on the Rate of Hydrolysis**

An Arrhenius plot of the reaction rate at different temperatures gave a straight line over the temperature range studied, as shown in Fig. 8.

13) J.R. Whitaker, *Anal. Chem.*, 35, 1950 (1963).

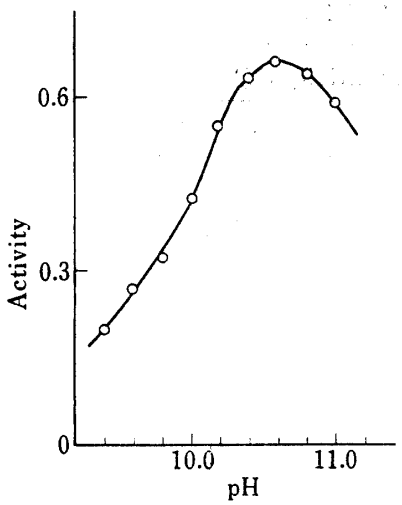


Fig. 6. Effect of pH on Alkaline Phosphatase Activity
buffer: 0.1M, glycine-KCl-KOH

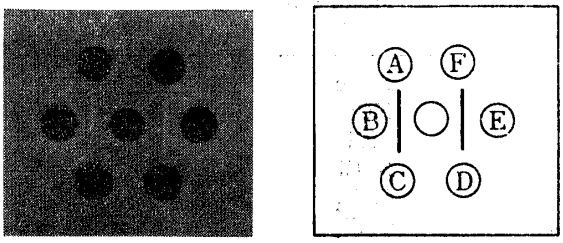


Fig. 7. Micro-Ouchterlony Immunodiffu sion Pattern of Purified Human Liver Alkaline Phosphatase
A and D: purified human placental alkaline phosphatase
B and E: purified human liver alkaline phosphatase
C and F: purified human intestinal alkaline phosphatase

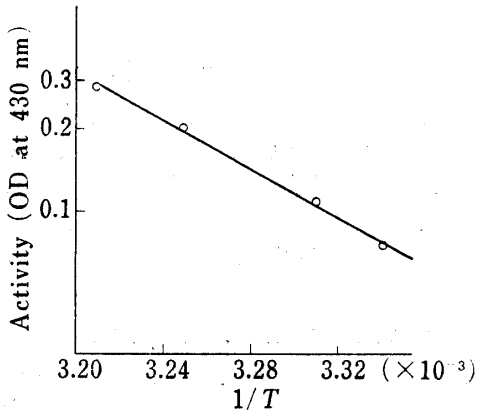


Fig. 8. Effect of Temperature on the Rate of Hydrolysis of *p*-Nitrophenyl Phosphate by Human Liver Alkaline Phosphatase

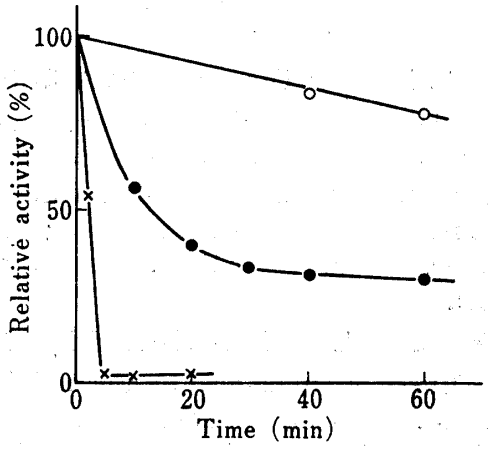


Fig. 9. Effect of Temperature on the Activity of Alkaline Phosphatase from Human Liver
—○—: 0°, —●—: 37°, —×—: 50°

The value for the heat of activation was 21000 cal/mole. This value was larger than that for human placental and intestinal[7] alkaline phosphatase.

**Effect of Temperature on the Activity and Stability**

Alkaline phosphatase from liver had a maximum activity at 40° in the standard assay system and activity was completely lost at 50° within 10 min, as shown in Fig. 9.

**Michaelis Constant**

In the standard assay system Michaelis constant of liver alkaline phosphatase for *p*-nitrophenyl phosphate was investigated and it was found to be 0.31 mM.

**Effect of Metal Salts on its Activity**

The solution of liver alkaline phosphatase was preincubated with metal salts at 37° for 30 min. After the mixture was diluted to 10-fold with a buffer, the remaining activity was assayed. The result was shown in Table III. Enzyme was inhibited by 0.1 mM of $CdCl_2$ and $HgCl_2$, and activated by 0.1 mM of $ZnCl_2$ and $MgCl_2$.

Table III. Effect of Metal Salts on the Activity

| Metal salts | Relative activity (%) | |
|---|---|---|
| | 0.1 mM | 0.01 mM |
| None | 100 | 100 |
| KCl | 82 | 112 |
| LiCl | 109 | 92 |
| NaCl | 74 | 84 |
| $CaCl_2$ | 97 | 106 |
| $CoCl_2$ | 89 | 88 |
| $BaCl_2$ | 99 | 94 |
| $ZnCl_2$ | 130 | 107 |
| $HgCl_2$ | 55 | 84 |
| $CdCl_2$ | 17 | 41 |
| $CuCl_2$ | 73 | 82 |
| $MgCl_2$ | 140 | 101 |
| $MnCl_2$ | 64 | 82 |
| $NiCl_2$ | 81 | 92 |
| $FeCl_3$ | 74 | 76 |

The enzyme was preincubated at 37° with metal salts for 30 min (10 mM Tris–HCl, pH 7.4) and diluted with same buffer. Remaining activity was measured as described in Materials and Methods.

### Effect of Various Compound the Activity of Alkaline Phosphatase in the Enzyme System

The effect of various compounds on the enzyme activity was examined. The solution of liver alkaline phosphatase was incubated with various compounds at 37° for 30 min. After the mixture was diluted 10-fold with a buffer solution, the remaining activity was assayed. The results as follow were obtained; The activity inhibited by 1 mM of N-bromosuccinimide, iodoacetic acid and EDTA. However it was not inhibited by *p*-chloromercuribenzoate or KCN.

### Effect of Amino Acid on the Activity of Alkaline Phosphatase in the Reaction System

Effect of amino acid on the activity of alkaline phosphatase in the reaction system was studied. As shown in Table IV, the enzyme was inhibited by 1 mM of L-cysteine and sensitive to homoarginine, but it was not inhibited by L-phenylalanine.

Table IV. Effect of Amino Acids on the Activity in the Reaction System

| Amino acid | Relative activity (%) | |
|---|---|---|
| | 1 mM | 0.1 mM |
| None | 100 | 100 |
| L-Cysteine | 0 | 51 |
| L-Cystine | 68 | 78 |
| L-Phenylalanine | 96 | 100 |
| L-Tyrosine | 87 | 98 |
| L-Tryptophan | 80 | 99 |
| L-Histidine | 72 | 83 |
| Imidazole | 82 | 88 |
| Homoarginine | 57 | 73 |

## Discussion

The purification of alkaline phosphatase from human liver was performed and properties were investigated. The authors used butanol extraction, acetone precipitation, ammonium sulfate fractionation, DEAE-cellulose, CM-cellulose and Sephadex G-200 for purification,

and the resulting alkaline phosphatase was purified up to about 8900-fold. The homogeneity of purified enzyme was confirmed by disc electrophoresis.

Fishman, *et al.*[14] had reported that there was variant alkaline phosphatase in human liver, which was precipitated at the concentration of ammonium sulfate 0—30% saturation. However, the authors could not recognized its existence in human liver.

The properties of purified liver alkaline phosphatase was found to be similar to those of human placental, intestinal, and biliary alkaline phosphatase in respect to its effect on various compound, optimum pH, Michaelis constant and pH stability. Isoelectric point of purified liver alkaline phosphatase was pI 4.7 and this value was similar to those of placental, intestinal, and biliary alkaline phosphatase, pI 4.5, pI 4.6, pI 4.75, respectively. Molecular weight of liver alkaline phosphatase was calculated to be 180000 and this value was identical to that of human biliary alkaline phosphatase.

In the effect of amino acid on enzyme, liver alkaline phosphatase was inhibited by homoarginine which was specific inhibitor for liver and bone, and this data was similar to that of biliary alkaline phosphatase, and also thermal stability, effect of metal salts on the purified enzyme were similar to those of human biliary alkaline phosphatase. From these results, it was suggested that biliary alkaline phosphatase will be originated from liver.

Activation energy of the purified enzyme for the hydrolysis of *p*-nitrophenyl phosphate as substrate was calculated as 21000 cal/mole. In immunological studies, antiserum of human liver alkaline phosphatase was specific for it, and it was found that liver alkaline phosphatase was different from placental and intestinal alkaline phosphatase.

Detailed immunological comparison among human liver, biliary, placental and intestinal alkaline phosphatase will be reported successively.

14) L. Fishman, N.R. Inglis, and W.H. Fishman, *Clin. Chim. Acta*, **34**, 393 (1971).