Z. Klin. Chem. Klin. Biochem.
11. Jg. 1973, S. 167—171

Quantitative Differentiation of Human Serum Alkaline Phosphatase Isoenzymes with Polyacrylamide Disc Gel Electrophoresis

By P. G. Dingjan, T. Postma and J. A. P. Stroes

Department of Clinical Chemistry, Stichting Samenwerking Delftse Ziekenhuizen, Delft, The Netherlands

(Eingegangen am 31. August 1972/24. Januar 1973)

The separation of alkaline phosphatase (EC 3.1.3.1) isoenzymes by means of polyacrylamide disc gel electrophoresis is described. The electrophoretic quantitation of the alkaline phosphatase isoenzymes and the ratio of bone-type fraction/liver-type fraction gives more clinical information than the heat-inactivation method.
It is demonstrated that the described electrophoretic method together with the determination of γ-glutamyl-transpeptidase (EC 2.3.2.1) and 5′-nucleotidase (EC 3.1.3.5) is a reliable and a satisfactory method in the routine hospital laboratory for the differentiation of bone and hepatobiliary diseases.

Die Trennung der Isoenzyme der alkalischen Phosphatase (EC 3.1.3.1) mit der Polyacrylamidgel-Disc-Elektrophorese wird beschrieben. Die elektrophoretische Bestimmung der Isoenzyme der alkalischen Phosphatase und des Quotienten Knochentyp-Fraktion/Lebertyp-Fraktion gibt mehr Information für die Klinik als die Hitze-Inaktivierungs-Methode.
Es wird gezeigt, daß die beschriebene elektrophoretische Methode zusammen mit der Bestimmung der γ-Glutamyltranspeptidase (EC 2.3.2.1) und 5′-Nucleotidase (EC 3.1.3.5) eine zuverlässige und befriedigende Methode zur Differenzierung von Knochen- und Leber-Galle-Erkrankungen im klinischen Routinelaboratorium ist.

The determination of alkaline phosphatase (EC 3.1.3.1) is one of the most frequently requested analyses in the hospital laboratory.
An elevated alkaline phosphatase is usually the result of hepatobiliary or bone diseases.
The 5′-nucleotidase (EC 3.1.3.5) and γ-glutamyltranspeptidase (EC 2.3.2.1) determinations can, of course, be used to differentiate between bone and liver diseases, but methods for differentiating alkaline phosphatase isoenzymes as a basis for diagnosis frequently appear in the literature (1—7).
In healthy normal non-pregnant subjects, only the small intestine-type alkaline phosphatase, bone-type alkaline phosphatase and liver-type alkaline phosphatase appear in serum.
In healthy pregnant subjects in the third trimester of gravidity, an extra placenta-type alkaline phosphatase appears together with an increase of total alkaline phosphatase activity.
Patients with intra- or extrahepatic obstruction very often have an extra cholestatic type (bile-type) alkaline phosphatase (3, 4).
Occasionally, other alkaline phosphatase isoenzyme types are found in human serum (3, 5).
Electrophoresis on paper, agar, starch or cellulose-acetate and column chromatography (1, 2) are either difficult to interprete or time-consuming.
Heat-inactivation combined with stereospecific inhibition of serum with L-phenylalanine (6) is a convenient, rapid technique, but in our opinion, it gives less information about the alkaline phosphatase isoenzymes.
Of the electrophoretic techniques, the polyacrylamide disc gel method is the most suitable (7, 8).
In the literature, the results obtained by electrophoresis are rarely submitted to a quantitative evaluation. This could be due to the bad separation and staining techniques used.

Materials and Methods

Selection of sera

Normal sera were gathered from 95 donors. All sera had normal alkaline phosphatase activity. Only three presented elevated γ-glutamyltranspeptidase and 5′-nucleotidase and were not examined further. The remaining 92 subjects (39 female and 53 male) ranged in age from 21 to 65 years with a median age of 34 years.
Abnormal sera were selected from patients, which had elevated alkaline phosphatase activity (over 65 IU/l) and in which the clinical diagnosis was confirmed by histological, roentgenological and radiological examination, or laporatomy or autopsy.
They were grouped according to their clinical disorder as follows:
1. 25 patients with hepatobiliary diseases: liver metastases (9); cholecystitis chronica (4); cholelithiasis (4); obstructive jaundice (3); extrahepatic obstruction (2); cholecystitis acuta (1); cirrhosis of liver (1); non-specific liver degeneration (1). (Minimum age 47 and maximum age 87 years with a median of 62 years.)
2. 15 patients with bone diseases: malignant bone diseases with osteoblastic activity (9); healing phase of bone fractures (2); Paget's disease (2); Kahler's disease (1); osteomalacia (1). (Minimum age 51 and maximum age 81 with a median of 61 years.)
3. 1 patient (47 years) liver and bone metastases.
4. 4 patients (51, 61, 21 and 74 years old) with diseases other than bone or liver diseases.

Polyacrylamide disc gel electrophoresis

25 µl serum samples were electrophoresed in a discontinuous buffer and gel system in glass tubes (5 mm × 75 mm; cleaned with Tween-20) with a separating gel of 45 mm for one hour

at 3 mA/gel at room temperature according to the instructions of the manufacturer (acrylamide 7%; bisacrylamide 0.18%) (9).
It is possible to prepare, run and stain 12 samples simultaneously in about $4^1/_2$ h.
The substrate solution for staining the alkaline phosphatase fractions after electrophoresis consisted of *p*-toluidinium-5-bromo-4-chloro-3-indolyl phosphate, dimethylformamide, and a buffer.
It gave weak to very intense blue bands, with no background colour. The bands were very stable in 7% acetic acid at room temperature.
It is important to take an aliquot part of the serum, i. e. reduce the sample load, when the alkaline phosphatase activity is above 100 IU/l. This avoids uneven staining and helps in the interpretation of the electrophoretic pattern. The incubation time must be standardized. This method does not influence the relative ratio between the isoenzyme fractions.
The bone-type/liver-type alkaline phosphatase ratio was calculated densitometrically.
Quantitation of the alkaline phosphatase isoenzyme fractions was carried out by means of the Kipp Model DD2 Microdensitometer at 580—650 nm and the Kipp Model BC1 Integrator (Kipp & Zonen, Delft, Holland).
The gels were scanned in glass tubes filled with 7% acetic acid solution and were placed horizontally on a flat perspex holder. The linear velocity past the light source was 20 mm/min. The integrator was started and stopped manually. For overlapping peaks the approximation was used that the area between two imaginary perpendicular lines between the minima of two peaks was representative of the relative activity of the isoenzyme fractions.
Although the quantitative differentiation of incompletely separated curves is theoretically difficult, it was evident that the above mentioned approximation was satisfactory on a practical basis (see Table 1).
For identification of the bands, tissue extracts were made with *n*-butanol: 500 mg tissue, (frozen at —60°C before use) was homogenised, according to a modified procedure of Morton (8, 10), for about 10 min in 2 ml of 20% *n*-butanol solution at 0°C. (Tri-R homogeniser S 220, with a glass reinforced teflon pestle with a clearance of 0.11—0.15 mm; Tri-R Instruments, Inc., 48 Merrick Road, 11570 Rockville Centre N. Y., USA.)
After centrifugation and storage overnight at 4°C, the supernatant was electrophoresed.
This treatment when applied to sera, caused no detectable change in the isoenzyme pattern or in the relative isoenzyme electrophoretic mobilities.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured by incubating 0.1 ml serum for exactly 15 min at 37°C in 1 ml of substrate-buffer (0.61 mol/l 2-amino-2-methyl-propan-1-ol, 0.018 mol/l 4-nitrophenyl disodium orthophosphate and 0.001 mol/l $MgCl_2 \cdot 6\ H_2O$ at pH 10.5).
The reaction was stopped with 10 ml 1 mol/l NaOH solution and absorbance was measured at 405 nm.
The results were expressed in IU/l.
The normal values for adults were 37 ± 19.8 IU/l ($N = 92$; $\bar{x} \pm 2$ SD).

Heat-inactivation-L-phenylalanine inhibition (Q-value)

Sera were heat-inactivated as follows:
0.5 ml serum was pipetted into 53 mm × 9.5 mm test tubes at room temperature and stoppered.
The tubes were placed in a suitable incubating rack, then immersed in a waterbath at 56 ± 0.5°C for exactly 15 min.
The rack with tubes was immediately placed in an ice-water-bath for several minutes, then allowed to remain at room temperature.
The alkaline phosphatase activity was measured within 1 h. The alkaline phosphatase activity for the serum before and after heat-inactivation was measured in the substrate-buffer solution containing 0.005 mol/l L-phenylalanine (6).

The Q-value is defined as:

$$Q = \frac{\text{Alkaline phosphatase activity after heat-inactivation with L-phenylalanine}}{\text{Alkaline phosphatase activity before heat-inactivation with L-phenylalanine}}$$

This Q-value gives the ratio between the heat-labile bone-type phosphatase and the relatively heat-stable liver-type phosphatase. L-phenylalanine inhibits the small intestine-type phosphatase.

5'-Nucleotidase

5'-nucleotidase was measured with the manual method of Persijn and van der Slik et al. (11).
Normal values: 3.5—11.0 IU/l.

γ-Glutamyltranspeptidase

The γ-glutamyltranspeptidase was measured by the manual method of Szasz (12).
Normal values: male 6—28 IU/l
female 6—18 IU/l.

Results

Precision

The precision of the electrophoretic method was tested with two sera which were different in the ratio of bone-type to liver-type alkaline phosphatase isoenzymes (Table 1).

Heat-inactivation (Q-value)

The precision of the heat-inactivation method was investigated with a normal serum.
For results see Table 2.

Normal Values

Polyacrylamide disc gel electrophoresis

The normal values of the alkaline phosphatase isoenzyme fractions (in %) were calculated from the results of 92 sera. Discrimination was made between sera without and with an intestine-type alkaline phosphatase fraction in the electropherogram, because when an intestinal fraction is present the percentages of the bone and liver-type fraction will be lower.
For results see Table 3.

Tab. 1
Precision of the polyacrylamide disc gel electrophoresis
Serum Ia: n = 40, total alkaline phosphatase activity 50 IU/l
Serum Ib: n = 40, total alkaline phosphatase activity 195 IU/l

		Serum Ia $\bar{x} \pm$SD	Serum Ib $\bar{x} \pm$SD
Bone-type fraction	[%]	43 ±3.3	68 ±2.4
Liver-type fraction	[%]	57 ±3.3	32 ±2.4
bone-type fraction / liver-type fraction	ratio	0.8 ±0.10	2.1 ±0.24

Tab. 2
Precision of the heat-inactivation (Q-value)
Normal serum: n = 40, total alkaline phosphatase activity 30 IU/l
bone fraction 51%, liver fraction 49%

	Q-value $\bar{x} \pm$SD
Normal serum	0.16 ±0.008

Tab. 3
Normal values for alkaline phosphatase from disc gel electrophoresis
a) sera without intestine-type fraction; n = 59
b) sera with intestine-type fraction; n = 33

		a $\bar{x} \pm SD$	b $\bar{x} \pm SD$
Intestine-type fraction	[%]		13 ± 6.2
Bone-type fraction	[%]	50 ± 8.3	42 ± 9.6
Liver-type fraction	[%]	50 ± 8.3	45 ± 9.3
Bone-type fraction / Liver-type fraction	ratio	1.1 ± 0.35	1.0 ± 0.42

The mean total alkaline phosphatase activity was 16—55 IU/l ($\bar{x} \pm 2$ SD) for series 3a and 21—59 IU/l ($\bar{x} \pm 2$ SD) for series 3b.

As can be seen from Table 3 the bone/liver type ratios of the two groups do not differ significantly. Examples are given in Figure 1a and 1b, of the electrophoretic results from normal sera with and without intestine-type phosphatase. A bile-type alkaline phosphatase fraction could be detected in none of the 92 normal sera.

The small response at 1 in Figure 1 is due to the change in optical density caused by the interface of the separating gel/stacking gel.

Fraction 2 is a very small and weak band often seen in normal and abnormal sera.

Fraction 3 is a weak intestine-type alkaline phosphatase isoenzyme that appeared in 36.8% of these sera.

Fraction 4 is the bone-type alkaline phosphatase isoenzyme. Fraction 5 is the liver-type alkaline phosphatase isoenzyme.

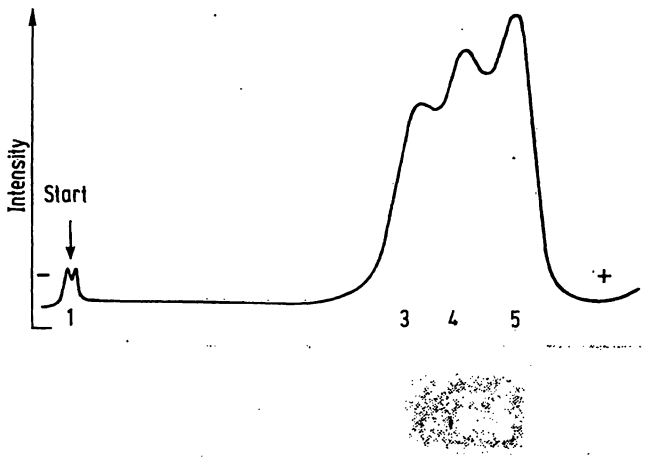

Fig. 1a
Electropherogram and densitogram of a normal serum with an intestine-type fraction

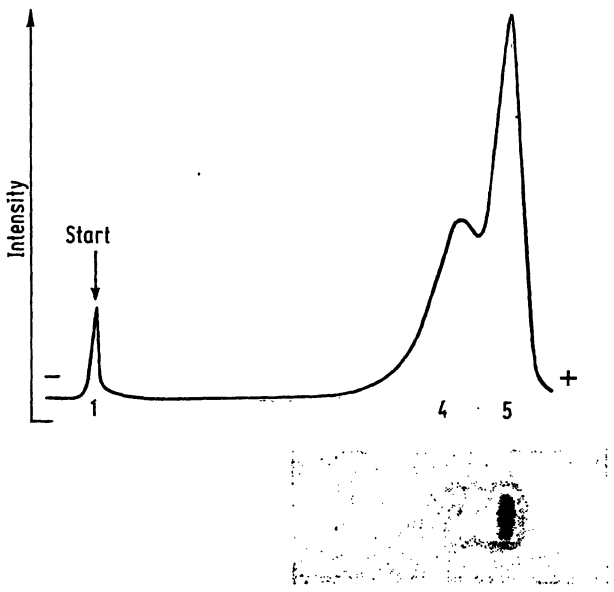

Fig. 1b
Electropherogram and densitogram of a normal serum without an intestine-type fraction

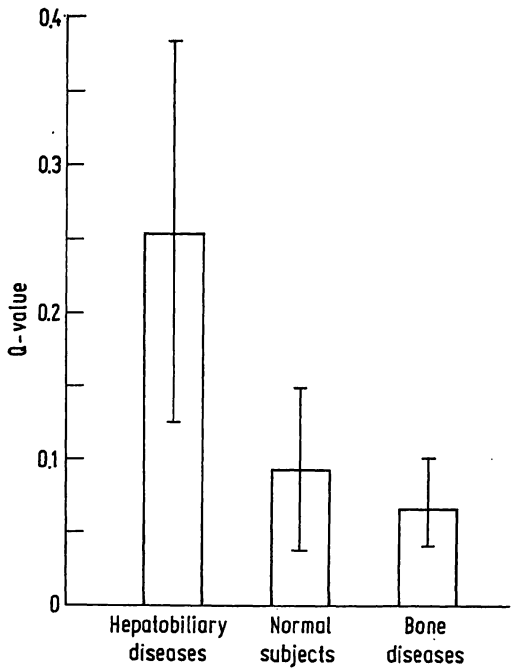

Fig. 2
Q-values ($\bar{x} \pm 2$ SD) in patients with hepatobiliary diseases (n = 25), n normal subjects (n = 38) and in patients with bone diseases (n = 15)

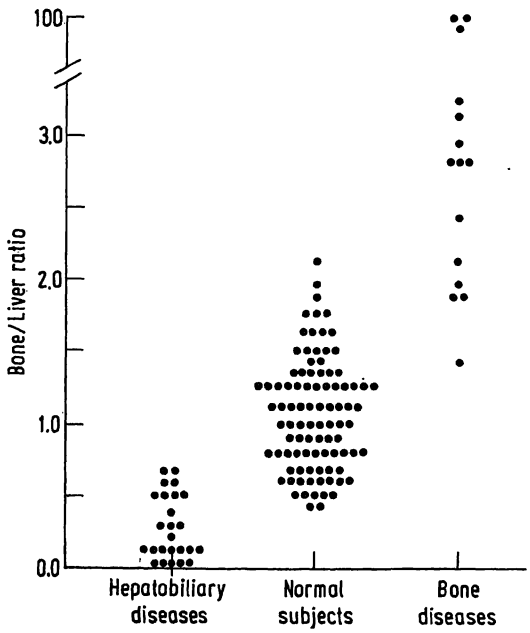

Fig. 3
Bone/liver ratio values ($\bar{x} \pm 2$ SD) in patients with hepatobiliary diseases (n = 25), in normal subjects (n = 92) and in patients with bone diseases (n = 15)

Tab. 4

Examples of laboratory results from patients with different diseases

Serum Diagnosis No.	Alkaline phosphatase [IU/l]	5'-Nucleotidase [IU/l]	γ-Glutamyltranspeptidase [IU/l]	Q-value	Bone/liver ratio	Fraction: 1 Type: bile [%]	3 intestine [%]	4 bone [%]	5 liver [%]
Hepatobiliary diseases									
1. liver metastases	427	45.2	71	0.28	0.5	+	—	34	66
2. occlusion of bile duct	305	50.0	181	0.32	0.0	16	—	—	84
3. cirrhosis of liver	180	49.5	186	0.31	0.6	—	—	38	62
4. adenomacarcinoma of liver	740	182.0	320	0.30	0.1	24	—	5	71
5. cholelithiasis	92	75.3	136	0.24	0.6	4	—	36	60
Bone diseases									
6. skeletal metastases	217	9.4	10	0.08	2.8	—	—	73	27
7. skeletal metastases	217	14.4	38	0.07	1.9	—	—	66	34
8. KAHLER's disease	73	6.9	10	0.04	100.0	—	—	100	—
9. PAGET's disease of bone	85	3.8	22	0.11	1.8	—	—	64	36
10. skeletal fractures	250	9.7	36	0.04	3.2	—	—	76	24
Bone and Liver diseases									
11. metastases in liver and bone	706	27.5	158	0.04	3.0	—	—	75	25
Miscellaneous diseases									
12. bronchial carcinoma	103	11.4	45	0.29	0.1	18	—	8	74
13. metastases in lung	72	9.4	41	0.21	0.0	+	—	—	100
14. HODGKIN's disease (stage II)	87	5.7	28	0.23	0.2	—	—	15	85
15 aortic stenosis	132	19.9	213	0.25	0.2	+	15	12	73

Heat-inactivation

For 38 normal sera the mean Q-value was 0.09 with a standard deviation of 0.027. See also Figure 2.

Applications of the described methods

Hepatobiliary diseases

Examination of sera from 25 patients with different hepatobiliary diseases gave the following results: Alkaline phosphatase 80—900 IU/l, γ-glutamyltranspeptidase 28—363 IU/l, 5'-nucleotidase 11.5—182.0 IU/l, a mean Q-value of 0.26 with a standard deviation of 0.066 and a bone/liver ratio 0.0—0.7. See Figures 2 and 3. Examples (sera 1—5) are shown in Table 4.

Figure 4a shows the electrophoretic pattern of a serum with a high liver-type fraction and positive bile-type fraction.

Bone diseases

Examination of sera from 15 patients with different bone diseases yielded the following results: alkaline

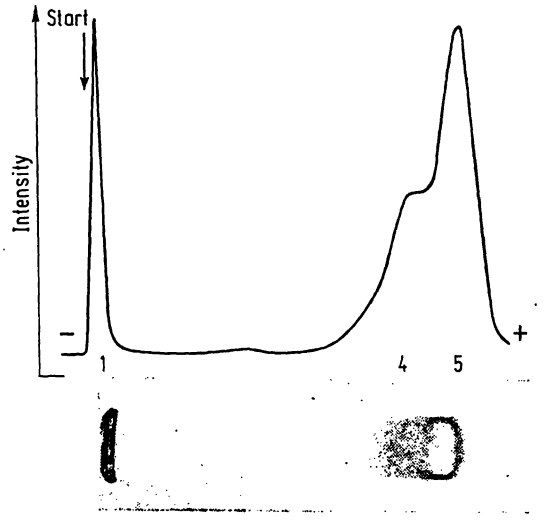

Fig. 4a

Electropherogram of a serum from a 39 year-old man with a T-drain, ten days after cholecystectomy and appendectomy; alkaline phosphatase 112 IU/l, 5'-nucleotidase 19.5 IU/l, γ-glutamyltranspeptidase 40 IU/l, bone/liver ratio 0.6, fraction 1 = 13%, fraction 4 = 33%, fraction 5 = 54%

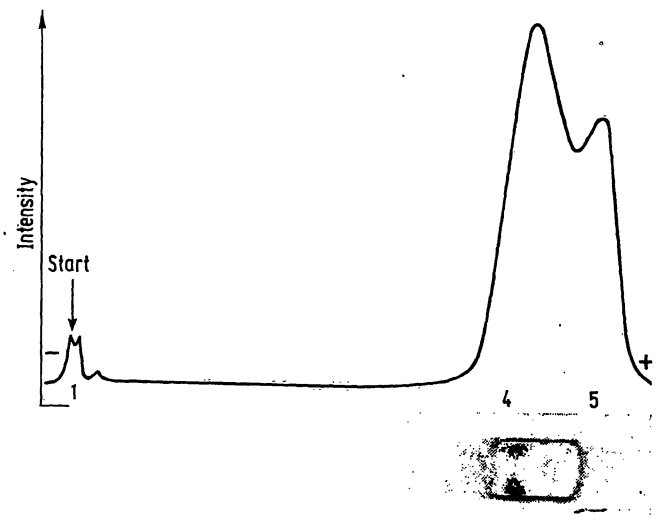

Fig. 4b

Electropherogram of a serum from a 61 year-old woman with generalized skeletal metastases; alkaline phosphatase 90 IU/l, 5'-nucleotidase 7.0 IU/l, γ-glutamyltranspeptidase 24 IU/l, bone/liver ratio 2.1, fraction 4 = 68%, fraction 5 = 32%

Tab. 5
Progress of the illness of a woman, 67 years old, with bone metastases

Analysis date	Alkaline phosphatase [IU/l]	5′-Nucleotidase [IU/l]	γ-Glutamyl-Transpeptidase [IU/l]	Q-value	Bone/liver ratio	Bone-type [%]	Liver-type [%]
1.2.'72	103	7.8	41	0.15	0.9	48	52
24.2.'72	118	14.5	30	0.13	1.1	52	48
8.3.'72	102	5.1	14	0.13	2.4	71	29

phosphatase 80—267 IU/l, γ-glutamyltranspeptidase 8—40 IU/l, 5′-nucleotidase 3.8—17.3 IU/l, a mean Q-value of 0.07 with a standard deviation of 0.018, and bone/liver ratios from 1.8 to 100.
Examples (sera 6—10) are shown in Table 4.
Figure 4b shows the electrophoretic pattern of a serum with a high bone-type fraction.

Liver and bone diseases

It is also possible to encounter patients with both liver and bone diseases. In these cases, the interpretation is difficult on the basis of the Q-value or bone/liver ratio alone; other indices of liver functions are needed. An example (serum 11) is given in Table 4.

Miscellaneous diseases

Cases also exist where the electrophoretic pattern reveals a high liver fraction, with no clinical evidence for liver disease.
Examples (sera 12—15) are shown in Table 4.

Following the progress of disease

Table 5 shows an example of a change in alkaline phosphatase isoenzyme pattern which took place over 6 weeks. On 1. 2. '72 it was difficult to decide whether the bone-type fraction was elevated or not (bone-type fraction 48%, bone/liver ratio 0.9).
On 8. 3. '72 the bone-type fraction was clearly elevated (bone-type fraction 71%, bone/liver ratio 2.4).

Discussion and Conclusion

A clinician is often interested in determining the origin of elevated alkaline phosphatases in serum (13). In our laboratory, we could never obtain satisfactory results with the frequently used method of HAIJE and DE JONG (14) in an agar medium.
After introducing the method of polyacrylamide disc gel electrophoresis in our laboratory for screening high alkaline phosphatase sera, together with 5′-nucleotidase (15, 16) and γ-glutamyltranspeptidase (17) and also with the Q-value, we obtained very good results, indeed. This electrophoresis method is a good diagnostic aid for screening and following (Table 5) patients with bone and hepatobiliary diseases.
The quantitation of the disc electrophoresis and the expression of the results in terms of a bone/liver ratio are especially valuable.
In most cases, there are no difficulties at all in differentiating between bone and liver diseases when using this ratio (Fig. 3).
There are, of course, cases involving both bone and liver. In these cases disc electrophoresis gives valuable information, since the bone fraction is raised, together with a rise in γ-glutamyltranspeptidase and 5′-nucleotidase.

Acknowledgement

We wish to express our gratitude to Miss VAN ESSEN, Blood Bank Rotterdam, for providing normal sera and to Miss INEKE VAN BOVEN and Mrs. JOKE CORNELISSEN for their technical assistance.

References

1. FISHMAN, W. H. & GHOS, N. K. (1970), in Advances in Clinical Chemistry (BODANSKY, O. & STEWART, C. P. eds.), vol. 10, p. 255—370, Academic Press, New York-London. — 2. WILKINSON, J. H. (1970), Isoenzymes, Chapman & Hall Ltd., London. 3. KLEIN, U. E. (1969), Deut. Med. Wochenschr. *94*, 526—529. — 4. GERLACH, U., HIBY, W. & PAUL, L. (1970), Med. Welt *21*, 1252—1258. — 5. STREIFLER, C., SCHNITZER, N. & HARELL, A (1972), Clin. Chim. Acta, *38*, 244—246. — 6. OHLEN, J., PAUSE, H. & RICHTER, J. (1971), Eur. J. Clin. Invest. *1*, 445—451. — 7. JOHNSON, R. B. Jr., ELLINGBOE, K. & GIBBS, PH. (1972), Clin. Chem. *18*, 110—115. — 8. WALKER, A. W. & POLLARD, A. C. (1971), Clin. Chim. Acta *34*, 19—29. — 9. Canalco Diagnostic Products Group (1970), in QDA specific reagent kit instructions for quick disc gel electrophoresis of alkaline phosphatase isoenzymes, Rockville USA. — 10. MORTON, R. K. (1954), Biochem. J. *57*, 595—603. — 11. PERSIJN, J. P., VAN DER SLIK, W., TIMMER, C. J. & REIJNTJES, C. M. (1970), this J. *8*, 398—402. — 12. SZASZ, G. (1969), Clin. Chem. *15*, 124—136. — 13. KAPLAN, M. M. (1972), Gastroenterology *62*, 452—468. — 14. HAIJE, W. G. & DE JONG, M (1963), Clin. Chim. Acta *8*, 620—623. — 15. CONNELL, M. D. & DINWOODIE, A. J. (1970), Clin. Chim. Acta *30*, 235—241. — 16. PHELAN, M. B., NEALE, G. & MOSS, D. W. (1971), Clin. Chim. Acta *32*, 95—102. — 17. SZASZ, G., ROSENTHAL, P. & FRITSCHE, W. (1969), Deut. Med. Wochenschr. *94*, 1911—1917.

Dr. J. A. P. Stroes
Stichting Samenwerking
Delftse Ziekenhuizen
Reynier de Graefweg 7
Delft / The Netherlands