ZONE ELECTROPHORETIC STUDIES OF SERUM ALKALINE PHOSPHATASE * †

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The serum alkaline phosphatase activity is increased in bone diseases associated with increased osteoblastic activity and in certain disorders of the liver and biliary tract, particularly those characterized by biliary tract obstruction or by hepatic infiltration with neoplastic or granulomatous processes (1, 2). Although there is much experimental evidence in support of the view that bone is the source of the serum alkaline phosphatase and that the bile serves as an important channel for its excretion (3, 4), the hepatic retention theory fails to provide a completely satisfactory explanation for the occurrence, in certain hepatobiliary disorders, of increased serum phosphatase activity without hyperbilirubinemia, nor does it account for the normal serum phosphatase values found in many cases of severe parenchymatous liver disease. It has been suggested on the basis of experimental (5) and clinical (6) observations that under certain circumstances the liver may, by its secretory activity, actively contribute to the increase in serum alkaline phosphatase. Although studies of the kinetics of the serum phosphatase activity (7) and of the effects of enzyme inhibitors (8) have failed to reveal differences between the serum phosphatase activity in cases of bone and biliary disease, it seemed of interest to explore further the possible occurrence of qualitatively distinct serum alkaline phosphatases. Since zone electrophoretic methods have proven useful in a variety of enzyme separations, a study was made by this technique of the alkaline phosphatase patterns of sera obtained from patients suffering from disorders associated with increased serum phosphatase activity. The results suggest the occurrence in some cases of more than one serum alkaline phosphatase.

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MATERIALS AND METHODS

Most of the cases studied were observed in the wards of the Boston City Hospital. The clinical diagnoses were supported in many instances by anatomic study of surgical, biopsy or necropsy material.

Venous blood samples from the patients were obtained without regard to the time of day or recent ingestion of meals. After the blood had clotted, the separated serum was stored at 4° C. and generally used for electrophoretic study within a few days; in many cases, serum samples were stored frozen and used weeks or months later. Electrophoretic patterns of protein and of enzymatic activity were little affected by such storage, as compared with fresh, unfrozen serum. Zone electrophoresis of serum was performed with starch blocks as the supporting medium, the general technique described by Kunkel (9) being used. The starch blocks were prepared from slurries of potato starch which prior to use had been repeatedly washed by decantation in pH 8.6 barbital buffer. Matheson, Coleman and Bell and Merck brands of starch were satisfactory. The blocks were prepared in a rectangular lucite trough ($40 \times 10 \times 2$ cm.) lined with wax paper; the trough also supported the blocks during electrophoresis. Usually three parallel blocks, each containing 70 Gm. of starch and each 35 cm. long, 3.3 cm. wide, and 1 cm. thick, were prepared in the same trough, the sides of their wax paper envelopes separating one from the other, and electrophoresis of three serum samples was performed simultaneously. For preparative work a single large block (210 Gm. starch and 10 cm. wide) was used. Buffer-saturated plastic sponges and filter paper strips provided electrical continuity between the blocks and the buffer vessels, and the latter were connected by buffer-filled inverted U-tubes to the vessels containing platinum electrodes. The buffer was barbital-sodium barbital, pH 8.6, ionic strength 0.1. Test samples, diluted with an equal volume of buffer, were delivered from the needle of a syringe to a transverse trough (approximately one mm. wide) cut in the starch block midway between the center and cathode end. The volume of material applied was 0.7 ml. in the case of the small blocks, and 3.5 to 5 ml. for the larger blocks. The solution in the buffer vessels was used repeatedly for a number of experiments before being discarded, the polarity of the electrodes being reversed after each run. The starch block and associated vessels were kept in a refrigerator at 4° C. throughout the period of electrophoresis. A potential difference of 2.5 to 3 volts per cm. was maintained between

the ends of the block and the current was 1.4 mA. per cm. width.

At the end of the electrophoretic period of 36 to 48 hours, which under these conditions permitted albumin migration of 15 to 20 cm., the block was sectioned transversely into serial segments 5 mm. wide from a point 1 or 2 cm. on the cathode side of the origin to a point just beyond the albumin zone, which was generally visible because of its yellowish color (9). A section to be used as a blank was taken from a region close to the cathode end of the block well outside the range of protein migration. Each starch segment was suspended in 5.0 ml. water, the mixture was shaken gently and then filtered through Munktell 00 paper. Aliquots of the filtrates were analyzed for proteins (10) and for alkaline phosphatase by the nitrophenylphosphate method of Bessey, Lowry and Brock (11). For phosphatase determination 0.5 to 2.0 ml. of filtrate was added to an equal volume of buffered substrate solution and incubated at 38° C. for 30 to 120 minutes, depending upon the activity of the samples. Aliquot volumes and incubation times were the same for all sections of a particular block. At the end of the incubation period, NaOH solution (0.05 to 0.10 M) was added to each tube to make the total volume 7 ml. and the optical density measured at 410 mμ (Coleman Junior spectrophotometer) before and after acidification with two or three drops of concentrated HCl. From the calibration curve relating *p*-nitrophenol concentration to optical density, phosphatase activity was expressed as μM nitrophenol liberated per hour per ml. filtrate. The buffer-substrate solution was a mixture of equal volumes of 0.3 per cent aqueous solution of *p*-nitrophenylphosphate disodium (Aldrich Chemical Co.) and of pH 10.3 buffer and contained magnesium chloride at a concentration of 5×10^{-4} M. In the earlier experiments 0.1 M glycine-NaOH was used as the buffer (11), while in the later work the more satisfactory 2-amino 2-methyl-1-propanol-HCl (0.5 M) was used (12). Comparison of phosphatase and protein zones was made by plotting the phosphatase and protein concentration of each filtrate as ordinates and the migration distances as abscissa. The completeness of recovery of phosphatase activity from the block after serum electrophoresis is discussed under Results.

Acid phosphatase activity in the eluates of several starch blocks was determined by incubating aliquots with equal volumes of a mixture of *p*-nitrophenylphosphate solution and acetate buffer, pH 5.4 (13). In three experiments I^{131}-labeled thyroxine (Abbott) was added to serum samples prior to electrophoresis and the distribution of radioactivity in the block subsequently determined by measuring aliquots of the filtrate in a well-type scintillation counter.

Serum alkaline phosphatase activity was determined by the King-Armstrong method (14). Serum protein-bound hexose was determined by the orcinol method described by Winzler (15).

RESULTS

The electrophoretic patterns showed that in every case the major zone of alkaline phosphatase activity was in the region of the alpha-2 globulins; the peak of activity sometimes coincided with this protein peak and sometimes the mobility of the active material was slightly less than that of the alpha-2 protein peak. In some cases, particularly those with hepatobiliary disease, a second zone of phosphatase activity was present in the region of the alpha-1 globulins. In general no phosphatase was detected in the gamma globulin or albumin zones. Phosphatase activity was usually present in the beta-globulin zone of the electrophoretogram whence the activity increased in the anodeward direction to form the ascending limb of the alpha-2 activity peak. Occasionally a broad plateau of appreciable activity was seen in the beta globulins and in several instances the occurrence of a zone of slightly decreased activity between this region and the alpha-2 activity suggested the presence of a beta phosphatase zone with a peak.

Since the alpha-2 and alpha-1 components of phosphatase activity appeared to be the most prominent, quantitative analysis of the electrophoretic phosphatase patterns was made on the basis of the relative proportions of these components. This was done by dividing the total phosphatase activity in the graphical representation of the electrophoretic patterns into what will be called "alpha-2" and "alpha-1" fractions. In those patterns in which two distinct phosphatase peaks were present, a perpendicular was drawn from the lowest point of the trough between these zones, and the phosphatase activity on the anode side of the line was considered to be alpha-1 activity, while that on the cathode side (which comprised the remainder of the total activity) was called the "alpha-2" fraction. In those cases where no distinct alpha-1 activity peak was apparent, but where the descending anodeward limb of the alpha-2 zone encroached upon the alpha-1 globulin region, a perpendicular was dropped from the nadir of the trough between alpha-2 and alpha-1 globulins, and division into alpha-2 and alpha-1 activities was made by considering activity on the anode side of the line to be "alpha-1." In effect, all the phosphatase activity of mobility less than that of the alpha-1 globulins was called the "alpha-2" fraction.

Table I summarizes the results in 63 cases studied by starch block electrophoresis; the di-

TABLE I

Clinical data and electrophoretic phosphatase patterns of cases studied

Case no.	Age	Sex	Diagnosis	Serum alkaline phosphatase (King-Armstrong)	Number of phosphatase peaks	Per cent of activity in	
						alpha-2	alpha-1
1	42	F	Ovarian carcinoma, hepatic metastases	37	2	85	15
2*	82	M	Gastric carcinoma, hepatic metastases	107	2	89	11
3*	75	F	Gallbladder carcinoma, hepatic metastases; cholangitis	30	2	87	13
4*	55	M	Hepatic duct carcinoma, hepatic metastases, biliary cirrhosis	373	2	68	32
5*	79	F	Carcinoma of colon, hepatic metastases	150	2	56	44
6*	44	F	Carcinoma of breast, hepatic metastases	123	2	81	19
7*	63	M	Hepatic carcinoma, primary site undetermined, pigment cirrhosis	51	2	88	12
8*	73	M	Sigmoid carcinoma, hepatic metastases	78	2	89	11
9*	75	F	Gall bladder carcinoma, hepatic metastases	242	2	80	20
10*	81	M	Hepatic duct carcinoma, biliary cirrhosis	69	1	96	4
11*	55	M	Metastatic hepatic carcinoma, ? primary site; portal cirrhosis	115	2	83	17
12	84	F	Carcinoma of pancreas, obstructive jaundice	245	2	80	20
13*	65	F	Gall bladder carcinoma, hepatic metastases	276	1	97	3
14*	55	F	Biliary cirrhosis, idiopathic	85	2	88	12
15*	52	F	Pericholangitis, biliary cirrhosis	158	1	100	0
16*	77	F	Biliary cirrhosis, idiopathic	101	2	79	21
17*	52	F	Cholangiolitic cirrhosis	141	2	84	16
18*	80	M	Biliary cirrhosis, extrahepatic obstruction	65	2	85	15
19*	62	F	Choledocholithiasis, obstructive jaundice	74	2	84	16
20*	74	M	Biliary cirrhosis, choledocholithiasis	25	2	76	24
21	73	M	Choledocholithiasis, cholangitis	182	2	74	26
22*	80	M	Stricture of common duct, cholangitis	114	2	86	14
23*	88	M	Choledocholithiasis, obstructive jaundice	304	2	75	25
24*	57	F	Choledocholithiasis, cholangitis	97	2	89	11
25*	55	M	Choledocholithiasis	26	2	95	5
26	45	M	Viral hepatitis	38	1	100	0
27*	58	F	Viral hepatitis	65	1	97	5
28	18	M	Viral hepatitis	21	2	97	3
29	23	F	Viral hepatitis	72	1	100	0
30*	55	M	Fatty nutritional cirrhosis, active	25	2	88	12
31	54	F	Fatty nutritional cirrhosis, active	47	1	96	4
32*	50	F	Fatty nutritional cirrhosis, active	167	1	92	8
33	49	F	Fatty nutritional cirrhosis, active	12	1	97	3
34*	80	F	Fatty nutritional cirrhosis, active	11	1	100	0
35	50	M	Fatty nutritional cirrhosis, active	8	1	100	0
36	65	F	Paget's disease	228	1	99	1
37	74	F	Paget's disease	77	1	98	2
38	73	M	Paget's disease	68	1	99	1
39	54	M	Paget's disease	475	1	99	1
40	61	F	Paget's disease	348	1	99	1
41	69	M	Paget's disease	123	1	99	1
42	62	M	Paget's disease	115	2	99	1
43	72	M	Paget's disease	46	1	98	2
44	70	M	Paget's disease	130	1	95	5
45	71	M	Bronchiogenic carcinoma, bone metastases	68	1	99	1
46	67	M	Prostatic carcinoma, bone metastases	204	1	98	2
47	63	M	Prostatic carcinoma, bone metastases	104	1	97	3
48*	75	M	Prostatic carcinoma, bone metastases	90	2	93	7
49	72	M	Prostatic carcinoma, bone metastases	157	1	100	0
50	77	M	Prostatic carcinoma, bone metastases	257	2	98	2
51	39	F	Breast carcinoma, bone metastases	49	2	95	5
52	52	F	Obesity	11	1	98	2
53	44	F	Non-toxic goiter	7	1	96	4
54	54	M	Myocardial infarction	8	1	100	0
55	35	F	Psychoneurosis	11	1	95	5

* Diagnosis confirmed by anatomical study (biopsy, operation or autopsy).

TABLE I—*Continued*

Case no.	Age	Sex	Diagnosis	Serum alkaline phosphatase (King-Armstrong)	Number of phosphatase peaks	Per cent of activity in alpha-2	Per cent of activity in alpha-1
56	23	F	Psychoneurosis	12	1	97	3
57	47	M	Psychoneurosis	13	1	97	3
58	15	F	Obesity	12	1	97	3
59	81	M	General arteriosclerosis	9	1	100	0
60	32	F	Hyperthyroidism, in remission	12	1	100	0
61	61	M	Arteriosclerotic heart disease	12	1	100	0
62	30	F	Idiopathic hirsutism	14	1	100	0
63	33	M	Myocardial infarction	12	1	100	0

		Serum alk. phosphatase Range	Serum alk. phosphatase Average	Number of cases	Number with 2 peaks	Alpha-1 phosphatase per cent of total (av. ± S.D.)	Number with alpha-1 phosphatase >10% of total
Group A	Obstructive and infiltrative biliary disease (Cases 1–25)	25–373	131	25	22	16.2 ± 9.6	21
Group B	Parenchymatous liver disease (Cases 26–35)	8–167	47	10	2	3.5 ± 4.0	1
Group C	Bone disease (Cases 36–51)	46–475	159	16	4	2.2 ± 1.9	0
Group D	Control group (Cases 52–63)	8–14	11	12	0	1.8 ± 1.8	0

agnosis, serum alkaline phosphatase activity, the number of phosphatase peaks, and the proportion of alpha-2 and alpha-1 activity are shown. The cases may be divided into four groups.

Group A (Cases 1 through 25). In this group are included 25 cases of hepatobiliary disease, 13 of which were cases of carcinomatous metastases to the liver, while the remainder included cases of biliary cirrhosis (idiopathic and secondary to extra-hepatic obstruction), choledocholithiasis and cholangitis.

Group B (Cases 26 through 35), consisted of 10 cases of predominantly hepatocellular disease, including four cases of viral hepatitis and six of fatty nutritional cirrhosis in an active phase.

Group C (Cases 36 through 51) was made up of 16 cases of bone disease associated with hyperphosphatasemia and included nine cases of Paget's disease and seven of carcinomatous metastases to bone.

Group D (Cases 52 through 63). This group included 12 subjects with normal serum alkaline phosphatase activity who were hospitalized for a variety of illnesses and were clinically free of liver or bone disease. These were considered normal controls for purposes of this study.

Group A (hepatobiliary disease)

The serum alkaline phosphatase was high in all 25 cases, ranging from 25 to 373 King-Armstrong units. In all but three instances (Cases 10, 13, 15) two distinct phosphatase peaks were present in the electrophoretic pattern, and in 21 of the 22 cases showing both alpha-1 and alpha-2 peaks, the alpha-1 phosphatase exceeded 10 per cent of the total activity. A typical pattern of a case from the group is seen in Figure 1, and also in Figures 2 and 3. The alpha-2 phosphatase was always the predominant component, the maximum value for alpha-1 activity being 44 per cent and the average value, 16.2 per cent. In several cases in this group appreciable activity was found in the beta-globulin zone and in two cases (Cases 7 and 18) a beta-globulin phosphatase peak was found (included in "alpha-2" in the classification). Studies in Case 14 (biliary cirrhosis) were performed at intervals over a 16 month period, during which progressive impairment of liver function occurred (increased gamma globulins, positive Hanger test) and increased fibrosis as revealed by serial liver biopsies; although the data listed in the table show that appreciable alpha-1

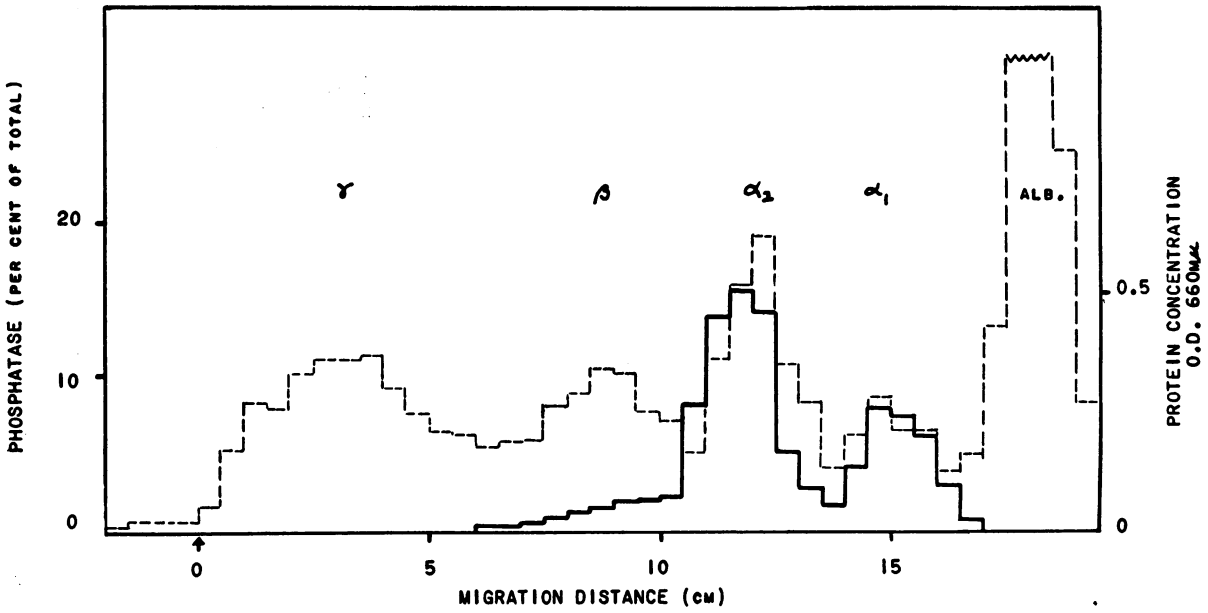

FIG. 1. SERUM ELECTROPHORETIC PATTERN, CHOLANGIOLITIC HEPATITIS (CASE 17)

In this and the remaining figures, the abscissa indicates the distance from the line of application of the sample, indicated by the arrow. The anodal direction is to the right. Phosphatase activity (solid lines) and protein concentrations (broken lines) are indicated by the height of adjacent vertical bars, of which only the noncontiguous portions are delineated. Each bar represents a single starch segment. Phosphatase is expressed as a fraction of the total activity in the pattern, protein concentration as optical density of starch filtrates as determined by the modified Folin method (10).

The serum specimen was obtained two months after surgical exploration which confirmed the diagnosis of cholangiolitic cirrhosis. The serum phosphatase was 113 King-Armstrong units. Two distinct phosphatase peaks are seen, the alpha-1 zone accounting for 29 per cent of the total. In the serum specimens obtained preoperatively, one month after onset of the disease, the alpha-1 phosphatase peak contributed 16 per cent of the total, as indicated in Table I.

activity was present in the first study, in two later studies no distinct alpha-1 peak was found, and only 5 to 8 per cent of the activity was in this region.

There seemed to be no correlation between the presence or degree of bilirubinemia and the value for the fraction of the total phosphatase activity in alpha-1, nor was this value related to the value of total alkaline phosphatase of the serum. Review of the clinical and laboratory data of the four cases with less than 10 per cent alpha-1 activity failed to show differences from the 21 cases in which alpha-1 activity exceeded 10 per cent.

Group B (hepatocellular disease)

Of the 10 cases, three had normal serum phosphatase values. In two of the 10, the electrophoretic phosphatase pattern revealed both alpha-1 and alpha-2 peaks, alpha-1 being 3 and 12 per cent. The average value for alpha-1 phosphatase in this group was 3.5 per cent. The serum protein pattern was abnormal in most of these cases, gamma globulins being increased and albumin decreased. In some patterns the phosphatase activity in alpha-2 had a broader base and the peak was not as prominent nor as sharp as in Groups A and C, and in two cases (31 and 33) a distinct peak of activity was present in the beta region.

Group C (bone disease)

Four of the 16 cases of hyperphosphatasemic bone disease had phosphatase patterns characterized by an alpha-1 as well as an alpha-2 peak, but the proportion of the total in the alpha-1 zone was small (average, 2.2 per cent; maximum, 7 per cent). The predominant alpha-2 phosphatase zone tended to have a sharp, distinct peak, with relatively less of a beta trail than was seen in the other groups, and the alpha-2 zone of activity was consequently more symmetrical. The protein pat-

tern revealed no consistent abnormalities although in several cases of Paget's disease the alpha-2 globulin fraction was more prominent than normal. An example of the pattern in a case of Paget's disease is shown in Figure 4.

Group D (control group)

The electrophoretic phosphatase patterns in the "control" cases were qualitatively similar to those in the bone disease cases. None of these cases showed a distinct alpha-1 phosphatase peak although a small fraction of the total activity (range, 2 to 5 percent; average, 1.8 per cent) was present in the alpha-1 globulin zone. An appreciable fraction of the "alpha-2" activity was in fact present as a trail in the beta globulins; this made the alpha-2 peaks less sharp and distinct than the corresponding peak in the bone cases. A normal pattern is shown in the lower panel of Figure 5. A possible explanation of the trailing might be that trailing or adsorption of a quantity of the alpha-2 phosphatase occurred in all cases, but a

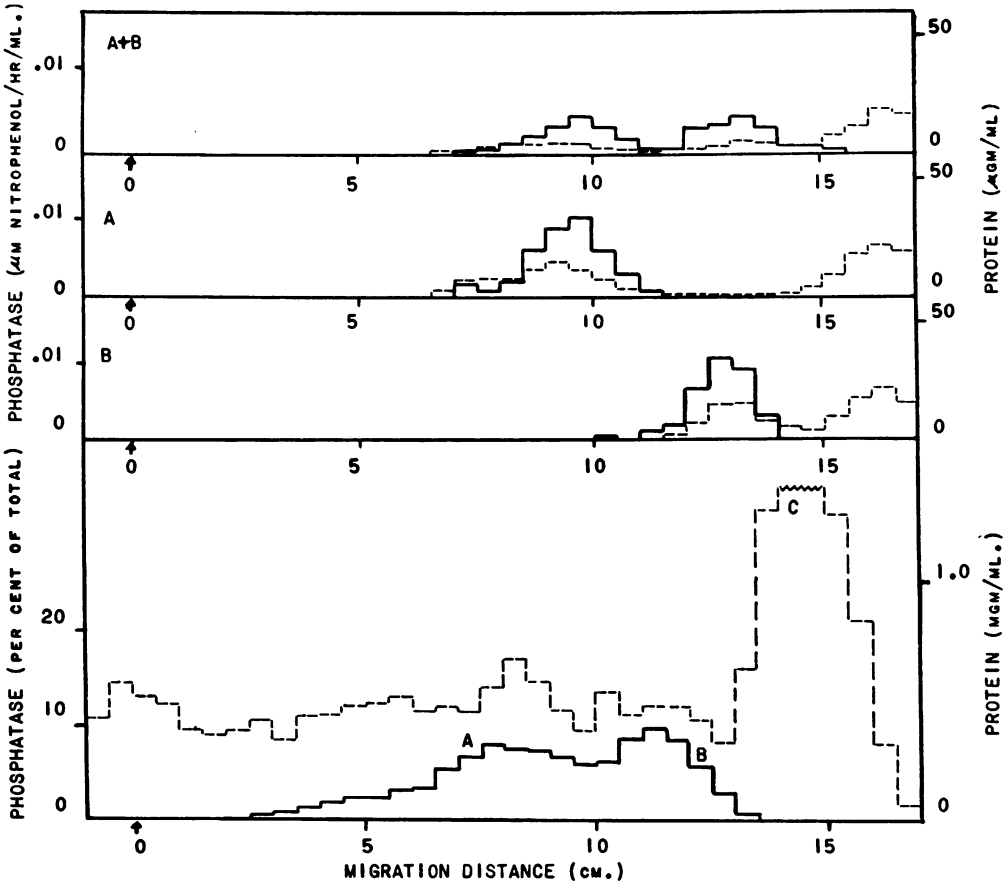

FIG. 2. ELECTROPHORETIC PATTERNS OF SERUM AND OF FRACTIONS OF THE ALPHA-2 AND ALPHA-1 GLOBULINS, CASE 5 (BOWEL CARCINOMA METASTATIC TO LIVER)

The electrophoretic pattern of the serum performed on a preparative scale is shown in the lower panel. Alpha-1 phosphatase is 42 per cent of the total. Samples of eluates of segments A (alpha-2) and B (alpha-1) and a mixture of the two (A plus B) were then run separately in small starch blocks simultaneously, a quantity of albumin (segment C eluate) having been added to each sample as a reference standard for mobility; the patterns shown in the upper panels, labeled A, B and A + B, correspond to the respective samples. In the mixture of A + B, 44 per cent of the phosphatase was derived from alpha-1 (B) and 56 per cent from alpha-2 (A); in the pattern of A + B, 53 per cent of the activity was in the alpha-1 zone. The characteristic mobilities of the two phosphatases observed in the electrophoresis of the individual fractions are preserved in the pattern of the mixture, and resemble those in the serum pattern.

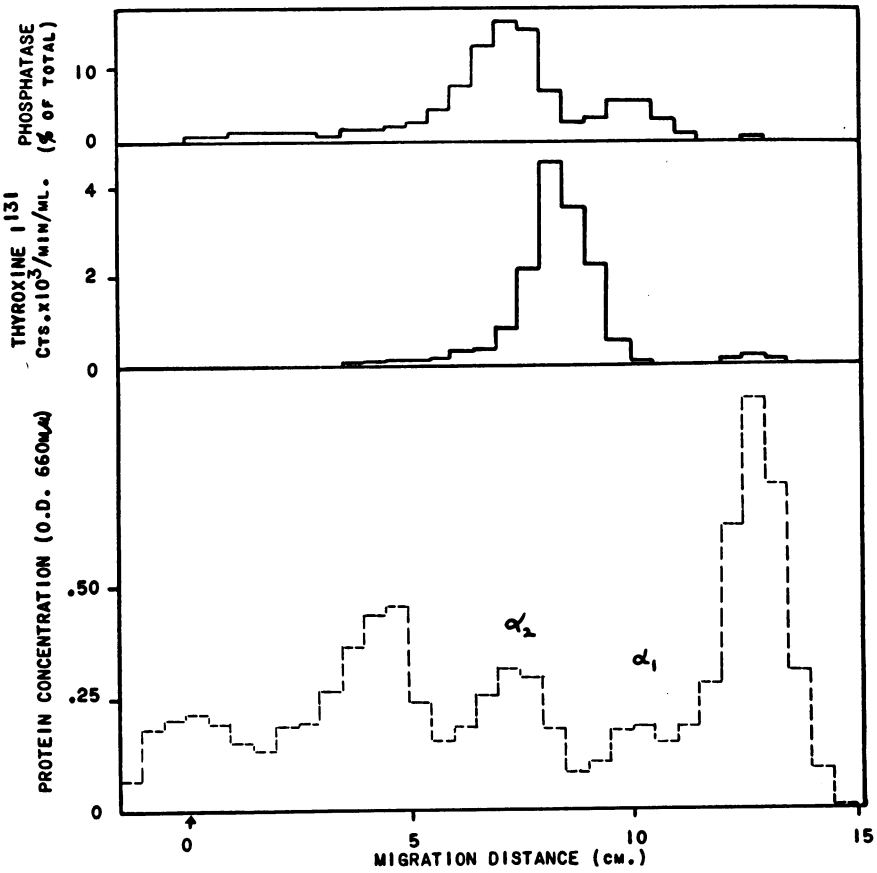

FIG. 3. ELECTROPHORETIC PATTERN OF SERUM, CASE 9 (CARCINOMA OF GALL BLADDER WITH HEPATIC METASTASES)

One-tenth ml. of I^{131}-labeled thyroxine (0.5 μg.) was added to a mixture of 0.5 ml. serum, and 0.5 ml. barbital buffer; 0.7 ml. of the resulting solution was used for electrophoresis. The lower panel shows the serum protein pattern, and the upper panel the alkaline phosphatase distributed in two well-resolved zones, the alpha-1 zone accounting for 20 per cent of the total. The middle panel shows the distribution of radioactive iodine in the pattern, with a large peak in the "inter-alpha" region and a small peak in albumin. The mobility of the thyroxine-binding protein is greater than the alpha-2 phosphatase and less than the alpha-1.

smaller proportion of the total activity was in the trail when the total serum alkaline phosphatase was high, as it was in the Group C cases.

The lower part of Table I summarizes the results in the four different groups studied. In group A, 84 per cent of the cases had more than 10 per cent of the total phosphatase activity in the alpha-1 zone, while only one of the 38 cases in the remaining three groups showed an alpha-1 zone exceeding this value.

The difference in the mobilities of the alpha-2 and alpha-1 phosphatase activities found in the serum electrophoretic patterns of most of the patients in Group A was apparent in two other types of experiments.

a). Hyperphosphatasemic sera from cases of bone disease showing only alpha-2 phosphatase in the electrophoretic patterns were mixed with sera from cases of hepatic cancer in which both alpha-2 and alpha-1 phosphatase zones were present. Electrophoresis of such mixtures (three cases) resulted in a phosphatase pattern suggestive of an additive effect, the pattern of the mixture being similar to that obtained by adding the values from the patterns of each serum alone. The resolution of the two peaks in the mixture was generally

inferior to that in the original serum containing the two activities, but alpha-1 phosphatase activity was evident in the mixture pattern.

b). In several experiments such as that shown in Figure 2, preparative scale electrophoresis was done with a serum sample containing alpha-2 and alpha-1 phosphatase peaks, and aliquots of the eluates from each phosphatase zone were then subjected to electrophoresis separately and as a mixture. The mobilities of the fractions separated from the serum and run independently differed distinctly from each other, and electrophoresis of the mixture of fractions effected complete resolution with preservation of the individual mobilities, which were similar to those observed in the original serum pattern; the proportion of each activity in the resolved mixture was comparable to the composition of the prepared mixture.

These results suggested that the occurrence of the two phosphatase zones in some sera was probably indicative of the presence of more than one alkaline phosphatase, rather than of a single phosphatase distributed into two electrophoretically distinct forms as a result of protein-protein interactions. Other experiments also failed to show such interaction. Thus, there seemed to be no relationship between the prominence of the alpha-1 globulin zone in the serum electrophoretic pattern and the magnitude of the alpha-1 phosphatase activity. In another study of possible interactions a serum sample from a case of bone disease (Case 47) was subjected to electrophoresis in a preparative block; the pattern showed almost all the alkaline phosphatase activity in the alpha-2 zone, and the alpha-1 globulins, although well resolved, had little activity. Electrophoresis was then performed with eluates of single segments of the alpha-2 zone, of the alpha-1 zone, and of a mixture prepared from both zones in such proportions that the concentration of alpha-1 globulin (phosphatase-poor) in the mixture was 2.5 times the concentration of the alpha-2 protein; the resulting patterns showed that the proteins were well resolved according to their characteristic mobilities, and the phosphatase activity was confined to the alpha-2 globulins in the patterns of the mixture as well as of the separate fractions. This again suggested that interaction of enzyme with inert protein carriers was not a major mechanism of the occurrence of the two electrophoretically distinct phosphatase activities.

The mobilities of the two phosphatases, corresponding closely but not always precisely with the peaks of alpha-2 and alpha-1 globulins, were studied further by comparison with the mobility of the "thyroxine-binding protein." A small quantity of I^{131} labeled thyroxine was added to serum before electrophoresis on a starch block and the distribution of radioactivity was compared with that of phosphatase. Three such experiments

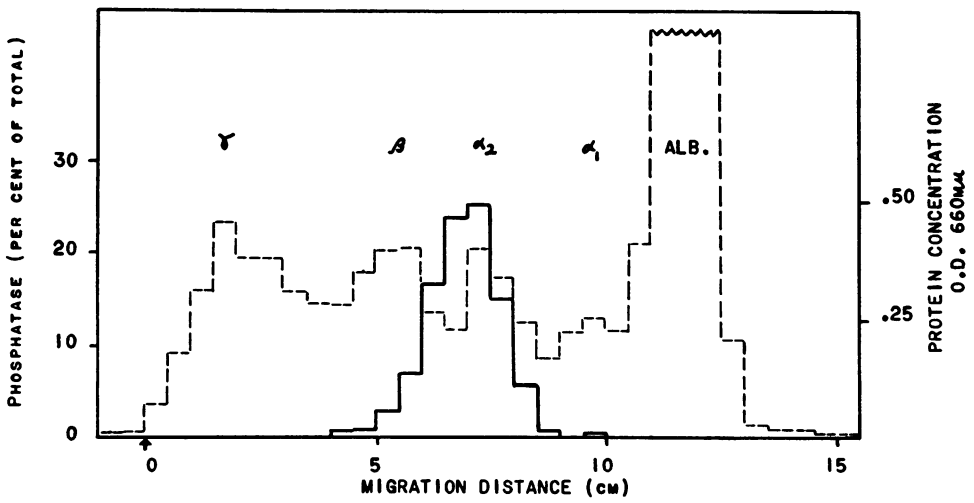

FIG. 4. SERUM ELECTROPHORETIC PATTERN, PAGET'S DISEASE (CASE 42)

Most of the phosphatase activity is in a single zone in the alpha-2 globulins, and only 1 per cent of the activity is in the alpha-1 globulin zone.

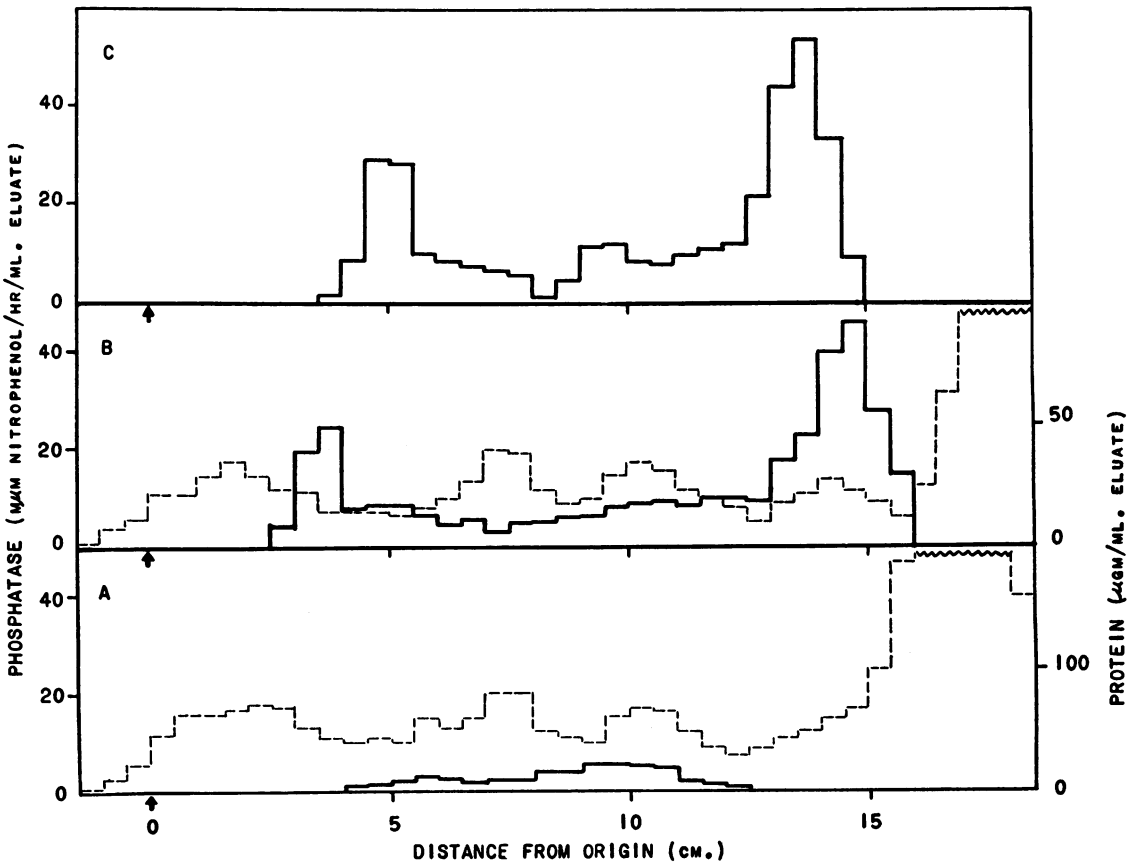

FIG. 5. ELECTROPHORETIC PATTERN OF BILE AND OF NORMAL SERUM (CASE 62)

The specimen of bile (alkaline phosphatase 164 King-Armstrong units) was obtained from a T-tube in the common duct of a patient eight days after operation for choledocholithiasis. The serum specimen had 14 K-A units alkaline phosphatase. The patterns were obtained by simultaneous electrophoresis in three parallel blocks.

Lower panel: serum electrophoretic pattern. The alpha-2 phosphatase is predominant, with a long beta trail.

Upper panel: electrophoretic phosphatase pattern of bile. The predominant activity corresponds to alpha-1 globulin in mobility, with smaller zones of activity at beta and alpha-2. The protein concentration was very small and is not indicated.

Middle panel: electrophoretic pattern of mixture of equal parts of bile and serum. Most of the phosphatase activity is contributed by the bile and the largest phosphatase zone corresponds to the alpha-1 globulins. There was considerable loss of activity in the electrophoresis of bile alone (C) and smaller losses during electrophoresis of the bile-serum mixture.

The phosphatase scale is the same in all three charts; the protein scale in the middle panel is twice that in the lower.

yielded results similar to the patterns shown in Figure 3. The thyroxine-binding protein, as judged by the position of the predominant band of radioactivity, was found to be in the "inter-alpha" postion of the electrophoretogram in confirmation of the findings of other (16, 17), and the alpha-2 and alpha-1 phosphatases were found on the cathodic and anodic sides, respectively, of the thyroxine-binding protein.

Studies of the pH optimum and of the effects of inhibitors upon enzyme activity have thus far failed to show differences between alpha-2 and alpha-1 phosphatase. In four experiments in which pH-phosphatase activity curves of eluates from the alpha-2 and alpha-1 segments of starch blocks were compared, no differences in the shape of the curves nor in the pH optimum of the two phosphatases were found (Figure 6). The value

of the pH optimum was approximately 9.8 in experiments using 2-methyl-2-amino-propanol as buffer and *p*-nitrophenylphosphate as substrate. Table II shows the effects of adding cyanide and fluoride to solutions of separated alpha-2 and alpha-1 phosphatases; both were almost equally inhibited by cyanide and insensitive to fluoride.

The effect of adding albumin to phosphatase fractions was studied since it has been reported that the addition of albumin to serum decreases serum alkaline phosphatase activity (18). Phosphatase-free albumin solutions, derived from the albumin zone of preparative starch block electrophoresis of serum, were added to solutions of similarly isolated alpha-2 and alpha-1 phosphatase fractions so that 80 per cent of the total protein in the mixtures was albumin, and the enzymatic activity of the mixtures was compared with that of the phosphatase fractions free of albumin. No inhibitory effect of albumin was observed, nor did gamma globulin have any effect in analogous experiments. Mixtures of alpha-2 and alpha-1 phosphatase fractions had enzymatic activity equal to the sum of the components separately. In one (Case 10) of the three cases in Group A in which little alpha-1 phosphatase was present, no effect on enzyme activity was observed when alpha-1 globulin obtained by zone electrophoresis of this serum was mixed with alpha-2 phosphatase from this case. This suggested that the absence of alpha-1 phosphatase in this case was not attributable to a phosphatase inhibitor in the alpha-1 globulins.

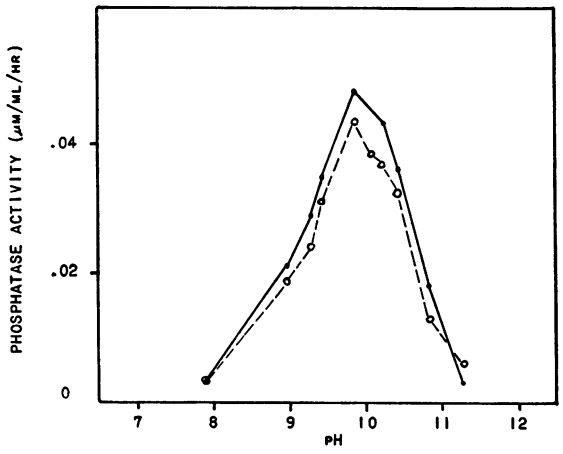

FIG. 6. PHOSPHATASE ACTIVITY-pH CURVES OF ALPHA-2 AND ALPHA-1 PHOSPHATASES

Eluates of single segments from the alpha-2 and alpha-1 zones of the serum electrophoresis in Case 21 were used; as in Figure 2, the segments were chosen from the cathodal limb of alpha-2 and the anodal limb of alpha-1 in order to minimize possible admixture due to incomplete electrophoretic resolution. Aliquots of each of the enzyme solutions were added to a series of tubes containing substrate-buffer mixtures of varying pH. The pH recorded is that of the mixture of enzyme with buffered substrate solution as determined in a parallel set of unincubated tubes. The pH optimum is approximately 9.8 for each phosphatase.

Unbroken line represents alpha-2; broken line, alpha-1.

TABLE II

Effect of cyanide and fluoride on alpha-2 and alpha-1 phosphatase *

Substance added	Final concentration	Phosphatase activity (% of control) alpha-2	alpha-1
None		100	100
Sodium cyanide	10^{-4} M	94	100
Sodium cyanide	10^{-3} M	59	64
Sodium cyanide	10^{-2} M	5	10
Sodium fluoride	10^{-4} M	97	100
Sodium fluoride	10^{-3} M	100	100
Sodium fluoride	10^{-2} M	97	100

* The solutions of alpha-2 and alpha-1 phosphatase, each containing 40 μg. protein per ml. and nearly equal phosphatase activity, were obtained from starch block electrophoresis of Case 5.

In one of the cases of prostatic cancer with bone metastases (Case 48) the serum acid phosphatase, as well as the serum alkaline phosphatase, was very high and the distribution of acid phosphatase activity in the starch filtrates was determined with the result shown in Figure 7. The acid phosphatase was found in a fairly discrete zone of greater mobility than the alpha-2 alkaline phosphatase and alpha-2 globulins but of lesser mobility than the alpha-1 phosphatase or alpha-1 globulin. The pattern in this case seemed to indicate clearly the separate nature of serum alkaline and acid phosphatases.

Bile phosphatase

Nine specimens of bile were studied; they were obtained either from the gall bladder at autopsy or from cholecystostomy and choledochostomy tubes of postoperative cases of biliary disease. The values of alkaline phosphatase in these samples ranged between 58 and 185 King-Armstrong units and averaged 127. The most satisfactory starch electrophoretic patterns were obtained with bile which had not been frozen and which was

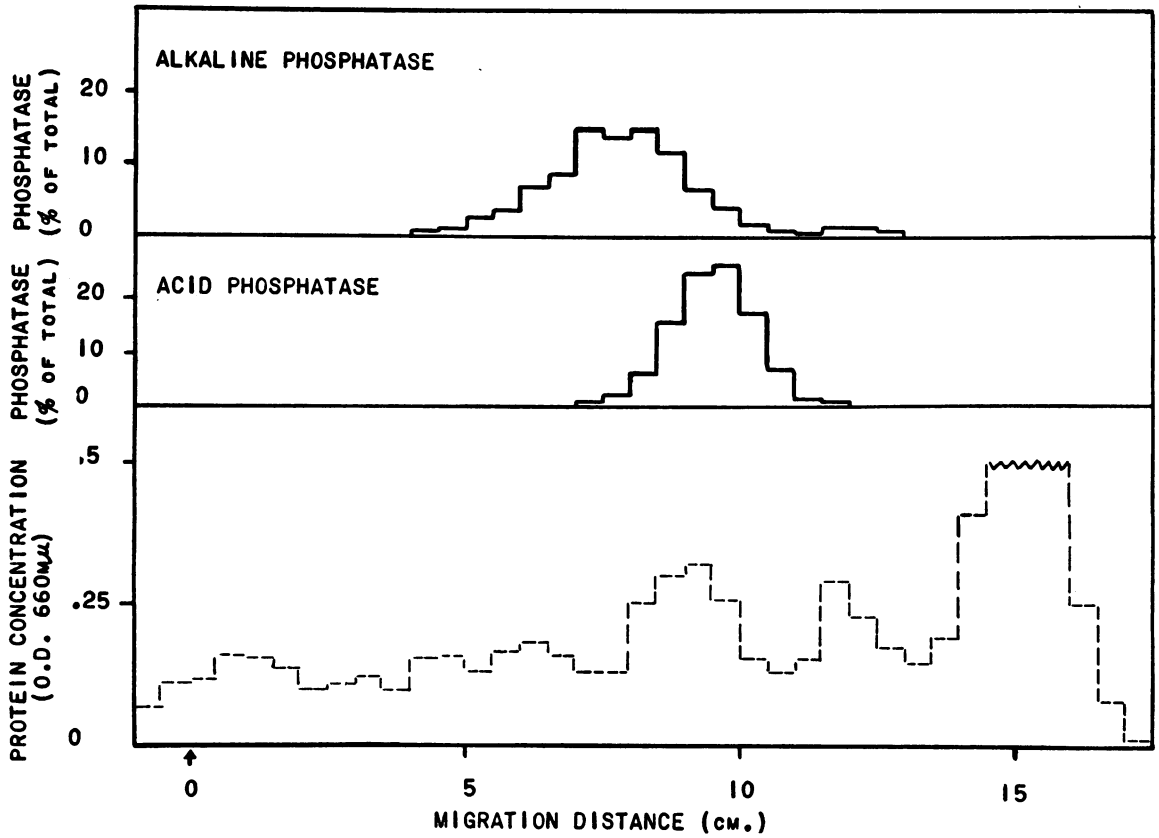

FIG. 7. ELECTROPHORESIS OF SERUM, CASE 48 (PROSTATIC CARCINOMA WITH SKELETAL METASTASES)

The serum acid phosphatase was over 2,000 King-Armstrong units. Lower panel, protein pattern; upper panel, alkaline phosphatase; middle panel, acid phosphatase. A small alpha-1 zone of alkaline phosphatase is present. The acid phosphatase peak lies between the two alkaline phosphatase zones.

filtered before use. The electrophoretic patterns of bile alone (six cases) were somewhat variable; a slow component of mobility similar to the beta globulins was seen in some, and in all cases a phosphatase zone of mobility approximating that of serum alpha-1 globulin was found as well as a phosphatase of alpha-2 globulin mobility. There was little protein in most of the bile samples and estimation of comparative mobilities was made by comparing the zones of enzymatic activity in the bile patterns with the protein bands of serum specimens run simultaneously in a parallel starch block. When bile was added to serum containing predominantly alpha-2 phosphatase, the electrophoretic pattern of the mixture resembled, in an exaggerated way, the alpha-2 plus alpha-1 pattern of most Group A cases. An example of this is seen in Figure 5, in which bile of high phosphatase activity was added to normal serum (Case 62); the predominant phosphatase in the mixture, as well as in bile alone, had the mobility of alpha-1 globulins, while the serum alone had no alpha-1 activity. The bile and bile-serum mixture also contained an appreciable beta zone activity, and there was some alpha-2 activity.

Phosphatase recovery and reproducibility of pattern

Between 51 and 68 (average, 60) per cent of the phosphatase in the serum sample subjected to electrophoresis was recovered in the starch filtrates (eight experiments). These estimates were subject to considerable error since they involved comparison of the phosphatase activity of the serum with the sum of the activities of 30 to 40 starch segment eluates. In the calculation, it was assumed that all the phosphatase in the starch segment was distributed in a volume equivalent

to the volume of water used for elution plus the water content of the damp segment (determined by weighing several segments before and after desiccation). These figures do indicate, however, that very large losses of activity probably did not occur during the electrophoresis. Satisfactory recoveries were also obtained when isolated eluates of the two phosphatases were re-run on blocks. Large losses (80 per cent) were encountered when bile was subjected to electrophoresis; addition of serum to the bile sample seemed to stabilize the activity, and losses were smaller.

The qualitative and quantitative aspects of the phosphatase patterns of a particular serum were reasonably reproducible. The difference between values for the proportion of alpha-1 phosphatase in duplicate runs was seldom greater than 20 per cent of the larger value.

Since the serum mucoprotein concentration is abnormally high in hepatic cancer and low in hepatocellular disease (19), since certain alkaline phosphatases contain carbohydrate (20), and since the fraction of phosphatase in alpha-1 was larger in hepatic cancer then in hepatitis, serum protein-bound hexose determinations were made in 40 cases in which the relative proportions of alpha-2 and alpha-1 phosphatase had been measured. There seemed to be no relationship between the serum glycoprotein concentration and the occurrence or magnitude of alpha-1 phosphatase activity.

DISCUSSION

The localization of serum alkaline phosphatase activity in the alpha globulin zone agrees both with the observation that phosphatase activity occurs in the alpha globulin prepared by alcohol fractionation of plasma (21) and also with results of paper electrophoretic studies of serum phosphatase in man (22–24). Eisfeld and Koch (22), using paper electrophoresis, reported that normally the serum alkaline phosphatase activity was in the alpha-2 globulin zone, while in hyperphosphatasemic states phosphatase activity was also present in the alpha-1 globulins; apparently they observed no difference between the phosphatase patterns in bone disease and in biliary obstruction. Baker and Pellegrino (23) also used paper electrophoresis and found alkaline phosphatase activity in the alpha-2 globulins, and in some cases observed an additional zone of activity in the beta globulins.

The present studies, in which starch block electrophoresis was used, yielded results which are not in complete agreement with those reported in the paper electrophoretic studies. Although the alpha-2 phosphatase was the predominant component found in all cases in the present study, an additional phosphatase in the alpha-1 globulin zone contributed an appreciable fraction of the total alkaline phosphatase activity in most hyperphosphatasemic cases of infiltrative and obstructive hepatobiliary disease. This contrasted with the normal cases and with the bone disease cases in which alpha-1 phosphatase activity was either absent or present as only a small fraction of the total (less than 10 per cent). It is concluded that the occurrence of an appreciable fraction of the serum phosphatase activity in the alpha-1 fraction is related to the nature of the clinical disorder associated with the increase in serum phosphatase activity rather than to the degree of the increase. Because of the qualitative similarity of the normal patterns and those of bone disease, it is believed that the pattern in which more than ten per cent of the phosphatase is in the alpha-1 fraction is abnormal. The occurrence of the abnormal alpha-1 phosphatase accounts for relatively little of the hyperphosphatasemia of biliary disease, the alpha-1 phosphatase being found to have an average value of only 16 per cent of the total serum alkaline phosphatase.

The results of the studies demonstrating the independence of the mobilities in mixtures of isolated fractions of alpha-2 and alpha-1 phosphatase and the failure to find modification of enzyme activity or mobility by addition of various protein fractions lead to the suggestion that the alpha-2 and alpha-1 phosphatase activities represent distinct enzymes, although thus far the difference in electrophoretic mobility has provided the only distinguishing feature.

It is of interest that in a recent study of serum fractionated by a column chromatographic procedure, evidence was presented suggesting the occurrence of two alkaline phosphatases in the serum of a patient with carcinoma metastatic to bone and liver (25). It has also been reported that after experimental biliary obstruction in the rat, an alpha-1 phosphatase is demonstrable by paper

electrophoresis of the serum, which normally contains an alpha-2 and a beta zone alkaline phosphatase (26).

The electrophoretic patterns of bile and of bile-serum mixtures showed that a phosphatase of mobility approximating alpha-1 globulin was present in bile and in some instances it was the major component. Although similarity of electrophoretic mobility is not proof of the identity of the alpha-1 phosphatase of bile and of serum, these observations are at least consistent with the idea that in hyperphosphatasemic biliary disease the serum may contain an appreciable quantity of an alkaline phosphatase which normally represents only a very small fraction of the total phosphatase of serum but which may be the predominant phosphatase in bile. The alpha-1 phosphatase of bile could represent a serum phosphatase (? alpha-2) which during the course of biliary excretion has undergone some transformation resulting in changed electrophoretic properties, or it might be a secretory product of hepatic origin excreted in bile. Regardless of the origin of the alpha-1 biliary phosphatase, the presence of an appreciable fraction of serum phosphatase of similar mobility is compatible with the view that the liver may in some cases contribute to the serum phosphatase activity. Another possible explanation might be that the alpha-2 and alpha-1 phosphatases are normally introduced into the circulation from the tissues of origin, but that the biliary excretion of alpha-1 phosphatase is more rapid or complete than the excretion of alpha-2; the lack of a prominent alpha-1 component in most cases of hepatocellular disease would make the latter explanation less likely. Although the serum alpha-1 phosphatase

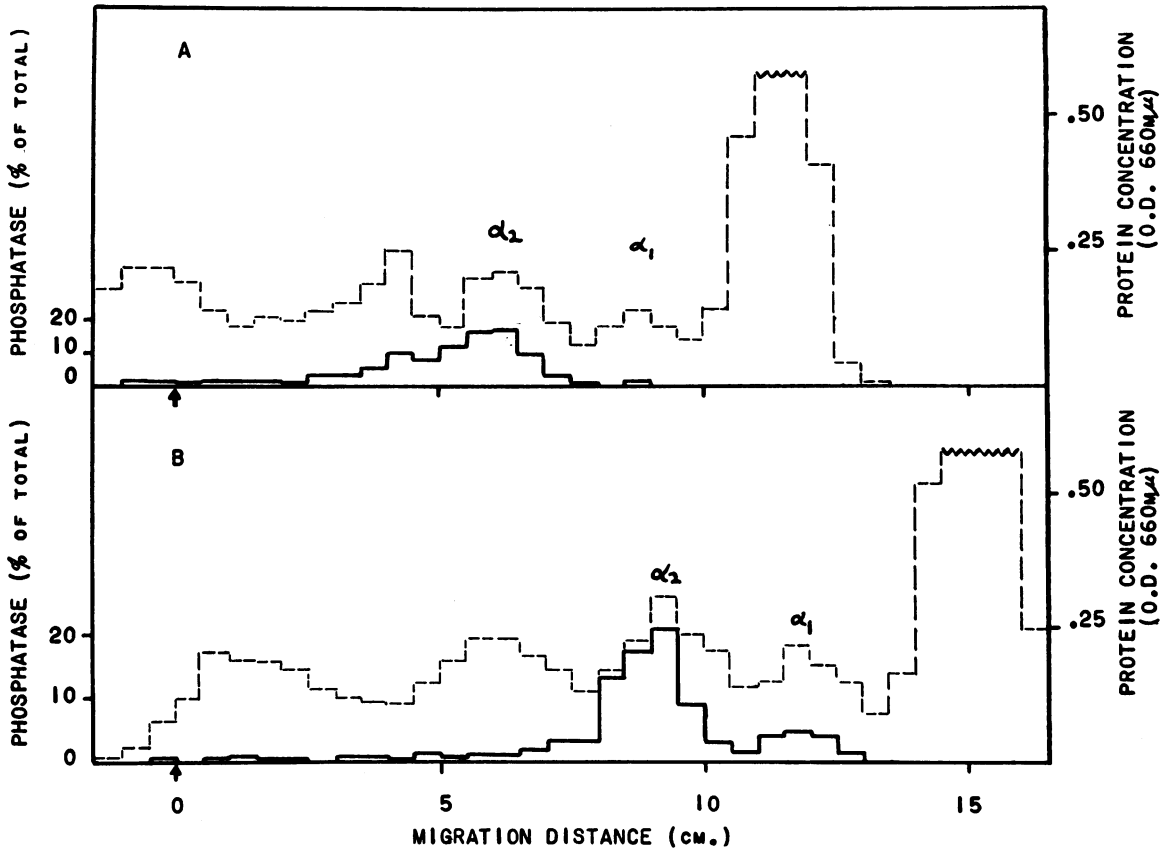

FIG. 8. SERUM ELECTROPHORETIC PATTERNS IN TWO CASES OF METASTATIC BREAST CARCINOMA WITH HYPERPHOSPHATASEMIA

The electrophoreses were not simultaneously performed, and the total traverse in both cases is not the same.
A. Case 51, bony metastases only: five per cent of the activity is in the alpha-1 region.
B. Case 6, metastases to bone, liver and other organs: 19 per cent of the activity is in the alpha-1 region.

was detected most regularly in cases of hepatic cancer, it would seem unlikely that it is of neoplastic origin, both because it was present in cases of cholangitis and noncarcinomatous biliary obstruction and also because it was absent in cases of disseminated cancer of the prostate and breast in which there were no hepatic metastases.

Although there was some overlap in phosphatase patterns between cases of hepatocellular disease and of obstructive or infiltrative liver disease, and although a few of the latter cases had patterns indistinguishable from those of bone cases, the general tendency toward the occurrence of a prominent alpha-1 phosphatase zone in obstructive and infiltrative biliary disease and its absence in the other cases studied suggests a possible clinical usefulness for this method of study in some cases of increased serum alkaline phosphatase of obscure nature. The method might be particularly useful in cases where hepatic metastatic lesions were suspected. Figure 8 shows the serum electrophoretic patterns in two cases of carcinoma of the breast with high serum alkaline phosphatase; in the case in which skeletal metastases were radiologically demonstrable and there was no evidence of hepatic metastases (upper panel) the pattern revealed a prominent alpha-2 phosphatase with some trail in the beta zone, and only a small alpha-1 phosphatase peak (5 per cent of the total), while in the case in which metastases had occurred in liver as well as bone and other tissues, a prominent alpha-1 zone was noted. Patterns obtained in two cases of sarcoidosis and in three cases of disseminated tuberculosis, in all of which the serum alkaline phosphatase activity was moderately increased, revealed appreciable alpha-1 as well as alpha-2 zones of activity; biopsy evidence was not available and, therefore, the suspicion that these patterns suggested hepatic involvement is not proven.

Evaluation of the possible clinical usefulness of this approach would be facilitated by the development of simpler methods for demonstrating abnormally high proportions of alpha-1 phosphatase.

SUMMARY

1. Electrophoresis on starch blocks of sera of increased alkaline phosphatase activity indicated the presence in some cases of two zones of alkaline phosphatase activity with mobilities corresponding to alpha-2 and alpha-1 globulins. The alpha-2 fraction was the larger in all cases. In bone disease the average value for the proportion of alpha-1 phosphatase was 2.2 per cent, while the corresponding value in cases of hepatobiliary infiltrative and obstructive disease was 16.2 per cent. Normal sera and those from cases of hepatocellular disease showed only a small percentage of the alkaline phosphatase activity in the alpha-1 zone, averaging 1.8 and 3.5 per cent, respectively.

2. Alpha-2 and alpha-1 phosphatases were similar in pH optimum, cyanide sensitivity and fluoride insensitivity. The difference in electrophoretic mobilities was preserved in mixtures of the two phosphatases.

3. An appreciable fraction of the alkaline phosphatase of bile had mobility similar to the alpha-1 globulins.

4. Zone electrophoretic study may be clinically useful in some cases of obscure hyperphosphatasemia.

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