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Profiles of Serum Isoenzymes of Alkaline Phosphatase in Hepatobiliary Disorders Using Cellulose Acetate Electrophoresis and Organ-Specific Inhibitors

DOUGLAS P. RHONE, M.S., M.D., FLORENCE M. MIZUNO, B.S., M.T. (ASCP), AND HELENE GIDASPOW, B.S., C (ASCP).

Section of Biochemistry, Department of Pathology, Illinois Masonic Medical Center, Chicago, IL 60657

ABSTRACT

The isoenzymes of alkaline phosphatase (AP) in the serum of 30 normal subjects and 90 patients with common hepatobiliary disorders are distinguished using electrophoresis on cellulose acetate. Physicochemical profiles of these mixtures of serum isoenzymes are presented using thermostability and organ-specific inhibitors (urea, L-phenylalanine, and L-homoarginine). The findings are reviewed and their implications discussed.

Introduction

The alkaline phosphates (orthophosphoric monoester phosphohydrolyases) are a group of enzymes which catalyze the hydrolysis of phosphate esters at an alkaline pH. The individual tissue and serum isoenzymes of alkaline phosphatase (AP) differ in certain physicochemical properties.[3,18,21] The placental isoenzyme is heat stable but the bone isoenzyme is heat labile.[6,20] There is an order of resistance to inactivation by urea,[10] and L-phenylalanine inhibits the intestinal and placental isoenzymes.[4,19] Both bone and liver isoenzymes are inactivated by incubation with L-homoarginine.[5]

The tissue and serum isoenzymes of AP can be separated by electrophoresis using a number of support media.[7,8,13,15,17,21,26] In a cellulose acetate system employing a substrate-gel imprint technique, the organ sources of the serum isoenzymes have been verified by comparing mobilities with extracts of tissue AP and inhibition with heat, urea, and amino acids. There are two liver isoenzyme bands in the serum which migrate in the α_1 and α_2 areas. The bone isoenzyme appears as a wide band in the pre-β area and is entirely inactivated by heat. A narrow, heat stable band in the pre-β area represents the placental isoenzyme in serum. The intestinal isoenzyme of AP migrates in the early γ region and appears as a narrow distinct band.[21]

Using organ-specific inhibitors, heat inactivation and electrophoresis on cellulose acetate, this investigation was carried out to study the isoenzymes of AP present in the sera of patients with hepatobiliary dis-

ease. The possibility that a particular profile may be diagnostic for a specific disease process was also considered. The physicochemical behavior of specific mixtures of AP isoenzymes in serum was also studied and results compared. The ability of physicochemical testing to detect specific mixtures of isoenzymes which were identified by electrophoresis was also investigated.

Materials and Methods

Serum Samples

Fasting blood samples were obtained by venipuncture from laboratory workers, who served as healthy subjects, and from hospitalized patients with elevated levels of AP in the serum. The diagnoses were verified by the usual clinical and laboratory criteria and, in many cases, by liver biopsy or autopsy. After a clot had formed, the serum was separated by centrifugation and stored at −20°. The specimens were defrosted on the day of use and tested immediately.

Isoenzyme Electrophoresis

Electrophoresis on cellulose acetate and development of colored isoenzyme bands were carried out essentially as previously described.[21,22] The concentrations of both beta-naphthyl phosphate and Fast violet-B salt were increased from 60 mg to 120 mg in 30 ml of buffer. Preparation of the substrate-gel plates was timed to coincide with the completion of electrophoresis, so that the strips could be imprinted immediately on the substrate gel. These changes resulted in maximum sensitivity of the staining method to lower levels of AP.

Isoenzyme bands were classified according to their location on electrophoretic strips when compared with the major serum protein fractions: albumin, α_1, α_2, β, and γ-globulins.

Physicochemical Testing

All studies except those of resistance to L-homoarginine were performed using an AP isoenzyme kit, which is commercially available.* Total AP activity was determined by the Hansen modification of the Kind-King method.[9,14] Thermostability was determined by heating the serum in a water bath at 56 ± 0.5° for 15 minutes, immersing it immediately in ice cold water, and measuring the remaining total AP activity. Resistances to urea[10] and L-homoarginine[5] were measured by preincubating 0.1 ml of serum with either 1.5 ml of urea solution (3.6 mM per ml) or 1.0 ml of L-homoarginine solution (0.25 mM per ml) for 10 minutes at pH 8. Resistance to L-phenylalanine was determined by carrying out the enzyme reaction in the presence of 10 mM of L-phenylalanine at pH 10.[4] The percent of total enzyme activity resistant to heat, urea, L-phenylalanine and L-homoarginine was calculated and recorded for each specimen.

Evaluation and Results

Mean values and their standard deviations were calculated for total AP and percent of total AP resistant to inactivation by heat, urea, and L-phenylalanine. Statistical significance of differences between groups was ascertained by means of the Student's *t* test. Electrophoretic patterns obtained for a given group were compared with the results of physicochemical testing.

Results

Healthy Subjects

The group of healthy subjects consisted of 14 male and 16 female laboratory workers. In table I are shown electrophoretic patterns and physicochemical studies obtained from testing these sera. Examples of electrophoretic patterns in this group are shown in figure 1. Only one showed activity due to the liver (α_2) isoenzyme alone. Seventeen healthy subjects showed activity in the (α_2) and the bone (pre-β) areas, while 12 showed an additional band in the

* Harleco, Philadelphia, PA 19143.

TABLE I

PROFILES OF ISOENZYMES OF ALKALINE PHOSPHATASE (AP) IN HEALTHY SUBJECTS [Mean ± SD (Range)]

Electrophoretic Pattern	No.	*Physiocochemical Testing* — *Total AP (KK units/ml)*	*Percent Total Resistant To:* *Heat*	*Urea*	*L-pheynl-alanine*	*L-homo-arginine*
α_2 only (Liver)	1	5.3	10.9	22.3	60.7	40.1
α_2/pre-β (Liver/Bone)	17	4.8 ± 1.4 (3.1 – 6.7)	25.0 ± 11.3 (13.0 – 53.0)	27.3 ± 7.0 (19.1 – 45.0)	59.2 ± 10.0 (41.6 – 88.0)	44.5 ± 8.1 (33.8 – 60.9)
α_2/pre-β/γ (Liver/Bone/Intestine)	12	7.2 ± 2.3 (3.3 – 11.3)	19.4 ± 5.6 (11.8 – 33.0)	34.6 ± 9.5 (24.6 – 59.8)	41.2 ± 9.3 (32.0 – 57.7)	55.5 ± 6.2 (43.4 – 64.9)

γ-globulin area which was intestinal in origin. When compared to the other healthy subjects those containing the additional intestinal isoenzyme showed greater total AP, lower resistances to heat and L-phenylalanine and greater resistances to urea and L-homoarginine. In this population, however, these differences were not statistically significant. There were no statistically significant differences in physicochemical testing between males and females.

The electrophoretic patterns were segregated according to sex and ABO blood groups. Seventy-two percent of those with the α_2/pre-β (liver/bone) electrophoretic pattern were females. The α_2/pre-β/γ (liver/bone/intestine) pattern was most commonly encountered in males with blood group O and B. Two controls with blood group A showed the mixture of three isoenzymes in their sera. The relationship of the electrophoretic pattern to the ABO blood group has been previously described.[24]

PATIENTS WITH HEPATOBILIARY DISEASE

Sera from 90 patients with hepatobiliary diseases were studied. The disorders included are shown in table II where the electrophoretic patterns are related to diagnosis. Examples of electrophoretic patterns are shown in figure 2. Some of the electrophoretic patterns illustrated show AP staining at the origin. This location of AP activity was not seen in the fresh specimens upon which the data in this report are based. Activity at the origin appeared after refreezing and rethawing of the specimen, which are known to alter total AP activity in serum. Intramolecular rearrangement leading to reversal of partial denaturation is one of the mechanisms proposed for this observation.[2] Such intramolecular

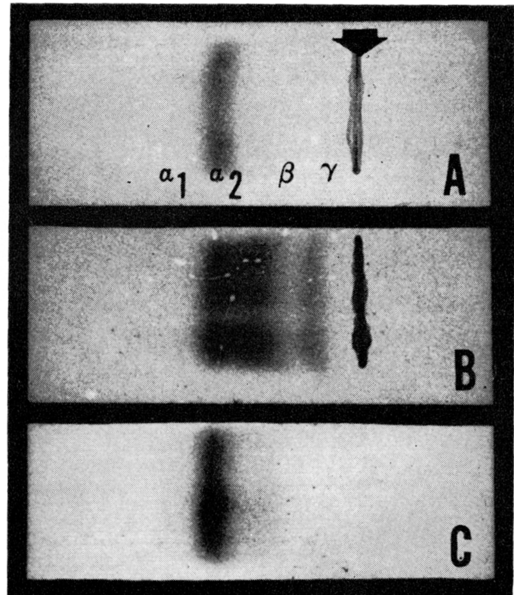

FIGURE 1. Electrophoretic patterns of AP isoenzymes in sera of healthy subjects. An arrow marks the point of application. A. Liver (α_2). B. Liver/Bone/Intestine (α_2/pre-β/γ). C. Liver/Bone (α_2/pre-β).

TABLE II

ELECTROPHORETIC PATTERNS OF SERUM AP ISOENZYMES IN HEPATOBILIARY DISORDERS

Disorder	Total No.	No. of Patients Showing Electrophoretic Pattern*					
		α_2/pre-β	α_2/γ	α_2/pre-β/γ	$\alpha_1\alpha_2$	$\alpha_1\alpha_2$/pre-β	α_2/pre-β/γ
Nutritional cirrhosis	14	0	1	1	6	6	0
Infectious hepatitis	16	10	0	2	2	2	0
Serum hepatitis	9	5	0	1	2	1	0
Metastatic tumors	23	1	0	0	13	8	1
Obstructive jaundice	20	0	0	0	10	9	1
Cholangitis	3	0	0	0	1	1	1
Chronic hepatic congestion	1	0	0	0	1	0	0
Hepatocellular carcinoma	1	0	0	0	1	0	0
Granulomatous hepatitis	1	0	0	0	1	0	0
Totals	88	16	1	4	37	27	3
Percent of total			23.8			76.2	

* α_1 and α_2 = liver; pre-β = bone; γ = intestine.

rearrangement might also cause altered electrophoretic properties and shifting of AP activity from the usual sites to the origin.

Twenty-one sera showed the α_2 liver band in combination with the bone and/or intestinal bands. Eighteen of the 21 sera with this pattern of liver AP were from patients with infectious or serum hepatitis. Two patients were diagnosed to have nutritional cirrhosis, and one patient to have squamous cell carcinoma of the uterine cervix metastatic to the liver.

Sixty-seven sera showed a couplet of liver isoenzymes in the α_1 and α_2 areas. This pattern was seen in 86 percent of those with nutritional cirrhosis, in all but one case of malignant tumor metastatic to liver, and in all of those with obstructive jaundice and cholangitis. One patient with each of the following disorders also showed the α_1 and α_2 liver couplet: chronic hepatic congestion, hepatocellular carcinoma, and granulomatous hepatitis.

Two patients whose information is not shown in table II, one with an adenocarcinoma of the colon metastatic to the liver and another with nutritional cirrhosis diagnosed by liver biopsy, showed heat stable pre-β bands, which were consistent with the Regan isoenzyme seen in malignant tumors and premalignant disorders, i.e., cirrhosis.[16]

In table III are shown the results of physicochemical studies on the groups of sera containing mixtures of isoenzymes. The mean total AP and mean resistance to heat inactivation were highest in the group with only the isoenzyme couplet (α_1 α_2) of liver AP. The latter observation can be explained by the fact that these sera did not contain bone isoenzyme (pre-β). The one serum which contained liver and intestinal AP (α_2/γ) showed greater resistance to urea and L-homoarginine with greater sensitivity to L-phenylalanine. These observations can be explained by the presence of rather large amounts of intestinal isoenzyme. The two sera containing large amounts of the Regan isoenzyme showed considerable resistance to inactivation by heat, urea, and L-homoarginine and marked sensitivity to L-phenylalanine. The other mixtures of liver, bone, and/or intestinal AP showed statistically similar responses to organ-specific inhibitors.

In table IV are shown the results of physicochemical testing of serum AP where the data are segregated by diagnosis. The highest mean level of total AP was seen in

those patients with malignant tumors metastatic to the liver. All other groups showed similar amounts of total AP. Greatest resistances to heat inactivation were shown by those sera from patients with metastatic tumors and hepatocellular carcinoma, which is due to the absence of bone isoenzyme in 50 percent of these sera. The lowest resistance to inactivation by heat was seen in the sera from patients with infectious hepatitis; this can be accounted for by the fact that all of the sixteen patients in this group showed a considerable amount of bone isoenzyme in their sera. The sera from all of the other patients with hepatobiliary disorders showed similar resistances to urea, L-homoarginine, and L-phenylalanine.

Discussion

These data indicate that a number of isoenzymes of AP can appear in various combinations in the sera of normal subjects and of patients with hepatobiliary disorders.

Healthy Subjects

All but one healthy subject showed both liver and bone isoenzymes (α_2, pre-β) in their sera and 40 percent of those studied showed additional activity due to the intestinal isoenzyme (γ). In two previous studies we did not frequently encounter the bone isoenzyme in sera of normal subjects.[21,22] The increased sensitivity of the staining technique used here probably accounts for this difference. Other investigators have described the bone isoenzyme in the sera of most normal subjects.[7,8]

Forty percent of healthy subjects showed the intestinal isoenzyme of AP in their sera. Using starch gel electrophoresis and a staining technique similar to ours, Green et al described the intestinal isoenzyme in about 77 percent of healthy subjects. When polyacrylamide gel electrophoresis with a different staining technique was performed on the same sera the percentage containing the intestinal isoenzyme was increased to 95 percent.[8] These differences have been attributed to decreased specificity of naph-

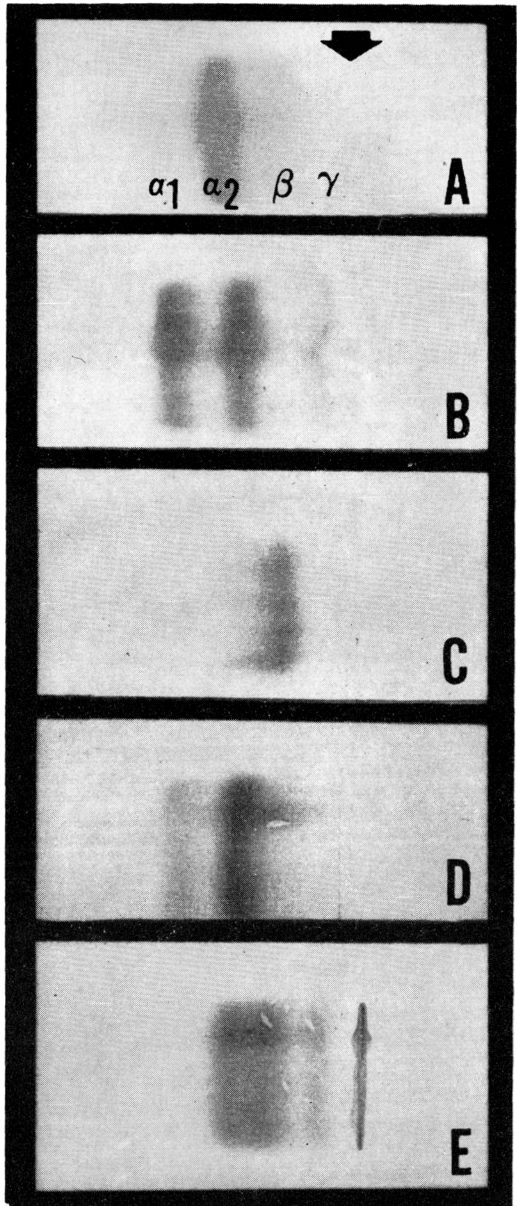

FIGURE 2. Electrophoretic patterns of AP isoenzymes in sera of patients with hepatobiliary disease. A. Liver/Bone (α_2/pre-β). B. Liver/Intestine (α_1 α_2/γ). C. Liver/Regan Isoenzyme (α_2/pre-β/heat stable). D. Liver/Bone (α_1 α_2/pre-β). E. Liver/Bone/Intestine (α_2/pre-β/γ).

TABLE III

PHYSIOCHEMICAL PROFILES OF MIXTURES OF AP ISOENZYMES IN HEPATOBILIARY DISEASE [Mean ± SD (Range)]

			Percent Total Resistant to Organ-Specific Inhibitors			
*Electrophoretic Pattern**	*No.*	*Total AP (KK units/ml)*	*Heat*	*Urea*	*L-phenyl-alanine*	*L-homo-arginine*
$\alpha_1\alpha_2$	37	30.8 ± 18.4 (9.9 – 94)	32.2 ± 14.5 (10 – 61.6)	31.6 ± 9.6 (11.0 – 49.4)	66.1 ± 9.0 (51.3 – 100.0)	23.5 ± 4.4 (17.3 – 39.8)
$\alpha_1\alpha_2$/pre-β	27	27.5 ± 10.0 (19.7 – 55.9)	28.0 ± 38.9 (7.8 – 38.9)	31.6 ± 7.6 (10.1 – 49.3)	68.6 ± 4.4 (61.1 – 76.3)	24.2 ± 7.4 (13.8 – 45.6)
$\alpha_1\alpha_2$/pre-β/γ	3	29.8 (28.4 – 31.2)	24.9 (17.8 – 31.9)	31.7 (28.8 – 34.5)	67.2 (64.9 – 69.4)	22.0 (17.1 – 27.0)
α_2/pre-β	16	22.8 ± 9.3 (13.5 – 39.5)	16.4 ± 6.7 (6.0 – 28.1)	28.1 ± 8.1 (10.0 – 43.2)	69.8 ± 5.7 (63.0 – 81.7)	24.9 ± 5.7 (15.1 – 33.9)
α_2/pre-β/γ	4	17.6 ± 4.5) (11.3 – 23.4)	15.0 ± 1.3 (13.6 – 16.4)	27.1 ± 5.0 (20.0 – 33.9)	62.0 ± 5.0 (54.5 – 67.3)	24.8 ± 11.4 (10.7 – 39.5)
α_2/γ	1	13.9	13.6	36.9	62.7	28.5
α_2/pre-β†	2	44.7 (33.6 – 55.7)	81.3 (68 – 94.7)	65.4 (59.5 – 71.2)	20.7 (19.1 – 22.4)	70.4 (65.4 – 75.4)

* α_1 and α_2 = liver; pre-β = bone; pre-β† = Regan; γ = intestine.

thyl phosphate for intestinal AP[25] and possibly imperfect staining by the coupling agent, Fast Blue BB.[8] Using electrophoresis on cellulose acetate, Fritsche described the occurrence of intestinal AP in normal sera as infrequent.[7]

The α_1 (liver) band which was seen in the sera of patients with hepatobiliary dis-

TABLE IV

PHYSICOCHEMICAL PROFILES OF SERUM AP ISOENZYMES IN SPECIFIC HEPATOBILIARY DISEASES [Mean ± SD (Range)]

			Percent Total Resistant to:			
Disorder	*No.*	*Total AP (KK units/ml)*	*Heat*	*Urea*	*L-homo-arginine*	*L-phenyl-alanine*
Metastatic tumors	23	33.7 ± 14.1 (9.2 – 50.1)	31.8 ± 16.6 (6.6 – 68.0)	33.0 ± 9.8 (10 – 49.4)	25.9 ± 10.3 (15.8 – 68.8)	63.7 ± 12.2 (22.4 – 81.7)
Infectious hepatitis	16	23.3 ± 10.4 (13.2 – 55.9)	17.6 ± 7.3 (7.8 – 27.7)	25.7 ± 6.3 (13.7 – 31.6)	25.2 ± 9.2 (10.7 – 45.6)	67.9 ± 5.5 (61.2 – 76.3)
Serum hepatitis	9	25.4 ± 11.0 (11.3 – 39.8)	26.3 ± 15.5 (8.6 – 36.0)	32.1 ± 4.7 (26.6 – 43.2)	25.3 ± 6.6 (17.2 – 36.1)	68.5 ± 3.9 (64 – 76.6)
Obstructive jaundice	20	25.2 ± 9.4 (13.6 – 42.8)	22.9 ± 12.3 (7.8 – 57.3)	32.3 ± 8.9 (14.2 – 49.3)	22.9 ± 6.1 (18.3 – 45.6)	68.4 ± 3.9 (61.7 – 76.3)
Nutritional cirrhosis	14	23.3 ± 12.8 (7.7 – 44.0)	26.7 ± 13.0 (13.6 – 56.1)	30.8 ± 7.2 (11.0 – 39.6)	24.4 ± 5.5 (17.2 – 34.5)	67.8 ± 10.0 (60.8 – 100)
Cholangitis	3	24.4 ± 5.0 (19.7 – 31.2)	19.3 ± 3.4 (16 – 24.0)	33.2 ± 3.6 (28.3 – 36.8)	22.6 ± 4.4 (18.2 – 27.0)	66.7 ± 1.6 (64.9 – 69.1)
Hepatocellular carcinoma	1	28.2	39.4	23.3	26.2	55.9

orders was not seen in any healthy subjects. In our previous work it was also not demonstrated in sera from newborn babies, pregnant females, and those with bone disease.[22] Its presence, therefore, is an abnormal finding and seems to indicate disease of the hepatobiliary system.

Although not statistically significant in this population of healthy subjects, those sera containing the intestinal isoenzyme did show higher total AP, greater resistances to urea and L-homoarginine, and greater sensitivity to L-phenylalanine. These findings are consistent with the influence of the intestinal isoenzyme on the physicochemical behavior of mixtures of isoenzymes of AP.[22] It is evident that the use of physicochemical inhibitors alone is not capable of differentiating the exact mixtures of AP isoenzymes present in the sera of healthy subjects. AP isoenzyme electrophoresis is able to identify the individual components clearly.

Hepatobiliary Disorders

In this study two distinct electrophoretic patterns of liver AP were encountered in sera of patients with hepatobiliary disease. In 22.8 percent of those studied only an α_2 band appeared, while the remaining 77.2 percent showed a couplet of liver isoenzymes (α_1 and α_2). This general distribution of the two isoenzyme patterns is consistent with that previously described in a preliminary investigation.[22]

When compared to the occurrence of the liver isoenzyme couplet, the isolated α_2 pattern of liver AP was most commonly seen in the sera of those patients with serum and infectious hepatitis. Seventy-two percent of patients with hepatitis showed the isolated α_2 pattern of liver AP. Whether or not this finding has predictive value in the laboratory work-up of patients with hepatobiliary disease will require further study.

The couplet of isoenzymes of liver AP has been described in sera from a few patients with hepatobiliary disease using electrophoresis on starch-gel[13,23] and cellulose acetate.[7,15,21,22] Its presence has been noted in a few patients with nutritional cirrhosis, chronic passive hepatic congestion, fatty metamorphosis of the liver, obstructive jaundice, malignant tumors metastatic to the liver, infectious hepatitis, and biliary cirrhosis.[7,13,15,21,22,23]

In this study, the α_1, α_2 couplet of liver isoenzymes was encountered in the majority of patients with all hepatobiliary disorders except infectious and serum hepatitis.

Using electrophoresis on polyacrylamide[12] and agar-gels[11] a couplet of fast and slow moving liver isoenzymes has been described in the sera of rats with experimental biliary obstruction, metastatic tumors and toxic hepatitis. The two bands seen in the experimental animal may represent the same couplet of liver AP isoenzymes described here in man.

Definitive characterization of these liver isoenzyme bands requires further study and is underway in our laboratory. Scanning of electrophoretograms with development of quantitative data, separation and purification of the individual bands and physicochemical testing, as well as comparison of AP isoenzyme patterns with other measurements of liver function may be helpful. Preliminary studies have shown that the α_1 band is somewhat more sensitive to inhibition by L-phenylalanine, while both bands are inhibited similarly by heat, urea, and L-homoarginine.

The bone isoenzyme was detected in 55 percent of the sera from patients with hepatobiliary disease and in 97 percent of healthy controls. This finding represents another observation contrary to the regurgitation theory for explaining elevations of AP in the serum in hepatobiliary disease.

The intestinal isoenzyme has been reported in 33 percent of those with chronic liver disease (nutritional cirrhosis and chronic active hepatitis) with blood groups O, B and A. It has not been seen in those with viral hepatitis, metastatic carcinomas, and obstructive jaundice.[1] In this study the intestinal isoenzyme was present in the sera of 8 percent of those studied and in patients with both chronic and acute hepatobiliary diseases. As previously mentioned, the diminished substrate specificity of naphthyl phosphate for intestinal AP[25] may explain this discrepancy.

Sera from 26 patients with malignant tumors metastatic only to the liver were studied. Only one serum in this group showed the Regan isoenzyme detectable by physicochemical testing and electrophoresis. Other sera may have contained this isoenzyme in such small quantities, however, that the use of immunodiffusion methods would have been required to detect it.[16]

Trends in physicochemical behavior are apparent in the data presented here to demonstrate that a single isoenzyme of AP retains its individual characteristics when mixed in the serum with other isoenzymes. The magnitude with which the physicochemical characteristics are expressed appear to be related to the concentrations of each isoenzyme in the mixture. This observation is best supported by the findings in the two sera which contained large amounts of the Regan isoenzyme in combination with intestinal AP. The sera which contained large amounts of bone isoenzyme in the presence of liver AP showed greater sensitivity to heat than those which contained liver alone. Sera from healthy subjects which contained the intestinal isoenzyme in combination with liver and bone AP showed greater sensitivity to heat and phenylalanine and resistance to urea and L-homoarginine. Except in the case of large amounts of Regan isoenzyme, however, these trends did not result in statistically significant figures.

These findings demonstrate that, in most cases, physicochemical testing alone is not capable of clearly differentiating specific mixtures of AP isoenzymes present in the sera of healthy subjects and patients with hepatobiliary disease. Electrophoresis, on the other hand, is capable of identifying the individual components of a mixture of AP isoenzymes in the serum. If quantitative data are necessary, densitometric scanning is probably the best method to use and an attempt is being made to develop such a procedure. It is also evident that a particular physicochemical profile using heat inactivation and organ-specific inhibitors is not peculiar to any hepatobiliary disorder investigated here.

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