CCA 5225

HEAT STABILITY OF HUMAN SERUM ALKALINE PHOSPHATASE IN BONE AND LIVER DISEASES

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(Received May 31, 1972)

SUMMARY

Heat stability studies on human serum alkaline phosphatase have been used as a means of identifying the organ origin of the enzyme contributing to the total enzyme activity in the serum. The present investigation was undertaken to evaluate the usefulness of these studies for the differential diagnosis of bone and liver diseases, particularly when elevated serum alkaline phosphatase levels were not associated with abnormal results of other biochemical tests for liver function.

Bone alkaline phosphatase was found to be more heat-labile than liver alkaline phosphatase and the two can be differentiated by pre-incubating the serum at 56° for 10 min before enzyme assay. After heat treatment, serum alkaline phosphatase levels decreased to less than 12% of the original activity in patients with bone diseases and to 20–40% of the original activity in patients with liver diseases.

INTRODUCTION

The question of whether a raised serum alkaline phosphatase (AP) activity is due to disease of bone or of liver has stimulated a great deal of interest in the laboratory identification of human organ-specific alkaline phosphatases[1]. Many attempts have been made to develop simple and reliable tests for the separation of bone and liver AP in serum.

Although electrophoretic techniques using a variety of supporting media have been used widely[2–6], conflicting results have been reported. Posen[7] using starch and cellulose acetate concluded that the migration rates of bone and liver alkaline phosphatase are so similar that electrophoresis is not a suitable diagnostic method for their differentiation. Immunological methods have been used with some success[8–10], but there are technical difficulties associated with these methods. Furthermore, methods involving the use of pure antigens are expensive for the routine laboratory. Inhibition studies have been attempted by some workers[11,12], but so far these have not been successful for the differentiation of bone and liver AP. The technique of heat inactivation[13–16] appears to be the most promising for the differentiation of bone

and liver enzyme. The present study was undertaken to evaluate the heat inactivation technique for such differentiation. The heat inactivation patterns of bone, liver and bile extracts and sera from subjects with normal and elevated alkaline phosphatase activity were studied.

MATERIALS AND METHODS

Autopsy specimens of rib bone, liver and bile were obtained from human subjects whose cause of death was either traffic accident or myocardial infarction. The tissues were homogenised in 0.9% saline and centrifuged. The supernatants were suitably diluted with saline for enzyme assays.

Sera from the following groups of subjects were studied: blood donors and hospital patients with normal serum AP activities and showing no evidence of either bone or liver diseases, patients with various bone diseases and with raised serum AP activities, patients with evidence of hepatobiliary disorders and with raised serum AP activities, pregnant patients with slightly elevated serum AP activities and children with neonatal hepatitis.

Serum and tissue extracts were heated in a water bath controlled at 56.0° ± 0.2° for 10 min, then cooled in ice bath immediately. One tenth milliliter aliquots of heated and unheated serum extract were incubated for exactly 10 min at 37° with 2 ml of buffered substrate consisting of 0.05 M $NaHCO_3$–Na_2CO_3 buffer, 5 mM Mg^{2+} and 2 mM *p*-nitrophenylphosphate. The reaction was stopped by adding 1.0 ml of 0.4 M sodium hydroxide containing 0.02 M EDTA to prevent the precipitation of $Mg(OH)_2$. The absorbances of the solutions were measured at 400 nm and enzyme activities expressed as King–Armstrong (K.A.) units per 100 ml. In normal and pregnancy cases where AP activity is low the reaction mixture was incubated for 20 min.

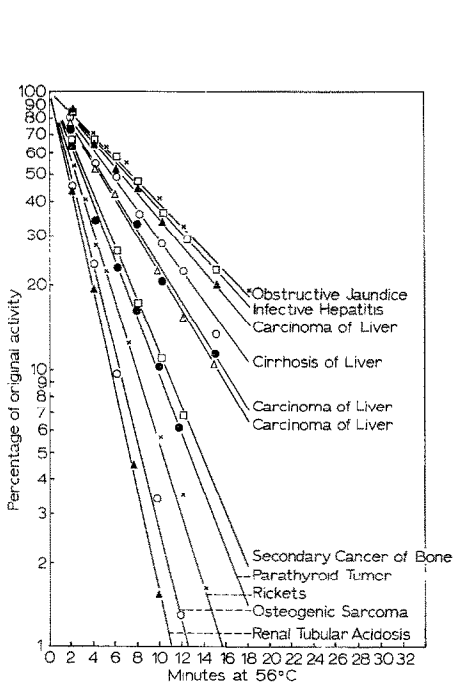
Heat inactivation curves were performed on extracts of liver, bone, bile and serum of patients with evidence of hepatobiliary disorders or bone diseases. As the study would be applied to sera of patients, the effect of serum on the heat inactivation pattern of the tissue APs was investigated. The tissue extracts in 0.9% saline were mixed in various proportions with pooled normal serum which had been preheated at 56° for 1 h to inactivate the serum AP. The inactivated pooled serum has AP activity of 0.5 K.A. units.

Five sera of patients with liver diseases were inactivated for 10 min at various temperatures to test the effect of temperature on enzyme activity.

RESULTS AND DISCUSSIONS

Fig. 1 shows some of the typical results of heat inactivation of AP in two groups of sera. Sera AP of patients with bone diseases are distinctly inactivated at a faster rate than sera AP of patients with hepatobiliary diseases. There is a clear separation of bone and liver cases.

Fig. 2 shows serum AP activity remaining after 10-min incubation at 56°. Serum AP from patients with bone diseases showed less than 12% of the original activity, while sera from patients with hepatobiliary diseases gave values of between 20% and 40%. AP in sera from pregnant women were particularly heat stable and can be distinguished from the other groups. This also means that it would be difficult to



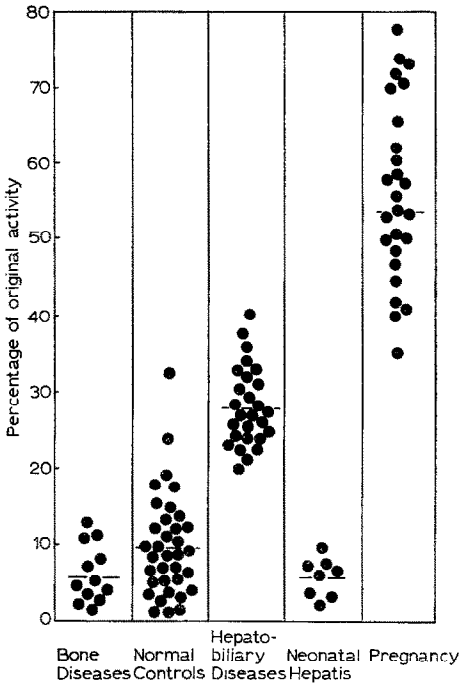


Fig. 1. Heat inactivation of serum alkaline phosphatase from patients with hepato-biliary and bone diseases.

Fig. 2. Serum alkaline phosphatase activity of sera heated at 56° for 10 min. The solid lines represent the means.

differentiate bone and liver AP in sera from pregnant women. Results on normal sera overlap considerably with those on sera from patients with bone diseases and to a lesser extent with those on sera from patients with hepatobiliary disorders.

Fig. 3 shows the AP results of all sera before and after heat inactivation. The normal and abnormal groups are shown to be distributed within well defined areas. In children with neonatal hepatitis, the pattern of heat inactivation differs from that shown by adults with various forms of liver disorder. The serum AP activity appears to be of bone origin.

Table I shows the diagnosis, liver function tests results and results of AP inactivation of patients with bone and liver diseases. Patients with bone diseases who had elevated AP values but otherwise normal liver function have low percentage of original AP activity after heat inactivation. Sera AP of patients with hepatobiliary diseases have much higher values after heat inactivation. Hence a combination of absolute serum AP and the percentage of AP remaining after 10 min heat inactivation is a simple and useful method for determining whether an elevated enzyme activity in serum is due to bone or hepatobiliary disease. However, this method is not suitable for the differentiation of neonatal hepatitis from bone disease, as serum AP in these conditions shows similar characteristics towards heat inactivation.

Figs. 4 and 5 show the results of typical heat inactivation patterns of bone/liver and of bile respectively. The pattern of heat inactivation at 56° for bone and liver

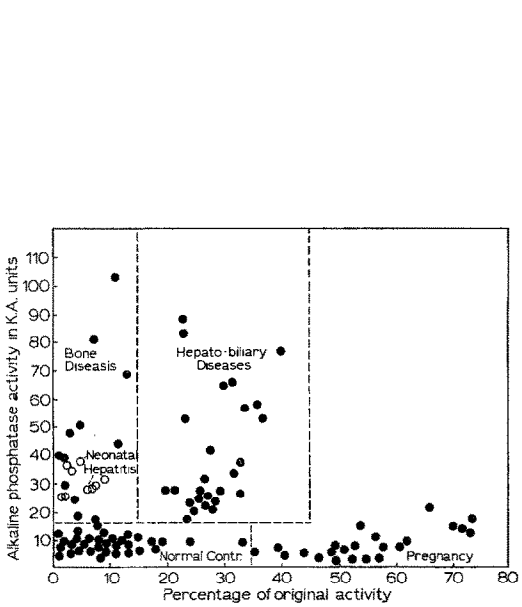
TABLE I

VALUES FROM PATIENTS WITH PREDOMINANTLY BONE OF HEPATO-BILIARY DISEASES

Diagnosis	*Bilirubin mg%*	*A/G ratio*	*SGPT (King's units)*	*AP activity*		
				heat treatment K.A. units		*% of original AP activity*
				before	*after*	
Hepato-biliary diseases						
Cirrhosis of liver	3.3	2.5/4.9	194	20.0	5.4	27.0
Cirrhosis of liver	1.2	2.1/4.3	127	26.6	5.4	20.3
Infective hepatitis	8.7	3.8/3.1	>400	26.0	8.6	33.1
Infective hepatitis	8.6	3.8/3.9	>400	22.2	5.2	23.4
Infective hepatitis	5.0	3.3/3.9	>400	16.4	3.8	23.2
Infective hepatitis	3.9	3.2/3.5	>400	21.7	5.7	26.3
Viral hepatitis	0.6	3.9/3.6	>400	43.0	15.5	36.0
Obstructive jaundice	11.8	2.7/4.3	200	25.5	7.5	29.4
Obstructive jaundice	23.6	2.4/4.1	360	83.2	32.8	39.4
Obstructive jaundice	26.0	2.8/4.2	200	63.6	19.2	30.2
Obstructive jaundice	19.1	2.6/3.6	>400	52.0	12.2	23.5
Ac. Cholecystitis	5.8	2.8/3.6	350	27.1	7.4	27.3
Ac. Cholecystitis	2.8	3.3/4.3	230	51.8	19.2	37.1
Ac. Cholecystitis	2.7	2.8/4.1	>400	21.7	5.9	27.2
Ac. Cholecystitis	3.9	3.3/4.0	>400	32.9	10.9	32.2
Ac. Cholecystitis	1.4	3.0/3.9	>400	19.5	4.8	24.6
Ac. Cholecystitis	0.9	3.4/3.1	>400	21.6	5.2	24.1
Cholecystitis	12.1	2.4/5.1	>400	41.0	11.4	27.8
Carcinoma of liver	1.4	3.2/3.5	212	87.9	19.9	22.6
Carcinoma of liver	2.8	2.4/3.7	220	31.0	8.3	26.9
Carcinoma of liver	2.9	2.3/2.8	170	28.2	5.9	20.9
Carcinoma of liver	2.2	2.2/4.7	355	81.9	18.6	22.7
Carcinoma of liver	4.0	2.7/5.3	230	37.4	12.4	33.2
Carcinoma of liver	17.7	3.0/3.0	375	25.4	6.8	26.8
Bone diseases						
Rickets	0.5	—	102	74.6	5.2	5.7
Parathyroid tumour	0.6	3.6/3.0	98	95.0	10.5	9.4
Osteogenic sarcoma	0.4	—	110	23.1	1.0	3.5
Renal tubular acidosis	0.2	3.5/2.9	78	21.0	0.1	1.5
Bone secondaries	0.1	3.0/3.7	75	46.8	5.2	11.1
Renal tubular acidosis	0.4	—	—	47.2	1.4	3.0
Multiple myeloma	0.3	3.1/7.1	95	27.0	0.6	12.2
Neonatal hepatitis						
	9.7	3.7/1.8	340	27.6	1.6	5.8
	7.2	4.3/1.7	>400	35.3	0.8	2.8
	9.7	4.6/2.5	>400	34.8	1.2	3.5
	7.0	4.1/1.6	>400	25.2	0.5	2.0
	9.0			27.8	1.2	7.2
	2.8			28.0	2.0	7.1
	6.6			28.0	1.8	6.5
	10.8			31.4	3.0	9.5

Note: Serum isocitrate dehydrogenase of all cases of neonatal hepatitis is greater than 1000 units.

Normal values:	
Serum bilirubin	$\leqslant$ 1 mg%
Serum albumin (A)	3.5–5.0 g%
Serum globulin (G)	2.1–3.5 g%
Alanine aminotransferase (SGPT)	35–125 King's units
Serum alkaline phosphatase	3.0–13.0 K.A. units
Isocitrate dehydrogenase (ICD)	15–480 units



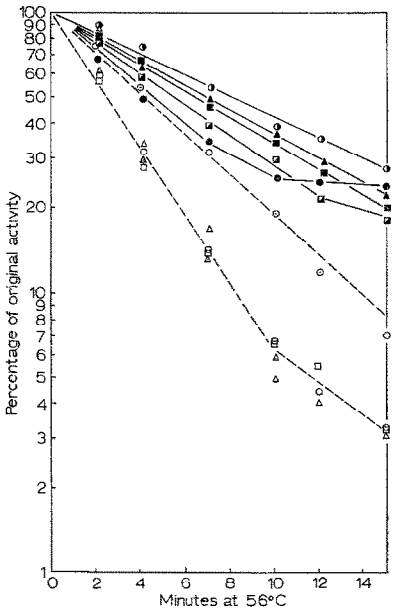
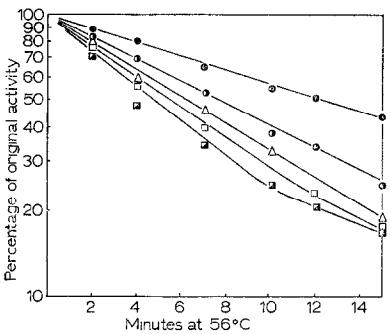


Fig. 3. Distribution of serum alkaline phosphatase activity after heat inactivation in relation to its original activity.

Fig. 4. Heat inactivation of alkaline phosphatase from bone and liver extracts and the effect of adding various amounts of serum to these extracts. Solid lines represent liver and dotted lines bone extract. ○●, pure extracts; ○◑, 3 parts extract, 1 part serum; △▲, 2 parts extract, 1 part serum; □■, 1 part extract, 1 part serum; △◪, 1 part extract, 2 parts serum.

extract is similar. After incubating for 10 min 15–20% of bone AP and liver AP remained. This similar behaviour of bone and liver AP towards heat inactivation is in agreement with the work of Posen *et al.*[13]. However, in Posen's work the actual values differ from the present study in that after heat inactivation 20–40% of original activity was obtained for bone extract and 35% obtained for liver extract. In our study, values varying between 57 and 70% were obtained for bile, while a value of 75% was observed by Posen *et al.*

The addition of serum to tissue extracts markedly altered the heat inactivation pattern of the tissue AP. Varying quantities of serum lower the bone AP heat stability to the same degree (Fig. 4). The AP activity remaining after 10 min heat inactivation was only 7% in the presence of serum as compared to 18% in the absence of serum. However, of the 4 liver extracts studied, 3 showed increase in stability, while one demonstrated a decrease in stability in the presence of serum. Further study is being carried out to investigate the variable effect of serum on liver extract. Addition of varying amounts of serum to bile reduced the bile AP heat stability from 57% to 25–40% of original activity (Fig. 5). This study demonstrates that AP of tissue extracts responds differently to heat inactivation in the presence and absence of blood serum, indicating that caution is needed in the interpretation of comparative study data on AP behaviour when pure extracts and serum are used. In fact it may be concluded that in any investigation of an enzyme in tissue extracts with the aim of establishing assay conditions for the serum enzyme it is essential to investigate the effect of serum on the extract.



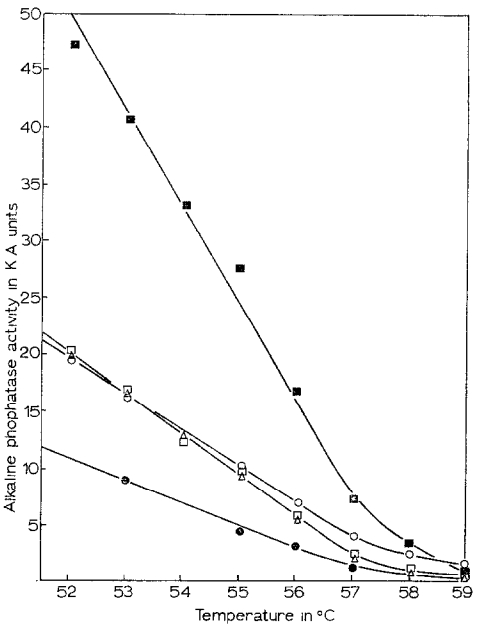


Fig. 5. Heat inactivation of bile alkaline phosphatase and the effect of adding various amounts of serum. ●, bile; ◑ 3 parts bile 1 part serum; △, 2 parts bile 1 part serum; □, 1 part bile 1 part serum ◪, 1 part bile 2 parts serum.

Fig. 6. Serum alkaline phosphatase activity after heat inactivation for 10 min at various temperatures. Each symbol represents a different serum.

Fig. 6 shows the effect of temperature on the stability of AP in five sera from patients with hepatobiliary disease. It is noted that a small change in temperature, even to the extent of $\pm$ 0.5°, results in a marked alteration in the heat stability of serum AP. Therefore in all heat inactivation studies on serum AP, the temperature of the incubation bath has to be well controlled.

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