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Serum-Cholinesterase Activity in Health and in Liver Disease

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It has frequently been reported that abnormally low values of serum cholinesterase are found in the sera of patients suffering from liver disease. Although there is a considerable variation in the cholinesterase activity in normal sera (Callaway, Davies & Rutland, 1951), as assayed by the manometric method of Ammon (1933), abnormally low activities have been found in liver disease which are statistically significant (McArdle, 1940; Wilson, Calvert & Geoghegan, 1952). McArdle (1940) used acetylcholine as the substrate for the enzyme estimation and Wilson *et al.* (1952) used benzoylcholine.

Human-serum cholinesterase is now a fairly well described enzyme although its physiological role is not understood. The optimum pH for the hydrolysis of acetylcholine by the enzyme was found by Glick (1937) to lie between pH 8.4 and 8.5 at 25°. The substrate-specificity pattern of the enzyme has been extensively studied. Augustinsson (1949) found that butyrylcholine was the most rapidly hydrolysed substrate when a series of acyl choline esters were compared. Adams & Whittaker (1949) examined a series of alkyl esters and found that the maximum rates of hydrolysis were obtained with the esters of 3:3-dimethylbutanol, the carbon analogue of choline. Electrophoretic and fractionation studies reported by Surgenor *et al.* (1949); Surgenor & Ellis (1954); Kekwick, Mackay & Martin (1953) and Kekwick (1955) have shown that the enzyme is probably an α -globulin.

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In view of this fairly extensive knowledge of the nature of the enzyme in normal serum, and because the activity of the enzyme was known to be decreased in liver disease, it was thought that a comparison of the properties of the enzyme found in the serum of persons having liver disease with those of the enzyme of normal serum might reveal some differences.

MATERIALS AND METHODS

Enzyme assay. The enzyme assays were carried out in the Warburg apparatus at 37° in a medium containing 0.023 M-sodium bicarbonate and 0.15 M-sodium chloride at pH 7.4, according to the method of Ammon (1933). The gas phase was $N_2 + CO_2$ (95:5). The substrates used were acetylcholine chloride and benzoylcholine chloride, both supplied by British Drug Houses Ltd., and the perchlorates of acetylcholine, propionylcholine and butyrylcholine, which were synthesized by the method of C. A. Lewis (personal communication). The concentration of acetylcholine, butyrylcholine and propionylcholine used was 0.06 M and that of benzoylcholine was 0.12 M. The concentrations were twice those found necessary by Mendel, Mundell & Rudney (1943) to give a maximal rate of hydrolysis of the substrate by the enzyme. These concentrations were also used in the study of the effect of temperature on the enzyme, in order to compensate for any change in the Michaelis constant with temperature. Estimations of enzyme activities at temperatures higher than 30° were obtained from measurements made during the first 30 min. after mixing serum and substrate; below this temperature it was necessary to prolong the period over which measurements were made owing to the lower reaction rates involved. The reactions proceeded linearly with time over the period of measurement in all cases, indicating that the enzyme was saturated with sub-

strate. The enzyme activity was measured as $\mu\text{l. of CO}_2$ evolved/hr./ml. of serum; in order to correct for any apparent change in the enzyme activity as a result of haemodilution, the enzyme activities of pathological sera have also been recorded as $\mu\text{l. of CO}_2$ evolved/hr./mg. of serum-protein nitrogen.

Estimation of protein nitrogen. Determinations of total nitrogen were carried out on serum by the micro-Kjeldahl procedure; to obtain protein nitrogen the blood urea was estimated and the corresponding nitrogen content was deducted from the total nitrogen found.

Determination of pH. The pH of the solutions used was measured by means of a glass electrode, 0.05 M-potassium hydrogen phthalate being used as standard at pH 4.0.

Sera studied. Normal sera were obtained from healthy persons aged between 18 and 50 years.

Pathological sera were obtained from patients suffering from liver disease classified in four groups. The group 'chronic liver damage' comprised patients with advanced hepatic insufficiency; a second group comprised patients whose conditions subsequently proved fatal, and in whom the diagnosis was confirmed at autopsy. The third group, those patients classified as having 'acute liver damage', were all in the acute phase of liver insufficiency, with fever, overt jaundice, a raised serum bilirubin, positive serum-thymol test and urobilinogen in the urine. Some of the patients whose sera were again assayed during the subsequent period of convalescence provided material for the fourth group, described as 'convalescent from acute liver damage'.

Some five sera from cases of chronic liver damage were selected for the specificity study; all of them had been observed for not less than 12 months and on four of them (1, 2, 3 and 4) the clinical diagnosis was confirmed at autopsy within 6 months of the analysis. The diagnosis of the remaining patient (5) was confirmed by biopsy.

Sera from six patients having acute liver damage were used for the specificity study: of these, three (6, 7 and 8) were examined within 14 days of the onset of jaundice, three (9, 10 and 11) were examined during a relapse occurring 4 weeks after the initial attack of infective hepatitis. Patient 10 had been confined to bed sick 1 year previously at a time when there was known to be an outbreak of hepatitis in his unit. Although at that time he had not become jaundiced it is possible that what was then regarded as 'abdominal flu' may have been a mild attack of infective hepatitis.

Sera were, if possible, assayed for activity as soon as they

were taken, or were, if necessary, stored at 2° for a few days. It was found that cholinesterase activity of sera remained constant after storage for 300 days at 2° without any special precautions to maintain sterility.

RESULTS

Serum-cholinesterase activities in normal sera and sera from patients with liver damage

A comparison of serum-cholinesterase activities in liver disease with those found in normal persons was made initially with acetyl- and benzoylcholine chloride as substrates, to provide a basis for a more extensive investigation of the behaviour of the enzyme in liver disease.

The results of the assays of enzyme activity with the chloride of acetyl- and benzoylcholine are shown in Table 1. In sera from both of the groups of patients with chronic liver damage the mean activity against either substrate was markedly lower than that of normal sera. All the sera in these two groups had enzyme activities, which, when expressed in $\mu\text{l. of CO}_2$ evolved/hr./ml. of serum, were below the normal range, but when these activities were expressed in terms of $\mu\text{l. of CO}_2$ evolved/hr./mg. of protein nitrogen some of them fell just within the normal range. The range of activities found in the sera from the group of persons suffering from acute liver damage was different. Although the mean activity against both the substrates was below that found for normal sera, 12 of the 17 sera studied had activities within the normal range. It is possible that these levels actually represented lowered values for individuals normally having rather a high serum-cholinesterase activity, in view of the considerable range of levels found in normal sera.

Patients convalescent from acute liver damage had serum-cholinesterase activities which were all within the range for normal sera. In all the sera studied, both normal and pathological, the rate of hydrolysis of benzoylcholine was always between 35 and 40 % of that of acetylcholine.

Table 1. *Hydrolysis of acetylcholine and benzoylcholine by normal and pathological human sera*

Group	No. in group	Acetylcholine				Benzoylcholine			
		CO ₂ evolved ($\mu\text{l./hr./ml.}$)		CO ₂ evolved ($\mu\text{l./mg. of N/hr.}$)		CO ₂ evolved ($\mu\text{l./hr./ml.}$)		CO ₂ evolved ($\mu\text{l./mg. of N/hr.}$)	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
Normal	20	5420	3180-7300	417	280-550	2050	1240-3100	186	109-250
Chronic liver damage	10	2150	1200-3050	208	136-352	815	500-1200	81	54-160
Chronic liver damage followed by death within 6 months	4	1647	500-3000	139	54-236	730	200-1500	54	25-91
Acute liver damage	17	4052	1130-6220	329	176-503	1612	515-3100	132	92-186
Convalescent from acute liver damage	5	5600	3800-7300	446	317-550	2073	1090-2180	165	91-212

*Substrate-specificity pattern of
serum cholinesterase*

Sera obtained from five patients having chronic liver damage which subsequently proved fatal, and from six patients having acute liver damage, were selected for comparison with four normal sera with respect to their specificity towards aliphatic choline esters. The results of assays of the activities of sera against the perchlorates of acetyl-, propionyl- and butyryl-choline are shown in the form of a specificity pattern in Fig. 1. The behaviour of four normal sera towards the three substrates was similar to that described by Augustinsson (1949) for a purified preparation of serum cholinesterase, butyrylcholine being hydrolysed the most rapidly. The relative rates of hydrolysis did show slight individual variations.

Sera from patients having chronic liver damage behaved quite differently; propionylcholine was always hydrolysed most rapidly.

In the group of sera from acute liver damage only two out of six showed this abnormal preference for propionylcholine, but one of these sera had an activity against acetylcholine which was within the normal range. Of the remaining sera in the group two hydrolysed propionyl- and butyryl-choline at the same rate, and one serum had a normal specificity pattern despite a very low activity against acetylcholine.

Fig. 1. Specificity of normal serum cholinesterase towards acetyl-, propionyl- and butyryl-choline. (a) Normal human serum: ○, NM; ●, RJK; □, CBC; △, RK. (b) Sera from patients (identified by numbers) with acute liver damage: ○, 6; △, 7; ▲, 8; □, 9; ●, 10; ■, 11. (c) Sera from patient (identified by numbers) with chronic liver damage: ○, 1; △, 2; □, 3; ●, 4; ▲, 5. Substrates: A, acetylcholine; P, propionylcholine; B, butyrylcholine.

*Effect of temperature and pH on
serum-cholinesterase activity*

The abnormal specificity pattern found for serum cholinesterase in some cases of liver damage suggested that in these pathological sera the enzyme might differ from the normal in some other characteristics. Consequently the variation of enzyme activity with temperature and pH in some pathological sera was compared with the normal in the hope that some further differences might be revealed.

Effect of temperature. Cholinesterase activities of sera towards acetyl- and benzoyl-choline chloride were measured over a range of temperatures from 2° to 60°; the reaction always proceeded linearly with time over the period of measurement; thus the enzyme always appeared saturated with the substrate. The rate of enzymic hydrolysis of acetylcholine was corrected for spontaneous hydrolysis, but this was negligible for benzoylcholine chloride.

The rates of hydrolysis of acetyl- and benzoylcholine by normal sera and by two sera from patients with chronic liver damage (both of which showed the abnormal specificity pattern) varied similarly with increase in temperature. The optimum temperature was, in all cases, 47° for both substrates. But a purified preparation of cholinesterase having a 20-fold increase in activity, which was obtained by the ether-fractionation technique of Kekwick & Mackay (1954) from pooled normal plasma (AP₁/P₃ of Table 2), had an optimum temperature at 45°. Since the enzyme reaction proceeded linearly with time in each measurement, no change of enzyme configuration could have occurred, and the optimum temperature is an indication of the thermal stability of the enzyme in the experimental environment.

As the enzyme was saturated with the substrate, the observed velocity (*v*) was maximal, and proportional to the velocity constant for the breakdown of the enzyme-substrate complex to give the reaction products.

Table 2. Activation energies for the hydrolysis of choline esters by normal and pathological human sera

	Activation energies (kcal./mole)	
	Acetylcholine	Benzoylcholine
(a) Normal sera		
NM	8.3	14.9
RK	9.2	12.5
RJM	9.3	13.2
Purified fraction AP ₁ /P ₃	8.4	12.7
(b) Chronic liver damage		
Patient 13	9.4	—
Patient 1 (died)	7.8	12.3

Substitution of the results obtained for the velocity of the reaction at different temperatures in the Arrhenius equation (1889) shows that the activation energy for the hydrolysis of acetylcholine by normal serum varies from 8.3 to 9.3 kcal./mole and for the hydrolysis of benzoylcholine from 12.5 to 14.9 kcal./mole (Table 2). One serum from chronic liver damage (1) had a very slightly lower value for the activation energy of the hydrolysis of both acetyl- and benzoylcholine, the other serum from chronic liver damage had a normal activation energy for the hydrolysis of acetylcholine. The purified preparation of serum-cholinesterase AP_1/P_3 had activation energies similar to normal serum. The activation energy for the hydrolysis of butyrylcholine by one normal serum (RK) was 8.5 kcal./mole.

All the values of the activation energies given were obtained from measurements made below 34°, because it was found that above this temperature the Arrhenius equation was no longer obeyed: the plot of $\log v$ against $1/T$ was no longer linear.

Effect of pH. The variation of serum-cholinesterase activity with pH at 37° was measured for both acetyl- and benzoylcholine chloride over the narrow range from pH 7.1 to 7.8 permitted by the bicarbonate buffer system. The behaviour of two normal and two pathological sera was studied; one

of the two pathological sera (4) was obtained from a patient having multiple myelomatosis but who showed clear evidence of extensive liver damage.

The results obtained are plotted in Fig. 2 (a); the optimum pH for the hydrolysis of acetylcholine is above pH 7.8 for normal serum, but for benzoylcholine it is about 7.4. The results obtained for pathological sera were similar but the variation in activity towards acetylcholine with pH was not so marked and the pH optimum for the hydrolysis of benzoylcholine by one serum (14) appears to be shifted to a more acid region.

It was thought that possibly an apparent shift in the optimum pH of the cholinesterase activity of serum might arise when the protein pattern of serum was grossly distorted, as in multiple myelomatosis. To test this, 5 ml. of a 4.2 % protein solution containing 90 % of γ -globulin prepared from normal pooled serum by the ether-fractionation technique of Kekwick & Mackay (1954) was added to 5 ml. of a normal serum (NM) containing 8.2 % of protein; this gave a solution containing 6.2 % of protein in which there was a high concentration of γ -globulin. The variation of cholinesterase activity with pH in the system is compared with that of the normal serum (NM) in Fig. 2 (c). The optimum pH for the hydrolysis of acetylcholine was shifted from above pH 7.8 to pH 7.4, but the optimum pH for the hydrolysis of benzoylcholine was not affected.

DISCUSSION

The values of the activation energy for the hydrolysis of acetylcholine by normal human serum are similar to those of Shukuya (1953), who also found that the Michaelis constant for this enzymic reaction is independent of temperature. This indicates that the same substrate concentration will give maximal hydrolysis at different temperatures, as was found.

The difference in the activation energies found for the hydrolysis of the aliphatic esters acetylcholine and butyrylcholine (about 8.5 kcal./mole) on the one hand and for benzoylcholine (13 kcal./mole) on the other, when considered with the observation that the optimum pH for the hydrolysis of acetylcholine is above pH 8.0, whereas that for benzoylcholine is about pH 7.4, suggests that the mechanism whereby benzoylcholine is hydrolysed is somewhat different from that of the aliphatic esters. For both normal and pathological sera the rate of hydrolysis of benzoylcholine was always a constant fraction of that of acetylcholine, which suggested that the same enzyme was always involved. Further evidence of the dissimilarity of the mechanisms involved comes from the observation of Augustinsson (1949), who has shown that

Fig. 2. Effect of pH on serum cholinesterase. (a) Normal serum: ○, NM hydrolysing acetylcholine (Ach); △, NM hydrolysing benzoylcholine (Beh); ●, RK hydrolysing Ach; ▲, RK hydrolysing Beh. (b) Serum from patients with chronic liver damage: ○, Serum 14 hydrolysing Ach; △, serum 14 hydrolysing Beh; ●, serum 4 hydrolysing Ach; ▲, serum 4 hydrolysing Beh. (c) Normal serum plus γ -globulin: ○, NM hydrolysing Ach; ●, NM + γ -globulin hydrolysing Ach; △, NM hydrolysing Beh; ▲, NM + γ -globulin hydrolysing Beh.

the hydrolysis of benzoylcholine, unlike that of acetylcholine, is inhibited by excess of substrate. Vincent, Lagreu & Parant (1953) have shown that the hydrolysis of benzoylcholine by human-serum cholinesterase is selectively inhibited by caffeine.

The estimations of serum cholinesterase in liver disease have shown that a very low enzyme activity is always found in chronic liver damage; although in patients with acute liver damage the assay frequently gave an indication of abnormality, it could not, in any sense, be used as a criterion of the function of the liver cells.

The change in specificity found in liver disease could in some cases be a more sensitive indication of liver dysfunction than the single assay against acetylcholine; but the sera from patients with acute liver damage in some instances behaved normally.

This change in specificity pattern observed in liver disease is probably due to a slight change in the configuration of the enzyme. It seems unlikely that there is present in human serum a family of enzymes having slightly different specificity patterns which are produced in different relative amounts in liver disease, since the specificity pattern shown by Augustinsson (1949) for a purified preparation of cholinesterase was similar to that of whole serum. Members of a family of enzymes might be expected to behave differently on fractionation; thus a purified preparation might show a different specificity pattern from that of whole serum.

As a result of these investigations it is suggested that a detailed comparison of the substrate specificity produced by diseased cells with that from normal cells offers a way of studying changes in the configuration of a protein which are more subtle than can be detected from other types of measurements.

SUMMARY

1. The rates of hydrolysis of acetyl- and benzoylcholine by normal sera, and by sera from patients having chronic or acute liver damage, have been compared. Activity/ml. of serum was always decreased in chronic liver damage but was frequently found to be within the normal range in acute liver damage.

2. A comparison of the relative rates of hydrolysis of acetyl-, propionyl- and butyrylcholine by

sera from normal persons and by sera from patients with chronic liver damage and from patients with acute liver damage showed that, in the five cases of chronic liver damage studied, the preferred substrate was always propionylcholine. This was so also in one case of acute liver damage, but the preferred substrate for the sera from the other five cases of acute liver damage studied was butyrylcholine. In all the sera examined, normal and pathological, the relative rates of hydrolysis of acetylcholine and butyrylcholine were constant.

3. The activation energies and pH optima for the hydrolysis of acetylcholine and benzoylcholine were measured. The values obtained on the sera from patients having chronic liver damage do not differ greatly from those obtained with normal sera.

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