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ATEe Esterase Activity of Human Serum. Concentration from Serum, Assay, Properties and Inhibitor. (30260)

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Benzoyl arginine methyl ester (BAMe), benzoyl arginine ethyl ester (BAEe), tosyl arginine methyl ester (TAMe), lysine methyl ester (LMe) and many other synthetic substrates(1) have been used widely for determination of the proteolytic activity of human serum. The hydrolysis of acetyl tyrosine ethyl ester (ATEe) has been found to be a reliable indicator of the activity of C′ 1 esterase of human serum(2,3,4). There is considerable interest in ATEe esterase because of its potential role in immune phenomena and in vascular permeability(5). A strong inhibitor in human serum, however, handicaps the study of the activity of the enzyme. Donaldson(2) has introduced a technic for determination of C′ 1 esterase activity, based on the hydrolysis of ATEe after treatment of serum with diethyl ether. The method, while accurate, cannot be applied to routine use in the clinical laboratory.

The present paper (a) describes a simple method for concentration of ATEe esterase activity of human serum; (b) confirms previous observations on some of the properties of ATEe esterase and of its inhibitor in human serum; (c) reports observations on the activity of the enzyme in some disease states.

Materials and methods. (a) *Substrates* used in this study were TAMe, BAEe, BAMe, LMe, ATEe, acetyl tryptophane ethyl ester (ATrEe) and acetyl phenylalanine ethyl ester (APhEe). ATEe,‡ ATrEe‡ and APhEe‡ were dissolved in 10% methyl cellosolve,§ while TAMe, BAEe and LMe were dissolved in distilled water to make a 0.02 M solution. The esterase activity of human serum and of its fractions was studied with a modification of the colorimetric method described by Brown(6) for the study of the BAEe esterase activity of human serum. A 0.1 M sodium barbital buffer (pH 7.8) was prepared

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§ Eastman-Kodak Co., Rochester, N. Y. Methyl cellosolve was freshly obtained and, after solution, was kept in dark colored bottle, stoppered, at 4°C. Methyl cellosolve allowed to age contains esters which are capable of reacting with hydroxylamine.

by dissolving 10.3 g sodium diethylbarbiturate to 500 ml with distilled water. 33.8 ml of 0.1 M HCl were added to 66.2 ml of barbital solution. Alkaline hydroxylamine solution was prepared by nearly neutralizing a stock solution (13.9% or 2 M) of hydroxylamine hydrochloride (Eastman Kodak Reagent No. 340)¶ with an equal volume of 14% (3.5 M) NaOH solution. The solution was used within 2 hours of preparation. $FeCl_3$ 10% solution (0.37 M) was prepared by dissolving $FeCl_3 \cdot 6\ H_2O$ (Merck and Co., Reagent Grade, No. 72741) in 0.1 N HCl. The esterolytic activity of serum was determined as follows: (a) 1 ml of each substrate solution (20 μM) and 1 ml of barbital buffer were mixed in each of two 16 $\times$ 100 mm test tubes (A and B). Both test tubes were incubated at 37°C for 10 minutes; (b) 0.5 ml of serum was added to tube A and incubation continued for one hour; (c) both test tubes were removed from the water bath and 1.5 ml of 10% solution of trichloracetic acid was added to tube A; (d) 0.5 ml of serum was added to tube B, followed by 1.5 ml of 10% solution of trichloracetic acid; (e) the contents of both test tubes were mixed thoroughly, allowed to stand for 5 minutes and then centrifuged at 2000 rpm for 10 minutes; (f) 2 ml of clear supernatant fluid were transferred from each test tube into Coleman cuvettes; (g) to cuvettes A and B (containing the reacting mixtures) and to cuvette C (containing 2 ml of distilled water) were added 2 ml of alkaline hydroxylamine solution and 1 ml of diluted HCl with thorough mixing; (h) 2 ml of $FeCl_3$ were added to each cuvette, which was allowed to stand at room temperature for exactly 15 minutes; (i) readings were made in Coleman spectrophotometer (model 14) at 540 mμ with the blank cuvette C set at 0 optical density. All determinations were done in triplicate and results averaged. A calibration curve was prepared from readings obtained with using 0 to 10 μM of a buffered solution of the various substrates(6). One unit of enzyme activity was considered equivalent to 1 μM of substrate hydrolyzed per hour by 1 ml of serum(6).

(b) *Streptokinase solution* was prepared from a commercial source (Veridase, Lederle Division, American Cyanamid Co., Pearl River, N. Y.) to contain 2,500 units per ml of distilled water.

(c) *Preparation of fractions of serum for study of ATEe esterase activity and activity of other esterolytic enzymes.* Human serum, stored at —20°C until used, was diluted with 19 volumes of distilled water. A 0.5% solution of acetic acid was added drop by drop and the pH was adjusted to the desired value by direct reading with an expanded scale Beckman pH meter (model 76). The precipitate which formed was separated by centrifugation at 2,000 rpm/15 min/4°C and resuspended in phosphate buffered saline solution (7) to a volume equal to that of the serum used originally.

(d) *Miscellaneous technics* are described in the following sections when indicated.

Results. (a) *Esterase activity of various fractions of human serum.* The fraction of serum precipitated at pH 5.2 digested TAMe, BAEe and BAMe. After adding 250 units streptokinase per ml, the fraction showed increased activity against TAMe, BAEe and BAMe and digested LMe also. The fractions of serum precipitated at pH values between 6.8 and 7.4 digested BAMe, BAEe, TAMe, ATEe (Fig. 1) and, to a lesser degree, ATrEe and APhEe. The activity of these fractions was not enhanced by addition of streptokinase. The fraction used for clinical studies was obtained at pH 7.0 Its dry weight varied from 7.2 to 11.5 mg/ml of human serum.

(b) *Average values of ATEe esterase activity in "healthy" individuals.* Determinations in 36 normal individuals gave a range of values between 18 and 26 units, with an arithmetic mean of 22.1 units (Fig. 2). Results were reproducible within 1 unit in multiple determinations.

Properties of serum ATEe esterase. The ATEe esterase activity was stable indefinitely in serum stored at —20°C. 50% activity was lost after 24 hours incubation at 4°C and after 12 hours incubation at 25°C.

The effect of temperature on activity and stability of ATEe esterase was studied in

¶ The stock solution of hydroxylamine was stored at 4°C.

TABLE I. Effect of Temperature on Activity of ATEe Esterase of Serum.*

Temperature of incubation, C	25°	30°	37°	40°	45°	56°
ATEe esterase activity, units/ml serum	16.1	19.4	22	16.9	3.7	0

* See text.

the following experiment. Fractions of serum precipitated at pH 7 were prepared as described. Aliquots of 0.5 ml were incubated at temperatures varying from 25° to 56°C for 30 minutes, with ATEe as substrate. The activity of the various aliquots incubated at different temperatures is shown in Table I. The ATEe esterase activity of serum was optimal at a temperature of 37°C.

In another experiment, equal volumes of various organic solvents were added to aliquots of serum. The mixtures were allowed to stand at room temperature for 15 minutes and the solvent was separated by aspiration. There was no appreciable change in ATEe esterase activity of serum after treatment with petroleum ether, but the activity was enhanced greatly after treatment with diethyl ether or acetone (Table II).

In additional experiments, 100 mg $BaSO_4$ (USP) (Mattheson, Coleman & Bell, Norwood, O.) or animal charcoal (Mallinckdrodt Chemical Works, St. Louis, Mo.) were added to 1 ml of a fraction of serum precipitated at pH 7 and reconstituted with saline solution, to contain 20 units of ATEe esterase activity per ml. After mixing, test tubes were kept at room temperature for 30 minutes. The absorbents were separated by centrifugation at 2,000 rpm/20 minutes. The supernatant fluid showed no change in ATEe esterase activity. Conversely, the elution of the cake of $BaSO_4$ or of the charcoal with buffered phosphate saline at pH 7.8 yielded no enzymatic activity.

TABLE II. Effects of Various Organic Solvents on ATEe Esterase Activity of Human Serum.*

	ATEe esterase activity, units/ml serum
Serum	.8
Serum, treated with	
Petroleum ether	1.2
Diethyl ether	13.4
Acetone	14.2

* See text.

Addition of varying amounts of ε-aminocaproic acid (American Cyanamid Co., Lederle Division, Pearl River, N. Y.) or soyabean inhibitor to serum caused no appreciable change in ATEe esterase activity. Di-isopropylfluorophosphate (DFP) was strongly inhibitory (Table III).

TABLE III. Inhibition of ATEe Esterase Activity of Human Serum by Diisopropylfluorophosphate (DFP).*

Concentration of DFP, M	ATEe esterase activity, units/ml
.005	0
.002	6.8
.001	13.6
.0005	16
.0001	22
—	22

* Source of esterase activity was 0.9 ml of the resuspended fraction of human serum precipitated at pH 7.0 (exhibiting an ATEe esterase activity of 22 units/ml). 0.1 ml of the solutions of DFP of various strength was added. Other technical details are: pH of mixture, 7.4; time of incubation, 10 min; temperature, 37°C.

(d) *Separation of an inhibitor of ATEe esterase activity in human serum.* Fresh human serum showed little ATEe esterase activity. This, however, was high in the resuspended precipitate of diluted serum at pH 7. The result suggested, among other possibilities, the separation of an active enzyme from an inhibitor, the existence of which was demonstrated in the following experiment. The pH of diluted serum was adjusted to pH 7.0 and the precipitate, containing ATEe esterase activity, was separated by centrifugation (see above). The pH of the supernatant fluid was then adjusted to pH 5.2 and the new precipitate also separated by centrifugation. Both precipitates were resuspended in a volume of phosphate buffered saline equal to that of serum originally used and the ATEe esterase activity was assayed in both (Table IV). The fraction precipitated at pH 7 had full ATEe esterase activity which was, however, sup-

pressed by addition of an equal volume of the fraction precipitated at pH 5.2. The mixture of the two precipitates was diluted with 9 volumes of distilled water and the pH adjusted to 7.0. A precipitate formed which, after resuspension in phosphate buffered saline, exhibited high ATEe esterase activity. These steps of combination and release of the enzymatic activity from the inhibitor could be repeated several times, suggesting that the enzyme combines reversibly with its inhibitor, without appreciable loss of activity. Fig. 3 shows the effects of addition of increasing amounts of the inhibitory fraction (pH 5.2) to a fixed volume of resuspended fraction containing ATEe esterase activity, indicating a possible quantitative relationship between enzymatic activity and its inhibitor.

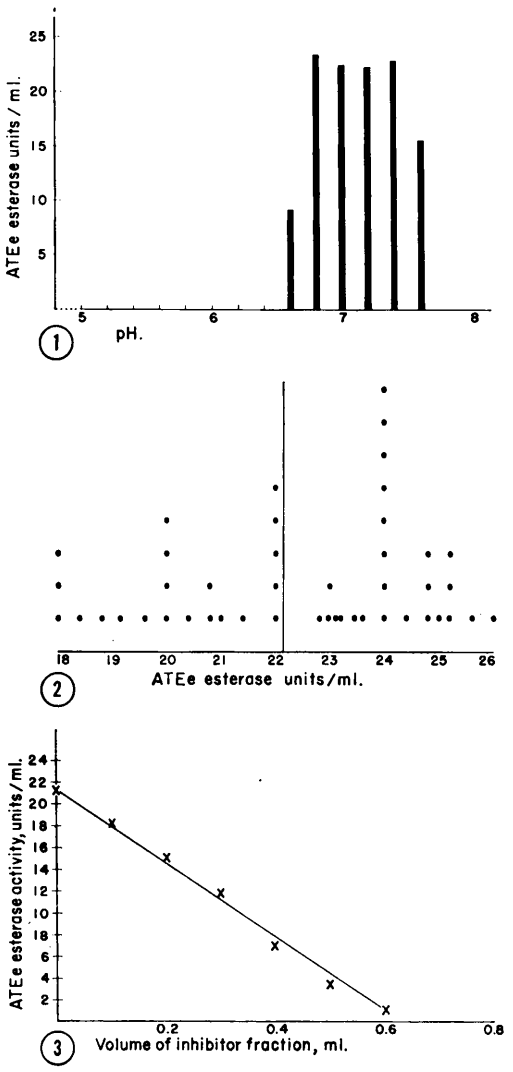


FIG. 1. ATEe esterase activity of serum fractions precipitated at different pH values.*

* See text. Maximum activity was found in the fractions precipitated at pH values between 6.8 and 7.4.

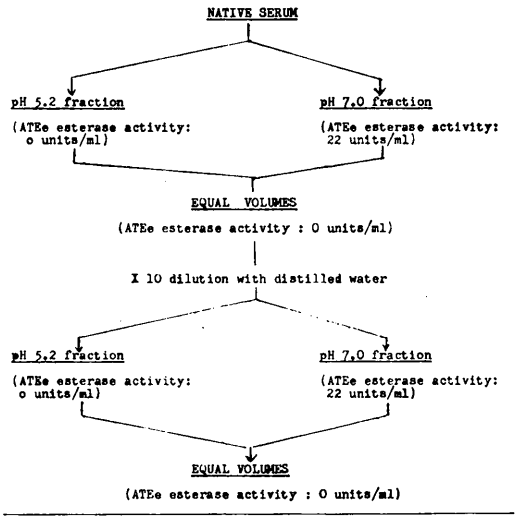
FIG. 2. ATEe esterase activity in serum from 36 healthy subjects.†

† See text. Vertical line represents arithmetic mean.

FIG. 3. Relationship of ATEe esterase to concentration of inhibitor.‡

‡ Increasing volumes of inhibitor fraction (from 0.1 to 0.6 ml) were added to 1 ml of a serum fraction with ATEe esterase activity and the total volume brought to 3 ml by adding phosphate saline buffer. The curve obtained, which is representative of 15 experiments, suggests that the reaction between the enzyme and its inhibitor proceeds according to a quantitative relationship.

TABLE IV. Reversible Binding of ATEe Esterase to Its Inhibitor.



(e) *Properties of inhibitor of ATEe esterase activity.* The inhibitor of ATEe esterase precipitated at pH 5.2 was destroyed by heating at 56°C for 30 minutes but was stable for 3 months when stored at −20°C. Prior treatment of serum with diethyl ether, chloroform or acetone resulted in destruction of the inhibitor. The activity of the inhibitor, however, was unaffected by treatment of serum with petroleum ether or benzene. The inhibitor was not absorbed from serum by treatment with barium sulfate or animal charcoal.

(f) *Preliminary clinical studies.* Table V

TABLE V. ATEe Esterase Activity of Human Serum in Some Disease States.*

Case #	Sex	Diagnosis	ATEe esterase activity, units/ml
1	♀	Portal cirrhosis of liver	0
2	♀	Diabetes mellitus, chronic alcoholism	0
4	♀	Malnutrition (also, lobar pneumonia)	0
5	♀	Diabetes mellitus	0
6	♀	Carcinoma of pancreas, with extensive involvement of liver	0
7	♀	Toxemia of pregnancy (4th month)	14
8	♀	Ependymoma of third ventricle; chronic pyelonephritis with uremia	10
11	♀	Portal cirrhosis of liver	2.8
13	♂	Diabetes mellitus	14.6
16	♀	Congestive heart failure with marked hepatomegaly	4
18	♂	Portal cirrhosis of liver	.5
19	♂	" " " "	0
20	♀	" " " "	0
22	♂	" " " "	0
23	♂	Cirrhosis of liver, posthepatitic	.4
25	♀	Adenocarcinoma of colon, metastatic to liver	1.5
28	♂	Cirrhosis of liver, posthepatitic	10.2
30	♀	" " " , postnecrotic	0
31	♀	Biliary cirrhosis	2.7
35	♂	Portal cirrhosis of liver	0
37	♀	Acute hepatitis	3.6
39	♂	" "	2.3
41	♂	Acute hepatocholangitis	3.9
45	♀	Obstructive jaundice (impacted calculi), long standing	17.2

* The list includes only the 24 cases where ATEe esterase activity of serum was below 18 units/ml, the lowest value ever found in our experience in healthy individuals. The remaining 26 cases showed values above 10 units/ml. None of these cases suffered from parenchymal liver disease or diabetes mellitus and represented a wide variety of medical and surgical conditions.

summarizes a series of determinations of ATEe esterase activity of the serum from 50 patients with various disorders. A striking decrease was observed in all patients with severe parenchymal liver disease, proven by a combination of clinical findings, laboratory tests and, in 3 instances, liver biopsy. The decrease in activity of ATEe esterase correlated with decrease in weight of the precipitate obtained from diluted serum at pH 7 which, in most instances, was below 5 mg per ml and as low as 2 mg per ml in 3 cases. A depletion of ATEe esterase activity was found also in the serum of many patients with long standing diabetes mellitus.

Discussion. There is considerable interest in the biologic activity and significance of esterases. Among these enzymes, perhaps the plasmin system and C′ 1 esterase have been investigated more carefully. C′ 1 esterase, an enzyme which is activated by the interaction of antigen-antibody complexes with C′ 1 of complement, hydrolyzes selected synthetic substrates and inactivates C′ 2 and C′ 4. It appears needed for the interaction of complexes of antigen, antibody and C′ 1 with C′ 2 and C′ 4. C′ 1 esterase of human serum has been purified and it is known to increase vascular permeability in guinea pigs(5). The enzyme probably possesses other physiologic functions. Its effects are inhibited by antihistaminics(5). An inhibitor of C′ 1 esterase has also been isolated as a heat-labile α-globulin fraction of serum(8).

The listing of properties of C′ 1 esterase seems to indicate that they are similar in many respects to those of ATEe esterase. Additional experiments have indicated also that ATEe esterase, like C′ 1 esterase, inactivates the fourth component of complement. ATEe esterase activity of fresh human serum is low and variable because of the existence of a specific inhibitor which combines reversibly with the enzyme.

While most observations indicate a similarity of properties between ATEe esterase and C′ 1 esterase, a few significant differences also exist. C′ 1 esterase activity, ac-

cording to Lepow *et al*(4), develops in an euglobulin fraction precipitated at pH 5.5 and the inhibitor of C′ 1 esterase is found in small amount only in the euglobulin fraction precipitated at pH 5.5 or pH 5.2. These striking differences in the behavior of C′ 1 esterase and ATEe esterase and their inhibitors cannot be explained readily, especially because of the differences in methods of concentration of the enzymatic activities and of their inhibitors.

We have developed a method of concentration of ATEe esterase activity of human serum which is simpler than previously proposed technics and yields reproducible results. It should facilitate studies in the changes of activity of serum ATEe esterase in pathologic conditions. Preliminary studies have shown a significant decrease in serum ATEe esterase activity in parenchymal liver disease and in long standing diabetes mellitus. It cannot be established whether the decrease of ATEe esterase activity in disease is related to true depletion of enzymatic activity, or to increased inhibitor activity or to a combination of both factors. In any event, these observations agree with those of others who have reported a decrease in BAEe esterase activity in the serum of patients with cirrhosis of the liver(9). Much additional work will be needed, of course, before any conclusion may be reached on the significance of variations in the activity of ATEe esterase of serum in clinical disorders.

Summary. ATEe esterase activity can be precipitated from fresh human serum by dilution of the serum with distilled water and adjustment of pH between 6.8 and 7.4. The activity of this fraction of serum is not enhanced by addition of streptokinase. Its properties are closely similar to those of C′ 1 esterase activity of serum. Human serum also contains an inhibitor, which is precipitated from diluted serum by adjusting the pH to 5.2. The inhibitor combines reversibly with ATEe esterase. Preliminary studies show striking depletion of the activity of ATEe esterase in parenchymal liver disease and in long standing diabetes mellitus.

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The Fate of Foreign Red Cells in Mice with Altered Reticuloendothelial Function.* (30261)

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Previous studies have demonstrated that it is possible selectively to stimulate phagocytic function, due to a pronounced increase in number and activity of Kupffer cells, by intravenous administration of the neutral polysaccharide glucan(1). Conversely, it has been demonstrated that intravenous administration of a methyl palmitate emulsion results in a profound depression of phagocytic function due, in part, to a failure in Kupffer cell phagocytosis(2).

Associated with an enhanced phagocytic function in glucan-treated mice, was a hyper-

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