SOME PROPERTIES OF AN ESTERASE DERIVED FROM PREPARATIONS OF THE FIRST COMPONENT OF COMPLEMENT*

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Several years ago, an hypothesis was proposed that the first of the four recognized components of complement was the precursor of an enzyme (1).

This view was based in part upon observations of the effect of proteolytic enzymes upon human complement. Streptokinase is an activator of plasmin, a proteolytic enzyme of plasma most effective at neutrality. The complement activity of human serum was inhibited by the addition of streptokinase (2). Analysis demonstrated that this loss of complement activity was primarily the result of inactivation of the first, second, and fourth components of complement. However, the inactivation of the second and fourth components occurred only in serums containing the first component (1). These experiments suggested that the streptokinase had activated plasmin which in turn had converted the first component of complement into an agent which inhibited the activity of the second and fourth components.

The possibility that the first component of human complement might be a proenzyme has found support in two separate lines of study. In the first group of experiments, it was observed that under certain conditions a partially purified preparation of the first component rapidly lost its hemolytic activity. In its stead, the preparation acquired the capacity to inactivate complement and to hydrolyze certain synthetic amino acid esters (3, 4). In the second group of experiments, aggregates of antigen and antibody were mixed with fresh human serum. This procedure resulted in a demonstrable loss from the serum of activity attributable to the first component. It was then possible to elute from the aggregates of antigen and antibody one or more factors which inactivated complement and hydrolyzed the same amino acid esters as the preparations derived from the partially purified first component (3, 5). These observations suggested that the first component of complement may have been the precursor of an enzyme tentatively identified as an esterase (3-5). The inhibition of the hemolytic action of guinea pig complement by diisopropyl fluorophosphate, an

agent known to inhibit other esterases, is consonant with this view (6, 7). Becker (7, 8), using an entirely different approach, has reached the same conclusion concerning the nature of the first component of guinea pig serum.

The present report describes some of the properties of an esterase derived from preparations of the first component of complement or eluted from aggregates of antigen and antibody which had been treated with fresh serum. The effect of the esterase was tested against a variety of possible substrates. N-acetyl-L-tyrosine ethyl ester was the one most readily digested. Using this substrate, an assay was devised with which to test certain aspects of the kinetics of the esterase. In addition, it was possible to demonstrate that human serum inhibited the esterase. The implications of these observations will be discussed.

Nomenclature and Materials

The method of preparation of partially purified *first component of human complement* (C'1) has been described previously. Rat and rabbit first component were made by the same technique. The partially purified human C'1 contained 17 to 25 per cent of the C'1 activity present in the original serum, with a purification of 30- to 50-fold, representing about 0.6 per cent of the original serum protein. It was heterogeneous in the ultracentrifuge, with at least three measurable components at pH 5.5 and ionic strength 0.30. There were no measurable amounts of the second and fourth components of complement, and only traces of the third component. In preparations concentrated 20-fold over the starting material, the concentration of properdin (9) was about 8 units per ml. when the first component was prepared from serum, and 1 unit or less per ml., when it wasprepared ffom serum from which the bulk of the properdin had been removed. Partially purified C'1 also contained appreciable amounts of plasminogen; the plasmin which formed upon the addition of streptokinase digested fibrin, casein, para-toluenesulfonyl-L-arginine methyl ester, and L-lysine ethyl ester, known substrates of plasmin (10). The recently described clot-promoting fractions, Christmas factor (plasma thromboplastin component), and Hageman factor were present in large amounts. In some preparations, traces of thrombin, and perhaps prothrombin or proconvertin were present. Neither proaccelerin, cholinesterase, nor acid or alkaline phosphatase could be detected. The preparation of partially purified C'1 was stored at −25°C. at pH 5.5 and ionic strength 0.30.

Partially purified C'1 rapidly lost its hemolytic properties and acquired complement-inactivating and esterase activities when adjusted to physiologic pH and ionic strength in the absence of inhibitors. This new product has been designated *activated C'1*, although it is recognized that the complement-inactivating and esterase properties are not necessarily derived from the first component of complement itself (5). In earlier studies the activated C'1 was referred to as "converted C'1." Activated C'1 was prepared by adding an equal volume of water to suspensions of partially purified C'1, and adjusting the pH to 7.0–7.8 by the addition of 0.05 N sodium hydroxide. The clear solution of activated C'1, now at ionic strength 0.15 and concentrated 10-fold with respect to serum, was incubated at 37°C. for 15 minutes to insure complete activation of the esterase. It was kept in ice water until used the same day, or stored at −25°C. for several weeks, without demonstrable loss of activity. However, when the frozen material was thawed, a precipitate formed so that the preparation required thorough stirring before use.

The term *eluate AE* is used to describe the eluate derived from aggregates of an antigen of pneumococcal type III specific soluble substance and type-specific rabbit antibody which had

been incubated with normal human serum. Its method of preparation had been described elsewhere (5). Eluate AE prepared from fresh human serum and concentrated 10-fold contained about 0.04 mg. of nitrogen per ml. of solution. It was dissolved in 0.15 N sodium chloride. Small amounts of Hageman factor and of streptokinase-activable plasminogen were present. It did not contain measurable amounts of the components of complement, properdin, acid or alkaline phosphatase, cholinesterase, prothrombin, proconvertin, proaccelerin, Christmas factor, or thrombin. Eluate AE had complement-inactivating and esterase activities qualitatively indistinguishable from activated C'1.

The *sodium phosphate buffer* used was composed of 1.82 grams of $NaH_2PO_4 \cdot H_2O$ and 16.75 grams of $Na_2HPO_4 \cdot 12H_2O$ per liter, and had a pH of 7.4 and ionic strength 0.15. The *barbital-saline buffer* was prepared by dissolving 2.76 grams of barbital, 2.06 grams of sodium barbital, and 7.40 grams of sodium chloride in one liter of water, and consisted of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5 and ionic strength 0.15. *Michaelis' acetate-barbital-saline buffers* were prepared according to published directions, and over the range of pH used were at ionic strength 0.15 (11).

Synthetic substrates, obtained from commercial sources or synthesized in laboratories of the Department of Chemistry, Western Reserve University, were dissolved in suitable amounts in a buffer solution of sodium phosphate at pH 7.4 unless otherwise indicated. The solutions were adjusted to pH 7.4 by the addition of 0.15 N sodium hydroxide or hydrochloric acid. Certain of the substrates were soluble only in the presence of suitable amounts of 2-methoxyethanol (methyl cellosolve). The synthetic substrates included esters or amides of amino acids or simple dipeptides or tripeptides.

Protein substrates were prepared in a variety of ways. *Casein* (Hammerstan quality, Nutritional Biochemicals Co.) was dissolved at a concentration of 5 per cent in 0.15 N sodium hydroxide and then dialyzed for 48 hours against barbital-saline buffer. A 2.2 per cent solution of *denatured hemoglobin* (Armour) was prepared by published methods (12). A 5 per cent solution of *bovine albumin* (Armour) was prepared by diluting a 30 per cent stock solution with 0.15 N soldium chloride. *Crystallized egg albumen* (Armour) was dissolved at a concentration of 1 per cent in sodium phosphate buffer. *Crystalline beta-lactoglobulin* (United States Department of Agriculture Eastern Utilization Research Branch) was dissolved at a concentration of 1 per cent in barbital-saline buffer. *Edestin* and *lactalbumin* (obtained from The Rockefeller Institute for Medical Research in 1946) were suspended in a concentration of 10 mg. per 5 ml. of 1 N sodium chloride. *Myometrium,* obtained from an intact surgically removed human uterus, was washed 20 times with 0.15 N solution of sodium chloride, crushed in a mortar, and pulverized in a hand homogenizer. It was then washed with 10 ml. of 0.15 N sodium chloride, centrifuged for 15 minutes at room temperature at 2000 R.P.M. and then resuspended, rehomogenized, and recentrifuged two more times. The myometrial suspension was then resuspended in barbital-saline buffer in a concentration of 5 grams per 100 ml. *Human serum,* when used as substrate, was diluted to one-tenth its original concentration with 0.15 N sodium chloride. *Human plasma,* obtained by centrifugation of blood rendered incoagulable by the addition of one-ninth part of 3.8 per cent sodium citrate, was diluted to one-tenth its original concentration by the addition of a mixture of 1 part of 3.8 per cent sodium citrate and 4 parts of 0.15 N sodium chloride, to inhibit clotting. *Gelatin* (Difco) was dissolved at 70–80°C. in phosphate buffer, in a concentration of 5 per cent and then maintained at 37°C. for immediate use.

Streptokinase ("high purity")[1], the filterable principle of cultures of beta-hemolytic streptococci which activates plasminogen, was dissolved in a concentration of 20,000 u./ml. of barbital-saline buffer.

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[1] Streptokinase was provided through the courtesy of the Lederle Laboratories Division, American Cyanamid Company.

Crystalline soy bean inhibitor (Nutritional Biochemicals Co.), *egg white inhibitor* (Nutritional Biochemicals Co.) and *heparin sodium* (Upjohn, 1,000 USP units per ml.) were each dissolved in a concentration of 10 mg. per ml. of 0.15 M sodium chloride, and *pancreatic trypsin inhibitor* (Nutritional Biochemicals Co.), in a concentration of 1 mg. per ml. N-acetyl-L-tyrosine when used as an inhibitor, disodium ethylene diamine tetraacetic acid[2] (*sequestrene Na2*), *sodium ascorbate, reduced glutathione, hydrogen peroxide,* and *thioglycollic acid* were neutralized with appropriate amounts of 0.15 N sodium hydroxide and dissolved in 0.1 M concentration in 0.15 N sodium chloride or phosphate buffer at ionic strength 0.15. *Oxidized glutathione* was dissolved in a concentration of 0.1 M in 0.2 N sodium hydroxide. A 2.5 M stock solution of *diisopropyl fluorophosphate* (Delta Chemical Works, New York, New York) was prepared in isopropyl alcohol, from which a 0.125 M emulsion in 0.12 M sodium bicarbonate was made just before use (6). Further dilutions of the emulsion were made in 0.15 M NaCl. *Human serum,* when used as an inhibitor, was diluted with one-half volume of 0.15 N sodium chloride. Preparations of human serum from which individual components of complement had been removed were prepared by published methods. The terms R1, R2, R3, and R4 will be used to designate serum from which the respective components of complement had been removed (13). *Complement-fixed serum* was prepared by the addition of fresh human serum of pneumococcal specific soluble substance, type III, and purified homologous rabbit antiserum. The mixture was incubated at 37°C. for 1 hour, the antigen-antibody aggregate removed by centrifugation at 0°C., and the supernatant (complement-fixed serum) tested for complement components. Eight micrograms of antigen and 200 micrograms of antibody nitrogen were used in a volume of 0.5 ml. for 1 ml. of human serum. The resulting treated serum contained 6 per cent of the original C'1, no second or fourth component, and 50 per cent of the original third component of complement.

Streptokinase-Treated Serum was prepared by adding 0.5 ml. of streptokinase (2,000 units/ ml.) to 1 ml. of fresh human serum and incubating at 37°C. for one hour. The resulting treated serum contained 4 per cent of the original C'1, no second or fourth component, and 60 per cent of the original third component of complement. In the final mixture of enzyme and substrate, the concentration of all the inhibitors tested was one-fifth of the original solutions.

Methods

Measurement of the hydrolysis of amino acid esters or amides or of peptides was performed by a minor modification of the technique of Troll, Sherry, and Wachman (10). Typically, 7 parts of a buffered solution of substrate were mixed with 2 parts of 0.15 M sodium chloride and 1 part of a solution of the enzyme to be tested, and incubated in a water bath at the desired temperature. In the case of the substrate N-acetyl-L-tyrosine ethyl ester, the buffer, sodium chloride solution and enzyme were first mixed, and the substrate, dissolved in methyl cellosolve equal to 5 per cent of the final mixture, was added last. The final concentration of methyl cellosolve in the assay mixture was 5 per cent. At the start and at appropriate intervals, samples of 1 ml. of the enzyme-substrate mixture were removed, mixed with 1 ml. of a 37 per cent solution of neutralized formaldehyde, and micro-titrated with 0.05 N sodium hydroxide using 2 drops of 1 per cent alcoholic phenolphthalein as the indicator. Suitable control experiments were performed, omitting enzyme or substrate from the mixture.

The influence of pH upon the action of the enzyme was tested by substituting Michaelis acetate-barbital buffer of varying pH in the ordinary assay procedure. Two drops of 5 per cent alcoholic solution of thymolphthalein were used as the indicator in those mixtures which were initially too alkaline for the use of phenolphthalein. The pH of the enzyme-substrate mixtures was tested at approximately 25°C. at the beginning of the experiment, using a Cambridge research model pH meter.

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[2] Sequestrene was provided through the courtesy of the Alrose Chemical Company.

The influence of ionic strength was tested in an enzyme-substrate mixture composed of 1.0 ml. of phosphate buffer, 0.25 ml. of activated C'1, 1.12 ml. of sodium chloride solution of varying concentration, and 0.12 ml. of 1.6 M N-acetyl-L-tyrosine ethyl ester in methyl cellosolve. The ionic strength of the substrate itself was ignored.

The digestion of N-acetyl-L-tyrosine ethyl ester, p-toluenesulfonyl-L-arginine methyl ester, benzoyl-L-arginine methyl ester, and L-lysine ethyl ester was also determined by Sherry's unpublished modification of the method of Hestrin (14).

Measurement of the digestion of protein substrates other than gelatin was performed in duplicate by mixing 0.1 ml. of the enzyme preparation with 0.4 ml. of phosphate buffer and 0.5 ml. of substrate solution, and incubating the mixture at 37°C. for approximately 24 hours. At the same time, two control mixtures were incubated, namely, a mixture of 0.1 ml. of enzyme and 0.4 ml. of buffer to which 0.5 ml. of substrate was added at the end of the period of incubation; and a mixture of 0.1 ml. of 0.15 M sodium chloride, 0.4 ml. of phosphate buffer, and 0.5 ml. of substrate. At the end of the period of incubation, 2.0 ml. of 0.3 N trichloracetic acid were added. After 30 minutes, the precipitate which formed was separated by centrifugation, and the tyrosine-like activity of an aliquot of 1 ml. of the supernatant solution was measured by adding 1 ml. of 2.5 N sodium hydroxide, 6 ml. of water, 3 ml. of 20 per cent sodium carbonate solution, and 1 ml. of Folin-Ciocalteau reagent. The color which resulted was measured in 10 mm. cuvettes in a Beckman Model B spectrophotometer at a wave length of 650 mμ. using as a standard a solution of tyrosine which had been treated in the same fashion. *Denatured hemoglobin* was incubated in the presence of 40 per cent urea in the enzyme-substrate mixture. Since the preparations of *lactalbumin* and *edestin* were poorly soluble, the mixtures containing these substrates were agitated on a mechanical shaker during the period of incubation.

The digestion of *gelatin* was measured by a modification of the viscosimetric methods described by Swyer and Emmens (15). One ml. of activated C'1 was added to 6.5 ml. of 5 per cent gelatin at 37°C. and the mixture transferred at once to an Ostwald viscosimeter in a 37°C. water bath. Flow times were measured at regular intervals to determine reduction of viscosity of gelatin by the enzyme. The flow time of water in the viscosimeters used was 71 to 74 seconds.

The effect of inhibitory agents was tested by preparing a series of tubes each containing a mixture of 1.62 ml. of phosphate buffer, 0.25 ml. of activated C'1, and 0.50 ml. of the solution to be tested or of 0.15 N sodium chloride. The tubes were then incubated at 37°C. and at appropriate intervals 0.12 ml. of 1.6 M N-acetyl-L-tyrosine ethyl ester in methyl cellosolve was added to each tube. The mixtures were then incubated for 15 additional minutes. One ml. aliquots were withdrawn just after the addition of substrate and after the 15 minute period, mixed with an equal volume of formaldehyde solution, and titrated in the manner described.

To test for a reversal of inhibition by thioglycolic acid, a technique suggested by the work of Carter and Warner (16) was used. A mixture was made of 2 parts of activated C'1 and 9 parts of phosphate buffer. A volume of 6.88 ml. of this mixture was incubated at 37°C. for 30 minutes with 2.5 ml. of phosphate buffer or 0.1 M neutralized thioglycolic acid in phosphate buffer. The mixture was then dialyzed at 4°C. for 5 hours in Visking no-jax cellophane casings (¼ inch in diameter) against phosphate buffer. In duplicate, 1.375 ml. aliquots were then incubated at 37°C. for 30 minutes with 0.5 ml. of phosphate buffer or of 0.1 M neutralized oxidized glutathione. Then 0.125 ml. of 1.6 M N-acetyl-L-tyrosine ethyl ester in methyl cellosolve was added and the amount digested at 37°C. in 15 minutes was determined in the usual manner. Concomitantly, the immediate effect of thioglycolic acid on activated C'1 was determined, omitting the dialysis.

Measurement of the effect of streptokinase and soy bean inhibitor on the digestion of casein was tested by a minor modification of the method used to test the digestion of other proteins. In duplicate, 0.9 ml. of eluate AE was incubated at 25°C. for 10 minutes with 0.3 ml. of streptokinase solution or barbital-saline buffer. Then, to one tube of each pair was added 0.3 ml. of a solution of 0.5 per cent soy bean inhibitor in barbital-saline buffer. Suitable amounts of buffer

were added to each tube to bring the volume to 1.5 ml., and the mixtures were reincubated at 25°C. for 30 minutes. A volume of 1.5 ml. of casein solution was then added to each tube and the mixtures were incubated at 37°C. for 24 hours. At that time, 1 ml. aliquots were removed and tested for tyrosine-like activity in the manner described; control mixtures, in which buffer was substituted for eluate AE, were tested simultaneously.

All experiments were first performed using activated C′1 as the enzyme. In most cases, the experiments were repeated with eluate AE only if the preparation of activated C′1 had enzymatic activity against the particular substrate tested.

Pyrex glassware, washed with chromic acid, was used throughout. When enzyme and substrate were incubated for longer than 1 hour, a drop of toluol was added to those mixtures not containing methyl cellosolve. All acidometric titrations were performed with a 1 ml. microsyringe (Micrometric Instrument Corporation, Cleveland).

RESULTS

1. Substrates of Activated C′1 and Eluate AE:

It was previously shown that converted C′1 and eluate AE hydrolyzed p-toluenesulfonyl-L-arginine methyl ester and N-acetyl-L-tyrosine ethyl ester (4, 5). A number of other synthetic amino acid esters, amides, and peptides were tested as possible substrates of human activated C′1, using the acidometric method (Table I). The concentration of substrate in the enzyme-substrate mixture was usually 0.02 M, and the initial pH approximately 7.5. Only benzoyl-L-arginine methyl ester, closely related to p-toluenesulfonyl-L-arginine methyl ester, N-acetyl-3,5, dinitro-L-tyrosine ethyl ester and N-acetyl-L-phenylalanine ethyl ester, both closely related to N-acetyl-L-tyrosine ethyl ester, and N-acetyl-L-tryptophan ethyl ester were appreciably digested by activated C′1 under these conditions. Benzoyl-L-tyrosine ethyl ester was not measurably hydrolyzed. Because of its relative insolubility, this ester could only be tested in the presence of 25 per cent methyl cellosolve. In this concentration, methyl cellosolve inhibited the action of activated C′1 as tested upon N-acetyl-L-tyrosine ethyl ester. For this reason it is not certain that under more suitable conditions, benzoyl-L-tyrosine ethyl ester might not be a susceptible substrate.

The relative degree of hydrolysis of 5 susceptible substrates was tested at 37°C. at a concentration of substrate of 0.02 M, ionic strength 0.15 and pH 7.5. (Table II) This experiment was performed at suboptimal concentrations of both N-acetyl-L-tyrosine ethyl ester and p-toluenesulfonyl-L-arginine methyl ester, because of the limited solubility of the dinitro derivative. N-acetyl-L-tyrosine ethyl ester appeared to be the most susceptible of these substrates. N-acetyl-L-tryptophan ethyl ester was not compared in this experiment because of its poor solubility; at a concentration of 0.005 M, it was not measurably digested during the same interval of time. All six substrates were also hydrolyzed by eluate AE.

The assays thus far described were performed by measuring the increase in titratable acidity in the enzyme-substrate mixture. Hydrolysis of N-acetyl-L-

TABLE I

Digestion of Synthetic Substrates by Activated C'1

Substrate	Concentration in enzyme-substrate mixture	Microequivalents of acid liberated in	
		1 hr.	24 hr.
	M		
N-Acetyl-L-tyrosine ethyl ester*· ‡	0.02**	18.0	—
N-Acetyl-3,5-dinitro-L-tyrosine ethyl ester§	0.02	1.2	11.9
N-Acetyl-L-tyrosine§	0.02	0	0
Benzoyl-L-tyrosine ethyl ester**	0.005‖‖	0	0
L-Tyrosine ethyl ester*	0.02§§	0	0
Diacetyl-L-tyrosine**	0.02	0	0
N-Acetyl-L-phenylalanine ethyl ester‡	0.02‡‡	2.9	12.0
N-Acetyl DL alanine ethyl ester‡	0.02	0	0
DL-Alanine ethyl ester‡	0.02	0	0
Benzoyl-DL-alanine ethyl ester§	0.02**	0	0
N-Acetylglycine ethyl ester‡	0.02	0	0
p-Toluenesulfonyl-L-arginine methyl ester§	0.02	14.2	—
Benzoyl-L-arginine methyl ester*	0.02	14.7	—
Benzoyl-L-arginineamide*	0.02**	0	0
N-Acetyl-L-tryptophan ethyl ester*	0.01§§	0.6	4.4
L-Tryptophan ethyl ester*	0.02	0	0
Glycine methyl ester‖	0.02	0	0
Benzoyl glycine methyl ester§	0.02	0	0
Glycylglycine ethyl ester*	0.02	0	0
L-Lysine methyl ester§	0.02	0	0
L-Lysine ethyl ester¶	0.04	0	0
DL-Serine ethyl ester‖	0.02	0	0
L-Cysteine ethyl ester*	0.02	0	0
L-Glutamic acid dimethyl ester*	0.02	0	0
Glycyl glycine*	0.02	0	0
DL-Leucyl glycine*	0.02	0	0
DL-Leucyl glycyl glycine*	0.02	0	0
Carbobenzoxy-alpha-L-glutamyl-L-tyrosine*	0.02	0	0
Carbobenzoxy-glycyl-L-phenylalanine*	0.02	0	0
Benzoyl glycine amide*	0.02	0	0
Carbobenzoxy-glycyl-L-phenylalanine amide*	0.005	0	0
L-Leucinamide*	0.02	0	0

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‡ Department of Chemistry, Western Reserve University.
§ H. M. Chemical Co., Santa Monica, California.
‖ Nutritional Biochemical Corp., Cleveland, Ohio.
¶ Dr. S. Sherry, St. Louis.
** 5 per cent methyl cellosolve in reaction mixture.
‡‡ 10 per cent methyl cellosolve in reaction mixture.
§§ 15 per cent methyl cellosolve in reaction mixture.
‖‖ 25 per cent methyl cellosolve in reaction mixture.

tyrosine ethyl ester, *p*-toluenesulfonyl-L-arginine methyl ester and benzoyl-L-arginine methyl ester was also observed by the Hestrin technique (14), which measures the concentration of ester linkages.

Activated C'1 of rat or rabbit origin also digested *N*-acetyl-L-tyrosine ethyl ester, as tested by the acidometric method, but the activity of these preparations was much weaker than that of human origin.

Thus far, no protein substrates of activated C'1 or eluate AE have been found. It had been shown earlier that there was no spontaneous hydrolysis of bovine fibrin by activated C'1 or eluate AE within an hour. However, fibrin was digested rapidly upon the addition of streptokinase by both these preparations, indicating that plasminogen was present in the mixture (4, 5). In the absence of streptokinase, neither enzyme preparation digested casein within

TABLE II

Relative Hydrolysis of Five Susceptible Substrates by Activated C'1

Substrate*	Microequi-valents of acid liberated in 15 minutes
N-Acetyl-L-tyrosine ethyl ester‡	3.6
p-Toluenesulfonyl-L-arginine methyl ester	1.8
Benzoyl-L-arginine methyl ester	0.7
N-Acetyl-3,5 dinitro-L-tyrosine ethyl ester	0.4
N-Acetyl-L-phenylalanine ethyl ester‡	0.0

* All at a concentration of 0.02 M.

‡ 10% methyl cellosolve in reaction mixture.

an hour (4, 5). In further experiments, both activated C'1 and eluate AE hydrolyzed appreciable amounts of casein during a period of 24 hours. The amount digested was greatly enhanced by the addition of streptokinase in a concentration of 2,000 Christensen units per ml. of enzyme-substrate mixture. The digestion of casein by eluate AE was inhibited by the presence of soy bean inhibitor in a concentration of 0.5 mg. per ml. of enzyme-substrate mixture. Soy bean inhibitor did not inhibit the digestion of *N*-acetyl-L-tyrosine ethyl ester by activated C'1 or eluate AE (Section 3). For this reason it seems likely that the digestion of casein could be attributed to the presence of plasminogen in these preparations.

Digestion of gelatin, egg albumen, bovine serum albumin, human plasma or serum, denatured hemoglobin, edestin, lactalbumin, beta-lactoglobulin, and human myometrium could not be detected by the methods used.

2. Some Studies of the Kinetics of Hydrolysis of N-Acetyl-L-Tyrosine Ethyl Ester by Activated C'1:

(*a*) *Substrate Concentration.*—A constant amount of *N*-acetyl-L-tyrosine ethyl ester was hydrolyzed by activated C'1 per unit time for the first 30

minutes of the reaction between enzyme and substrate, when the initial concentration of substrate was 0.08 M, the initial pH was 7.5, the ionic strength of the mixture was 0.15, and the amount of acid liberated did not exceed about 15 microequivalents per ml. (Fig. 1). At lower concentrations of substrate, the
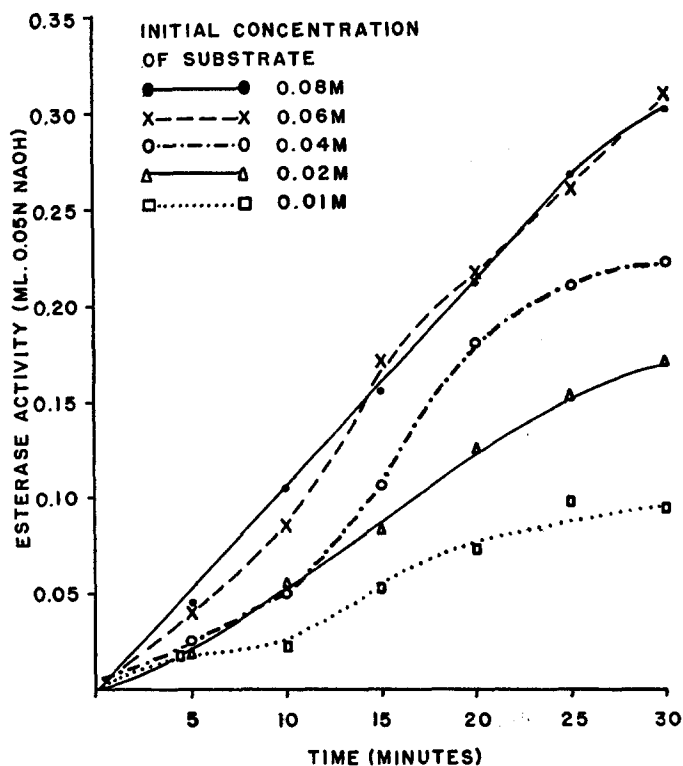

FIG. 1. The influence of substrate concentration on the rate of digestion of N-acetyl-L tyrosine ethyl ester by activated C'1. The experiment was performed at 37°C. at pH 7.5, ionic strength 0.15 (ignoring the substrate itself), and a concentration of 5 per cent methyl cellosolve.

degree of digestion was not a linear function of time. The values obtained were such that it was not feasible to calculate the Michaelis-Menten constant.

(b) *Enzyme Concentration.*—When the initial concentration of N-acetyl-L-tyrosine ethyl ester in the enzyme-substrate mixture was 0.08 M, the amount of acid liberated during a 20 minute period was proportional to the concentration of activated C'1 provided that the amount of acid liberated during this period did not exceed about 12.5 microequivalents per ml. In other words, under these conditions, zero order kinetics prevailed. With higher concentrations of enzyme, the assay was not linear, but the exact limits of linearity were not explored since a lesser amount of enzyme was used in most experiments. At the same initial

concentration of substrate, the degree of hydrolysis was also proportional to the concentration of enzyme when this substrate was allowed to react with eluate AE.

(c) *Optimal pH.*—When the initial concentrations of enzyme and substrate were kept constant, maximal hydrolysis of *N*-acetyl-L-tyrosine ethyl ester, at ionic strength 0.15 and substrate concentration 0.08 M, was observed between pH 7.5 and 8.2 (Fig. 2).

(d) *Optimal Temperature.*—The effect of temperature on the rate of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by activated C'1 was tested between 0° and 45°C. (Fig. 3). Between 25° and 38°, the logarithm of the amount of acid liberated was proportional to the reciprocal of the absolute temperature. Below
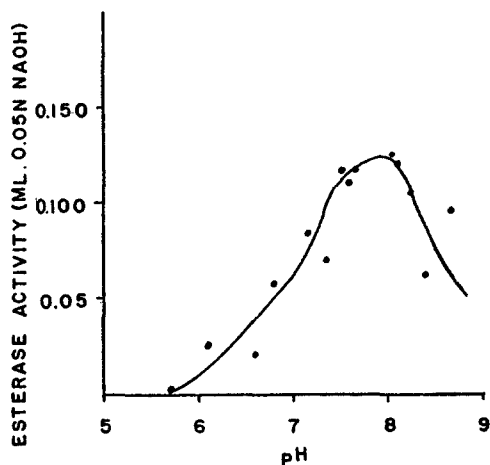

FIG. 2. The influence of pH on the rate of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by activated C'1 at 37°C. and ionic strength 0.15.

25°C., the amount of substrate hydrolyzed was relatively less, and no hydrolysis was measurable at 0°C. The maximal rate of hydrolysis was observed at 41°C. Above this temperature, the rate of digestion of substrate decreased. In other experiments, it was found that virtually no acid was liberated at 50°C. or 54°C. during a period of 30 minutes. The decrease in enzymatic activity at these higher temperatures was apparently the result of denaturation of the enzyme. Activated C'1 was heated at 53°C. for 30 minutes, and then tested for its ability to digest *N*-acetyl-L-tyrosine ethyl ester at 37°C. No measurable amount of enzymatic activity was demonstrable during a period of 15 minutes, although the unheated enzyme released 6.9 microequivalents of acid from the substrate during this time.

In one experiment, the effect of temperature on the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by eluate AE was found to parallel digestion by activated C'1, as tested at 25° and 37°C.

The apparent energy of activation for the hydrolysis of N-acetyl-L-tyrosine ethyl ester by activated $C'1$ was calculated from the data in Fig. 3, using the Arrhenius equation (17): $V = ze^{-\mu/RT}$ in which V is the velocity of the reaction, z is a constant, e is the base of natural logarithms, T is the absolute temperature, R is the gas constant, and μ is the critical thermal increment or energy of activation. In biological systems, the value μ has also been called "the temperature characteristic" (18) and may not be identical with the energy of acti-
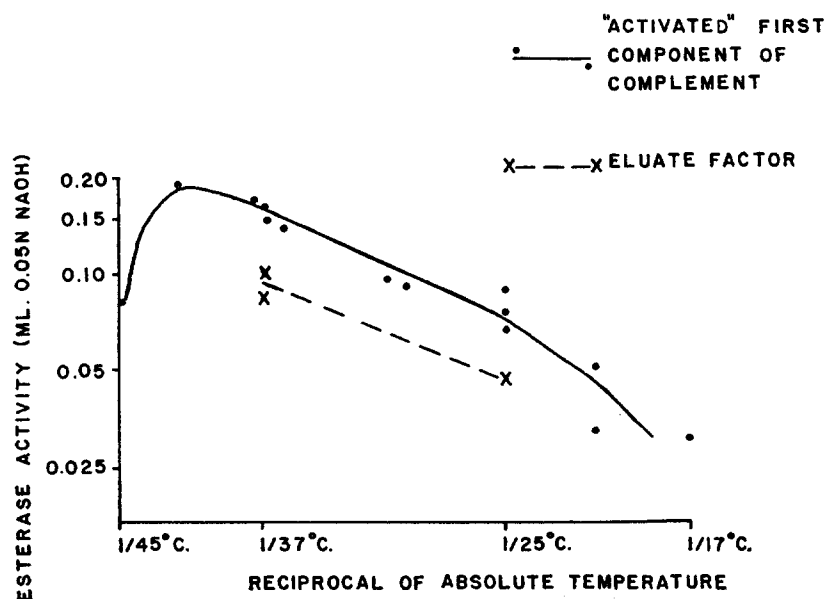

Fig. 3. The relationship between temperature and the rate of hydrolysis of N-acetyl-L-tyrosine ethyl ester by activated $C'1$ and by the eluate factor. The temperature is plotted as the reciprocal of the absolute temperature, but the legend appears in terms of the centrigrade scale for convenience.

vation. In Fig. 3, the method of least mean squares was used to determine the slope of the curve and a value for μ of 12,000 calories per mol. was calculated between 25° and 38°C.

(e) *Ionic Strength.*—The kinetic studies which have been described were performed at an ionic strength of approximately 0.15, ignoring the contribution of the substrate itself to ionic strength. The influence of ionic strength on the amount of acid liberated during the incubation of activated $C'1$ and N-acetyl-L-tyrosine ethyl ester was tested at 37°C. (Table III). Within the range tested, the rate of hydrolysis of the substrate was not appreciably influenced until the ionic strength of the mixture was increased above 0.250. At higher salt concentrations, enzymatic activity was moderately inhibited.

3. Inhibition of Activated C'1:

The influence of various substances on the enzymatic activity of activated C'1 was tested, using N-acetyl-L-tyrosine ethyl ester as substrate. *Human serum* inhibited the digestion of this substrate (Table IV). The inhibitory effect of serum could be demonstrated as soon as it was mixed with activated C'1. Incubation of serum and activated C'1 at 37°C. for 30 minutes prior to the addition of substrate did not increase inhibition of the enzyme. The inhibitory property was greatly diminished by heating fresh serum at 56°C. for 30 minutes, and to a lesser degree by preliminary incubation of the serum with streptokinase. The inhibitory property was non-dialyzable. It could not be identified with the known components of complement (Table IV). A fraction of serum deficient in the second component of complement (R2) lacked the inhibitory

TABLE III

The Influence of Ionic Strength on the Hydrolysis of N-acetyl-L-tyrosine Ethyl Ester by Activated C'1

Ionic strength of enzyme-substrate mixture	Microequivalents of acid liberated in 15 minutes
0.075	8.8
0.100	8.5
0.125	7.7
0.150	8.2
0.200	8.9
0.250	7.9
0.350	7.2
0.500	6.1

effect. However, complement-fixed serum, in which no detectable amount of the second component was present, had the same inhibitory effect as untreated serum. Presumably, then, the inhibitory property of serum was related to something which was deficient in the R2 fraction and yet was not inactivated by complement fixation. The fraction of serum responsible for the inhibition of the esterase did not survive the fractionation of human plasma by the method of Cohn, for fractions prepared in this manner lacked inhibitory activity. Indeed, fractions I and III appreciably hydrolyzed N-acetyl-L-tyrosine ethyl ester.

Pancreatic trypsin inhibitor, soy bean inhibitor, egg white trypsin inhibitor, heparin, and *disodium ethylenediamine tetraacetic acid* did not appear to affect the digestion of N-acetyl-L-tyrosine ethyl ester by activated C'1 in the concentrations tested. The preparation of *diisopropyl fluorophosphate* used inhibited the esterase only in a concentration of 1.25×10^{-3}M or higher; at this concentration, visible precipitation was observed.

Several reducing and oxidizing agents were tested for their effect on the

activity of the esterase. Although *ascorbate* did not appear to be inhibitory, *cysteine*, *reduced glutathione*, and *thioglycollic acid* all diminished the activity of the enzyme (Table IV C). The action of these substances appeared to be on the enzyme rather than on the substrate, since little or no effect was observed unless the inhibitor and enzyme were incubated together at 37°C. for a period of 30 minutes before the addition of substrate. On the other hand, *peroxide* and

TABLE IV

The Inhibitory Effect of Certain Agents on the Digestion of N-acetyl-L-tyrosine Ethyl Ester by Activated C'1

Agents	Microequivalents of acid liberated in 15 minutes
A. 1. 0.15 N sodium chloride..	6.9
2. Human serum...	0
3. 56°C. heated serum..	8.0
4. R1*...	0
5. R2*...	6.8
6. R3*...	0.2
7. R4*...	0.6
B. 1. 0.15 N sodium chloride.......................................	8.1
2. Human serum...	0.7
3. Human serum (diluted to 50 per cent).........................	3.5
4. Complement-fixed serum.....................................	1.0
5. Streptokinase treated serum.................................	3.5
C. 1. 0.15 N sodium chloride.......................................	7.9
2. 0.02 M sodium ascorbate.....................................	8.2
3. 0.02 M cysteine..	4.0
4. 0.02 M reduced glutathione..................................	4.5
5. 0.02 M sodium thioglycollate.................................	1.2

A, B, and C represent separate experiments.

* R1, 2, 3, and 4 represent preparations of serum deficient respectively in the first, second, third, and fourth components of complement.

oxidized glutathione did not inhibit digestion of N-acetyl-L-tyrosine ethyl ester under the conditions tested. The inhibition of activated C'1 by thioglycollic acid could not be reversed by subsequent incubation with oxidized glutathione.

The presumed end-product of the digestion of N-acetyl-L-tyrosine ethyl ester, *N-acetyl-L-tyrosine*, tested at a concentration of 0.02 M, did not inhibit activated C'1.

DISCUSSION

The possibility that complement exerts its hemolytic effect on sensitized red cells through an enzymatic process has intrigued investigators for many years.

This hypothesis was advanced as early as 1910 by Liefmann (19), and his ideas were apparently foreshadowed by Ehrlich. Others occasionally defended these views (1, 20–23), but the demonstration of the enzymatic nature of complement has been elusive. In 1954, we suggested on the basis of studies on the effect of plasmin that the first component of complement was the precursor of an enzyme (1). Investigation of the properties of preparations derived from the first component of complement has confirmed and extended this hypothesis, for such preparations digested certain synthetic amino acid esters (4, 5). Similarly Levine (6) and Becker (7) reported that hemolysis by guinea pig complement was inhibited by diisopropyl fluorophosphate, which is known to inhibit esterases and other hydrolytic enzymes. The inhibitory effect of diisopropyl fluorophosphate was directed primarily against the first component of complement, which was therefore thought to be the precursor of an esterase (7). Using different techniques, Becker (8) has confirmed our observation that the first component of complement is the precursor of an enzyme which will hydrolyze p-toluenesulfonyl-L-arginine methyl ester.

In the present study, the properties of the esterase derived from preparations of the first component of complement have been examined further. Four additional substrates have been found, all esters of amino acids (Table I). The hydrolysis of these compounds by the enzyme derived from the first component occurred at the ester linkage. No evidence has yet been adduced of hydrolysis of proteins, peptides, or amides. However, it is possible that in nature such compounds may be digested, for the esterase appears to inactivate the second and fourth components of complement by a mechanism which is not yet understood (4, 5). The spectrum of susceptible substrates seems to differentiate the estrase derived from complement from such other enzymes as trypsin, chymotrypsin, serum peptidase, serum acid or alkaline phosphatase, cholinesterase, plasmin, and thrombin.

Another idea old to immunology has been that antigen-antibody reactions in some manner release enzymatic activity (24–27), but again the evidence that has been offered has been controversial. In a preliminary report, it was demonstrated that antigen-antibody aggregates partially removed an enzyme or its precursor from serum (5). An esterase was eluted from such serum-treated aggregates which had properties remarkably similar to those of the enzyme derived from preparations of the first component of complement. In the present report, the parallelism between the properties of the esterase obtained from these two sources has been extended, for both hydrolyzed the same substrates, both inhibited the second and fourth components of complement, and both had the same apparent energy of activation. An esterase with similar properties could also be found in fractions I and III of human plasma, prepared by the method of Cohn.[3]

[3] Fractions of plasma prepared by the method of Cohn were provided through the courtesy of the late Dr. E. J. Cohn, Boston, and E. R. Squibb Division, Mathieson Chemical Company.

Experiments have also been described demonstrating that human serum inhibited the esterase (Table IV). The inhibitory property was heat labile and did not survive fractionation of plasma by the method of Cohn. The inhibitory property was also partially destroyed by the addition of streptokinase, but it could not be identified with any of the known components of complement. The enzyme could also be inhibited by sulfhydryl compounds, but this inhibition did not appear to result from the reversible reduction of disulfide bonds.

The data which have been reported support the hypothesis presented earlier (1) that the first component of complement is converted into an active form during the course of antigen-antibody reactions. At that time, it was suggested that streptokinase inhibited complement by converting the first component into an agent which in turn inhibited the second and fourth components (1). Experiments now in progress indicate that the addition of streptokinase to preparations of the first component does activate the esterase which has been described. This appears to be direct evidence in support of the foregoing hypothesis.

Finally it must be pointed out that no physiologic role for the esterase has been uncovered. It is tempting to suggest that this enzyme prepares the erythrocyte or other cells sensitized by specific antibody, for the hemolytic or cytotoxic action of one or another of the other components of complement. Experiments designed to test this hypothesis are under way.

SUMMARY

Studies on an esterase derived from partially purified preparations of the first component of complement are described. The esterase hydrolyzed certain synthetic amino acid esters, among which N-acetyl-L-tyrosine ethyl ester was most susceptible. This was hydrolyzed maximally between pH 7.5 and 8.2, and at 41°C. The esterase could not be identified with other previously described hydrolytic enzymes. An esterase with similar properties could also be eluted from antigen-antibody aggregates which had been treated with serum.

Human serum contained a heat-labile inhibitor of the esterase which could not be identified with any of the known components of complement. The esterase was also inhibited by certain reducing agents.

The experiments described support the early hypothesis that complement exerts its action enzymatically, but the physiological role of the esterase derived from preparations of complement is not yet clear.

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