

STUDIES ON THE ACTIVATION OF A PROESTERASE ASSOCIATED
WITH PARTIALLY PURIFIED FIRST COMPONENT OF
HUMAN COMPLEMENT*

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Previous investigations have indicated that the first of the four recognized components of complement exists in serum as a proesterase which may be activated by antigen-antibody aggregates (1-3), by plasmin (1), or, in the partially purified state, by adjustment of physico-chemical conditions (4).

The active esterase hydrolyzed certain synthetic amino acid esters, among which *N*-acetyl-L-tyrosine ethyl ester was most susceptible (3-5). This substrate was hydrolyzed maximally between pH 7.5 and 8.2 and at 41°C. The esterase was inhibited by a heat-labile factor in human serum which did not appear to be related to any of the known components of complement, and by certain sulfhydryl-containing reducing agents. The esterase could not be identified with other known hydrolytic enzymes of serum (5)

The possible role of the first component of complement (*C*'1) as a proenzyme was first suggested by studies of the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates (1, 2). Subsequently, two independent lines of evidence appeared to substantiate this hypothesis. In the first approach (3), an esterase with complement-inactivating activity could be eluted from antigen-antibody aggregates which had been treated with *C*'1-containing serum reagents. In the second approach (4), with which the present report is further concerned, *C*'1 was partially purified at pH 5.5 and at low temperatures and was brought into the soluble phase only at relatively high ionic strengths. Under these conditions, the preparation retained its hemolytic activity in the presence of the second, third, and fourth components of complement provided that it was maintained at pH 5.5, ionic strength 0.30 until just before assay. However, when the *C*'1 was first adjusted to physiological

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conditions, it lost its hemolytic activity and acquired instead the ability to inactivate the second and fourth components of complement and to hydrolyze certain synthetic amino acid esters. Preliminary experiments indicated that, under the conditions investigated, a correlation existed between the rate of disappearance of hemolytically active C'1 and the rate of appearance of complement-inactivating and esterase properties.

The kinetics of the spontaneous activation of the partially purified proesterase (C'1) have now been studied much more extensively. Activation has been found to follow the kinetics for second order autocatalysis and to occur optimally at pH 7.3-7.7 with an apparent energy of activation of 31,000 calories per mol. Activation was inhibited by small concentrations of ethylenediaminetetraacetate, by ionic strengths above 0.15, and by small concentrations of fresh serum. Experiments have also been performed which indicate that the proesterase may be activated by plasmin, thus substantiating an hypothesis presented earlier (1). The correlation obtained between disappearance of C'1 and appearance of esterase under the wide range of conditions investigated provides further direct evidence for the proesterase role of the first component of complement.

Nomenclature and Materials

First Component of Human Complement (C'1) was prepared by the procedure described previously (4). The partially purified 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 (6) was about 8 units per ml. when the first component was prepared from serum, and 1 unit or less per ml. when it was prepared from serum from which most of the properdin had been removed by treatment with zymosan. 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 (7). The recently described clot-promoting fractions, Christmas factor (plasma thromboplastin component) (8) and Hageman factor (9), 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 pH 5.5 and ionic strength 0.30 at -25°C .

Partially purified C'1 lost its hemolytic activity and acquired complement-inactivating and esterase activities when adjusted to certain conditions of 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 C'1 itself (3). In earlier studies, activated C'1 was referred to as "converted C'1" (3, 4).

Human serum reagents deficient in the first, second, third, or fourth components of complement, designated *R1*, *R2*, *R3*, *R4*, respectively, were prepared by conventional procedures described in detail elsewhere (10). *R1* and *R2* were prepared by dialysis of serum against pH 5.5 acetate buffer of ionic strength 0.02. *R3* was prepared by treatment of serum with zymosan (11), and *R4* by treatment with hydrazine (12). All reagents were diluted 1.5-fold with respect to serum.

Sheep blood was collected weekly in an equal volume of Alsever's solution (13) and maintained at 1°C. The red cells were washed two times with 0.15 M NaCl, finally with barbital-Ca⁺⁺-Mg⁺⁺ buffer, and then suspended in buffer containing 4 units of rabbit hemolysin per ml. to give a final suspension of *sensitized sheep cells* containing 2.5×10^6 cells/mm.³. This standardization was effected by reference to a standard curve relating cell count and optic density at 550 m μ .

Barbital-Ca⁺⁺-Mg⁺⁺ buffer (10), for use in complement assays, had a pH of 7.4 at ionic strength 0.15 and contained 1.5×10^{-4} M Ca⁺⁺ and 5×10^{-4} M Mg⁺⁺. *Sodium phosphate buffer* was composed of 1.82 gm. of NaH₂PO₄·H₂O and 16.75 gm. of Na₂HPO₄·12 H₂O per liter, and had a pH of 7.4 and ionic strength 0.15. *Barbital-saline buffer* was prepared by dissolving 2.76 gm. of barbital, 2.06 gm. of sodium barbital, and 7.40 gm. 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 over a wide range of pH at ionic strength 0.15 (14).

N-Acetyl-L-tyrosine ethyl ester, a substrate to test esterase activity, was synthesized in the Department of Chemistry, Western Reserve University. It was dissolved to a 1.6 M solution in 2-methoxyethanol (methyl cellosolve).

p-Toluenesulfonyl-L-arginine methyl ester (H. M. Chemical Co.), a substrate hydrolyzed by both esterase and plasmin, was dissolved in a concentration of 0.16 M in phosphate buffer.

Pneumococcal specific-soluble substance, Type III (S-III),¹ was dissolved in 0.15 M NaCl to a stock concentration of 1 mg. per ml.

Anti-pneumococcal rabbit serum, Type III (Anti-S-III),¹ a refined and concentrated anti-serum containing 5 mg. antibody nitrogen per ml. (10), was diluted as required in 0.15 M NaCl.

Complement-fixed (CF) serum was prepared by adding 200 μ g. of anti-S-III antibody nitrogen and 8 μ g. of S-III per ml. of serum in a final dilution of serum of 1.5-fold. The mixture was incubated at 37°C. for 1 hour, centrifuged at 0°C. at 4000 R.P.M. for 1 hour, and the clear supernatant solution retained at 0°C. for use on the same day.

Streptokinase ("varidase" or "high purity"),¹ the filterable principle of cultures of beta hemolytic streptococci which activates plasminogen, was dissolved in distilled water or sodium chloride solution to a suitable concentration.

Streptokinase-treated (SK) serum was prepared by adding one-half volume of a dilution of streptokinase containing 2000 units per ml. to one volume of serum and incubating at 37°C. for 1 hour. The resulting treated serum, diluted 1.5-fold with respect to the original serum, was held at 0°C. for use on the same day.

Amberlite IRC-50 (sodium cycle), adjusted to pH 7.7-7.8, was prepared from the hydrogen cycle (Fisher Scientific Company, Pittsburgh) as previously described (15).

Disodium ethylenediaminetetraacetic acid (EDTA) (Bersworth Chemical Company, Framingham, Massachusetts) was dissolved in distilled water and adjusted to pH 7.4 with sodium hydroxide at a final concentration of 0.15 M.

Crystalline soy bean inhibitor (Nutritional Biochemicals Company) was dissolved variously in phosphate buffer or in 0.15 M NaCl to a concentration of 10 mg. per ml.

Methods

A *unit of complement* is defined as the smallest amount of fresh serum which will cause complete hemolysis of 1 ml. of a standardized suspension of sensitized sheep cells in a final

¹ Provided through the courtesy of Lederle Laboratories Division, American Cyanamid Company.

volume of 1.5 ml. after 30 minutes at 37°C. (1, 10). The volume unit of R1, R2, R3, and R4 was based on the complement unit of the serum from which these reagents were prepared.

Titrations of individual components of complement were carried out according to general methods described elsewhere (1, 10, 16). The sample to be titrated was diluted serially in barbital-Ca⁺⁺-Mg⁺⁺ buffer in an ice bath. In the case of C'1 an initial dilution in an equal volume of R1 was made, prior to further dilution in buffer, in order to prevent activation of partially purified C'1 which could otherwise occur under the conditions of titration (4). To a series of 12 × 75 mm. tubes in an ice bath, 0.2 ml. of the sample dilutions, a constant amount of reagent (5 units of R1, 2 units of R2, 3 units of R3, or 3 units of R4, depending upon the component to be titrated) and 1 ml. of sensitized sheep cells were added. The tubes were mixed, incubated at 37°C. for 30 minutes, centrifuged at 2000 r.p.m. for 5 minutes, and compared visually with a 50 per cent hemolytic standard. The titer was expressed as the reciprocal of the sample dilution which gave 50 per cent hemolysis. This value multiplied by 5 gave the number of units of component per milliliter of sample. The endpoint was read to the nearest half-tube; for example, if a dilution of 1:64 gave 70 per cent hemolysis and 1:128 gave 30 per cent hemolysis, the endpoint was taken at 1:96 and the titer expressed as 480 units per ml. Criteria for the suitability of complement reagents and limitations of the double dilution titration procedure are discussed elsewhere (1, 10, 16, 17).

Esterase activity was measured by micro-formol titration of the acid liberated from *N*-acetyl-L-tyrosine ethyl ester after incubation with enzyme at 37°C. for 15 minutes at pH 7.4, ionic strength 0.30, and a substrate concentration of 0.08 M (3-5, 7). The relatively high ionic strength was chosen to inhibit further activation of C'1 during the assay procedure (4), without interfering seriously with esterase activity (5). Typically, 1.50 ml. of phosphate buffer, 0.5 ml. of M NaCl, and 0.375 ml. of an appropriate enzyme dilution were mixed and incubated at 37°C. At zero time, 0.125 ml. of 1.6 M *N*-acetyl-L-tyrosine ethyl ester in methyl cellosolve at 37°C. was added to the enzyme-buffer mixture. Samples of 1 ml. of the enzyme-buffer-substrate mixture were removed at the start and after 15 minutes of incubation at 37°C., 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. Esterase activity was expressed as the difference, in milliliters of 0.05 N NaOH, between the titrations for the 15 minute and zero time aliquots. All titrations were performed with a 1 ml. micro-syringe (Micrometric Instrument Corporation, Cleveland).

Nitrogen was determined by a modification of the micro-Kjeldahl procedure of Pregl.

Electrophoretic analyses were performed in a Perkin-Elmer electrophoresis apparatus in 2 ml. cells, using 0.5 per cent protein solution and buffer consisting of 0.28 M NaCl and 0.02 M sodium acetate-acetic acid buffer at pH 5.5.

RESULTS

1. *The Influence of Temperature on the Rate of Activation of Partially Purified C'1.*—

The rates of disappearance of hemolytically active, partially purified C'1 and of appearance of esterase activity were measured as a function of temperature at pH 7.1, ionic strength 0.15, and an initial concentration of C'1 in the final reaction mixture of 2500 units per ml. At the desired temperature, one part of partially purified C'1 at pH 5.5, ionic strength 0.30, was mixed with 2 parts of pH 7.4 phosphate buffer, ionic strength 0.075. Aliquots were withdrawn at frequent time intervals and assayed for C'1 titer, esterase activity, or both. Typical

rate curves for C'1 disappearance are shown in Fig. 1. The correlation between the time of C'1 disappearance and of esterase appearance is demonstrated in Table I.

Mixed temperature experiments revealed that the rate of activation at a given temperature could be reduced by lowering the temperature during the course of the reaction and could be restored to its initial value by re-establishing the original temperature.

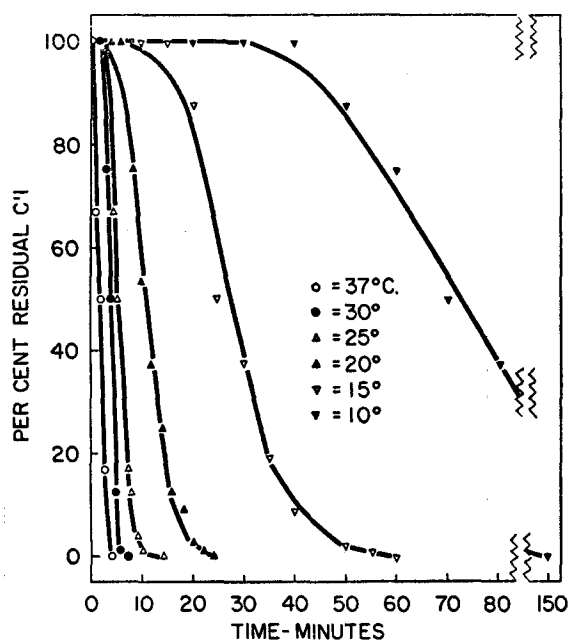


FIG. 1. The rate of disappearance of hemolytically active, partially purified C'1 as a function of temperature, at pH 7.1, ionic strength 0.15, initial concentration of C'1 of 2500 units per ml.

TABLE I

A Comparison of the Time Required for Complete Disappearance of Hemolytically Active, Partially Purified C'1 and for Complete Activation of Esterase Activity, as a Function of Temperature

Temperature	Time required for	
	Disappearance of C'1	Appearance of esterase
°C.	min.	min.
37	3.5-4	3-4
25	9-11	8-10
20	20-24	15-20
15	50-60	40-60

pH 7.1, ionic strength 0.15, initial concentration of C'1 of 2500 units per ml.

Within the limits of experimental error, the curves in Fig. 1 conform to the equation for second order autocatalysis. The integrated form of this equation (18),

$$k = \frac{1}{t(a+b)} \ln \frac{a(b+x)}{b(a-x)},$$

may be used to calculate k , the specific reaction rate. Such calculations may be made on the

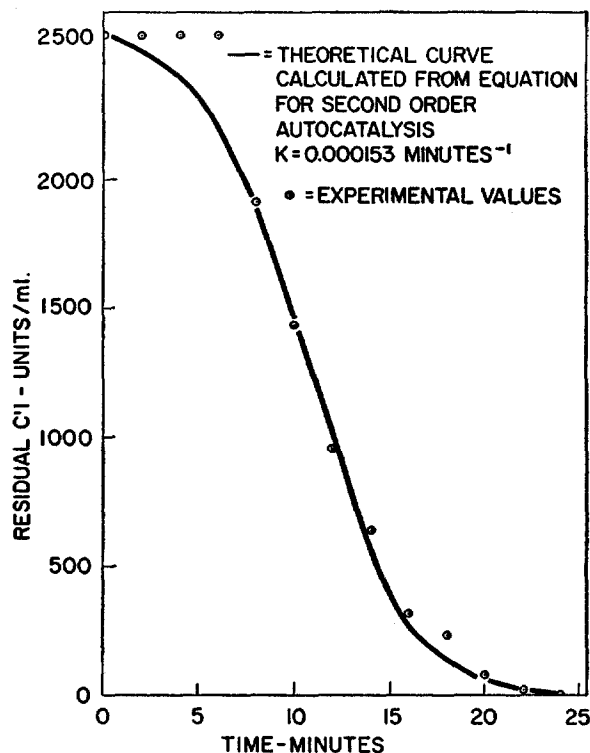


FIG. 2. A comparison of the theoretical curve calculated from the equation for second order autocatalysis with experimental values for the rate of disappearance of hemolytically active, partially purified C'1 at 20°C. and at pH 7.1, ionic strength 0.15, initial concentration of C'1 of 2500 units per ml.

following empiric basis:

a = units per milliliter of C'1 present at time t_1 .

b = (units per milliliter of C'1 present at time t_0) - (units per milliliter of C'1 present at time t_1).

x = (units per milliliter of C'1 present at time t_1) - (units per milliliter of C'1 present at time t_2).

t = $t_2 - t_1$, in minutes.

For example, employing the data represented in Fig. 1, the average value for k has been found to be 0.000153 min.^{-1} at 20°C. Comparison of the experimental and theoretical curves at this temperature is shown in Fig. 2. It will be noted that, except at the beginning of the

reaction, the experimental values fit the theoretical curve calculated from the equation for second order autocatalysis. The apparent discrepancy during the first 6 minutes is attributable to the inability to measure small changes in C'1 at high initial concentrations of C'1 by the assay procedure employed in this study. The kinetics of activation of C'1 to esterase, conforming to second order autocatalysis, are identical in type with those for the conversion of pepsinogen to pepsin (19) and trypsinogen to trypsin (20).

The energy of activation for the conversion of C'1 to esterase was calculated, using the

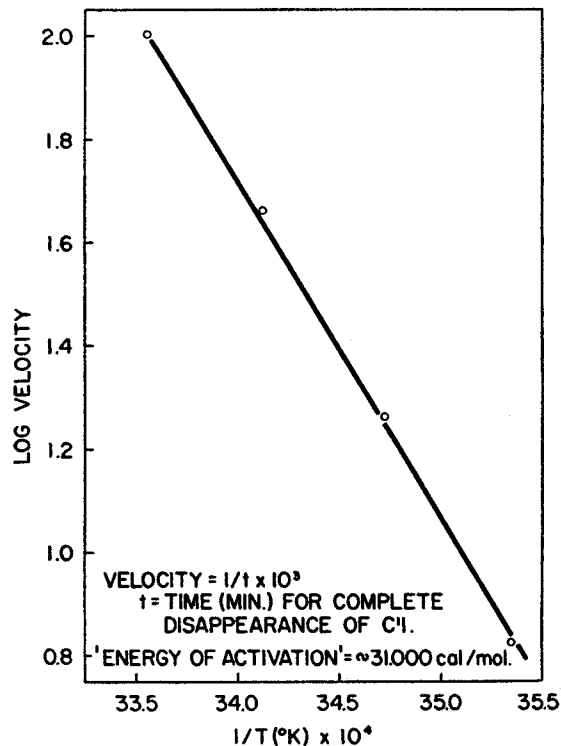


FIG. 3. Arrhenius plot of the data in Fig. 1.

Arrhenius equation (21):

$$V = ze^{-\mu/RT}$$

in which V = the velocity of the reaction, z = a constant, e = the base of natural logarithms, T = absolute temperature, R = the gas constant, and μ = the critical thermal increment or energy of activation. The velocity of the activation reaction at each temperature studied was defined as $\frac{1}{t} \times 10^3$, in which t = the time in minutes for complete disappearance of hemolytic C'1 activity. A plot of the logarithm of velocity *versus* the reciprocal of the absolute temperature gave a straight line (Fig. 3) at temperatures between 10° and 25°C. From the slope of this line, a value of about 31,000 calories per mol was calculated for the energy of activation. The slope of the line and the value for μ were unchanged by defining the velocity of the reaction in terms of 50 per cent disappearance of hemolytic C'1 ac-

tivity. In biological systems, the value μ has also been called "the temperature characteristic" (22) and may not be identical with the energy of activation. The value for μ of 31,000 calories per mol for the activation of C'1 to esterase may be compared with the value of 12,000 calories per mol for the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by the activated esterase (5).

2. The Influence of pH on the Rate of Activation of Partially Purified C'1.—

The rate of disappearance of hemolytically active, partially purified C'1 and of appearance of esterase activity was measured as a function of pH at ionic strength 0.15 and an

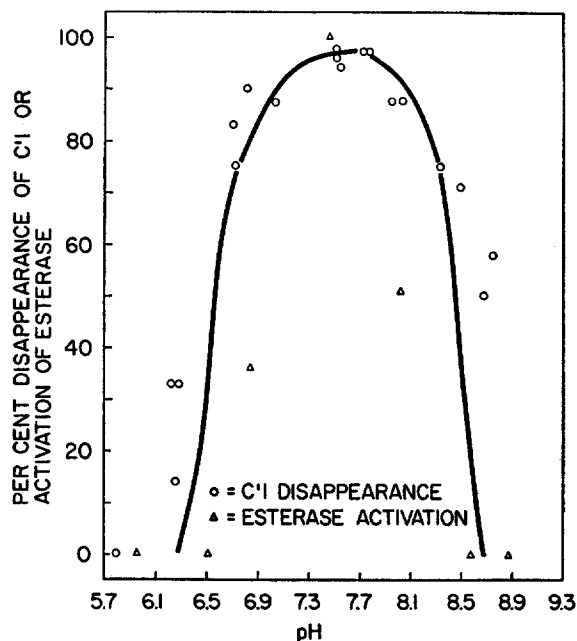


FIG. 4. The rate of disappearance of hemolytically active, partially purified C'1 and of activation of esterase as a function of pH, at 20°C. for 30 minutes, ionic strength 0.15, initial concentration of C'1 of 1000 units per ml.

initial concentration of C'1 of 1000 units per ml., during 30 minutes' incubation at 20°C. One part of partially purified C'1 at pH 5.5, ionic strength 0.30 was mixed with 4 parts of Michaelis' acetate-barbital-saline buffer at ionic strength 0.15 and at the desired pH, and with 1 part of distilled water. All reagents were pre-incubated at 20°C. prior to mixing. Aliquots were withdrawn at the start and after 30 minutes' incubation at 20°C. and assayed for C'1 titer, esterase activity, or both. The pH of the final reaction mixture was measured at room temperature at the end of the incubation period.

The results of these experiments are plotted in Fig. 4. It will be noted that the pH optimum both for disappearance of C'1 and for appearance of esterase activity lies at pH 7.3-7.7. Inhibition is progressively more marked on each

side of these pH values and is complete below pH 6 and above pH 9 under the conditions of the experiment. The pH optimum of 7.3–7.7 for the activation of C'1 to esterase may be compared with the pH optimum of 7.5–8.2 for the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by the activated esterase (5).

3. *The Influence of Ionic Strength on the Rate of Activation of Partially Purified C'1.*—

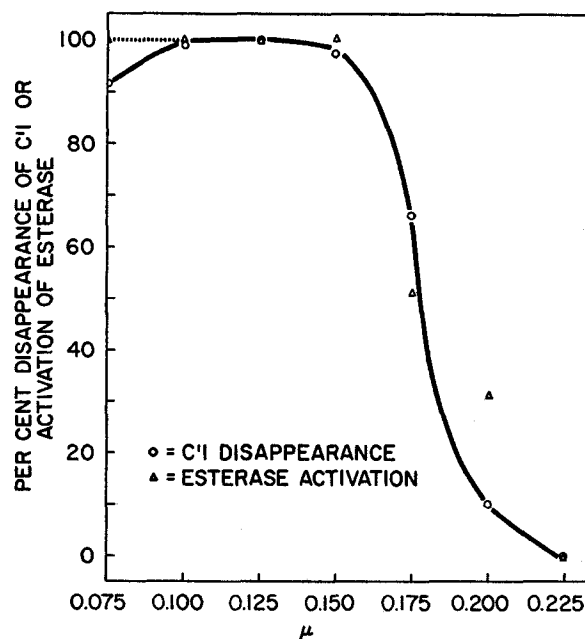


FIG. 5. The rate of disappearance of hemolytically active, partially purified C'1 and of activation of esterase as a function of ionic strength, at 20°C. for 30 minutes, pH 7.1, initial concentration of C'1 of 1500 units per ml.

The rate of disappearance of hemolytically active, partially purified C'1 and of appearance of esterase activity was measured as a function of ionic strength at pH 7.1 and an initial concentration of C'1 of 1500 units per ml., during 30 minutes' incubation at 20°C. Diluents of known ionic strength were prepared by mixing 1 part of pH 7.4 phosphate buffer of ionic strength 0.15 with 4 parts of distilled water or of a sodium chloride solution of appropriate ionic strength. One part of partially purified C'1 at pH 5.5, ionic strength 0.30 at 20°C. was mixed with 5 parts of diluent at 20°C. Aliquots were withdrawn at the start and after 30 minutes' incubation at 20°C. and assayed for C'1 titer, esterase activity, or both.

The results of these experiments, plotted in Fig. 5, demonstrate that activation depends upon the presence of a critical ionic strength. Inhibition both of C'1 disappearance and of esterase appearance occurred above ionic strength 0.15. In separate experiments, this was found to hold even if the activation re-

action were started at ionic strength 0.15 and the ionic strength subsequently elevated. Advantage has been taken of this observation in this investigation by performing assays for esterase activity at ionic strength 0.30, thus inhibiting further activation of C'1 during the procedure. Under the conditions of the experiments inhibition was complete at ionic strength 0.225. The marked effect of ionic strength on the activation of C'1 to esterase may be compared with the small and gradual inhibition of esterase activity at ionic strengths of 0.25 and above (5).

4. The Influence of Cation Exchange, EDTA, and Ca^{++} and Mg^{++} on the Rate of Activation of Partially Purified C'1.—

It was known from previous investigations (1, 2, 15) that the inactivation of complement both by antigen-antibody aggregates and by plasmin is potentiated by Ca^{++} and that this inactivation is very probably a result of activation of a proenzyme (C'1) (1-4). It was also known that divalent cations are not involved in the activity of activated C'1 against synthetic amino acid esters (5). Accordingly, it was of interest to study the effect of Ca^{++} on the activation of C'1 to esterase.

In the first group of experiments, reagents were cation-exchanged with amberlite IRC-50 in the sodium cycle (15). All glassware was scrupulously cleaned and copiously rinsed with ion-exchanged water with a resistance of at least 10^6 ohms (bantam demineralizer, Barnstead Still and Sterilizer Company, Boston, Massachusetts). At 20°C., one part of cation-exchanged, partially purified C'1 at pH 5.5, ionic strength 0.30 was mixed with 4 parts of cation-exchanged Michaelis' acetate-barbital-saline buffer at pH 7.9, ionic strength 0.15 and with 1 part of ion-exchanged water. The final pH of the reaction mixture was 7.5. The rate of disappearance of hemolytic C'1 activity from this mixture at 20°C. was compared with the rate of C'1 disappearance from similar mixtures containing 2.5×10^{-3} M Ca^{++} or 8×10^{-4} M Mg^{++} and with the rate of C'1 disappearance from non-exchanged mixtures with and without added Ca^{++} or Mg^{++} (10^{-6} to 10^{-3} M). No significant differences in rate could be detected among any of these mixtures.

Thus, although cation-exchanging with amberlite IRC-50 in the sodium cycle resulted in inhibition of inactivation of complement by plasmin and to a lesser extent by antigen-antibody aggregates (1, 2, 15), this procedure was without significant effect on the activation of partially purified C'1 to esterase.

In the second group of experiments, the same experimental conditions were employed, except that reagents were not cation-exchanged and, except for controls, one part of an appropriate concentration of EDTA was substituted for water. EDTA in concentrations as low as 5×10^{-5} M in the final mixture completely inhibited disappearance of C'1 and appearance of esterase during 30 minutes incubation at 20°C., at pH 7.5, ionic strength 0.15. The inhibition curve was extremely sharp; 1×10^{-5} M EDTA was without effect. The in-

hibition of C'1 disappearance by EDTA could be overcome by equimolar concentrations of Ca^{++} or by 10-fold greater concentrations of Mg^{++} , as shown in Table II.

TABLE II
Effect of EDTA and of Ca^{++} and Mg^{++} on the Rate of Disappearance of Hemolytically Active, Partially Purified C'1 and of Appearance of Esterase Activity

EDTA	Ca^{++}	Mg^{++}	Disappearance of C'1	Appearance of esterase
M	M	M	<i>per cent</i>	<i>per cent</i>
0	0	0	90	100
10^{-4}	0	0	0	0
10^{-4}	0	10^{-4}	0	—
10^{-4}	0	10^{-3}	80	—
10^{-4}	10^{-4}	0	90	—
10^{-4}	10^{-3}	0	95	—

pH 7.5, ionic strength 0.15, initial concentration of C'1 of 1300 units per ml., 20°C. for 30 minutes.

Inhibition by EDTA was completely reversible by dialysis. Partially purified C'1 at pH 5.5, ionic strength 0.30 was treated with EDTA to a final concentration of 10^{-3} M and dialyzed at 0°C. for 24 hours against 1000 volumes of a buffer mixture consisting of 0.29 M NaCl and 0.01 M acetate buffer of pH 5.5. A control sample without EDTA was dialyzed separately under the same conditions. The dialyzed samples were then brought to pH 7.5, ionic strength 0.15, and incubated at 20°C. for 30 minutes, as described above. No significant difference in the rate of C'1 disappearance could be detected between the two samples, indicating that EDTA inhibition had been overcome by dialysis, while readdition of EDTA to the dialyzed samples restored complete inhibition.

These experiments showed, therefore, that the activation of C'1 to esterase was inhibited by small concentrations of EDTA and that this inhibition could be reversed by equimolar concentrations of Ca^{++} , by larger concentrations of Mg^{++} , and by removing the EDTA by dialysis. It appeared possible that inhibition by EDTA was due to chelation of tightly bound Ca^{++} on the C'1 molecule, not dissociable by amberlite IRC-50. However, a second possibility exists that EDTA was directly bound to C'1 without a metal ion intermediary. Indeed, semi-quantitative spectrographic analysis of partially purified C'1 (National Spectrographic Laboratories, Inc., Cleveland) failed to reveal significant amounts of any polyvalent metal ion.

5. *The Influence of Initial Concentration of C'1 and of Esterase on the Rate of Activation of Partially Purified C'1.*—

The observation that the activation of C'1 to esterase followed the kinetics for second order autocatalysis suggested that the rate of activation would be

functions of initial concentrations of C'1 and of esterase. Experiments designed to test the validity of this prediction were therefore carried out.

The effect of initial C'1 concentration was studied at 20°C., pH 7.1, ionic strength 0.15. At 20°C., one part of partially purified C'1 at pH 5.5, ionic strength 0.30 was mixed with 2 parts of pH 7.4 phosphate buffer, ionic strength 0.075, and with appropriate volumes of 0.15 M NaCl to give initial C'1 titers of 1920, 960, and 240 units per ml. Aliquots were withdrawn at frequent intervals and titrated for C'1.

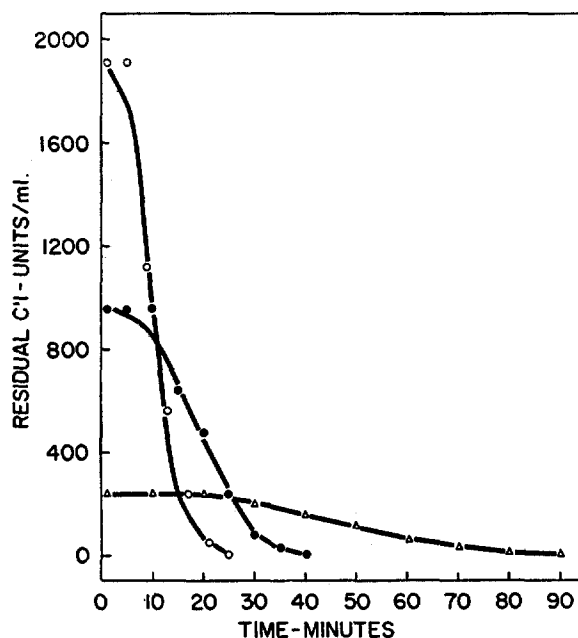


FIG. 6. The rate of disappearance of hemolytically active, partially purified C'1 as a function of initial C'1 concentration, at 20°C., pH 7.1, ionic strength 0.15.

The results given in Fig. 6 demonstrate that the rate of reaction does indeed increase with increasing initial C'1 concentration. However, the rate is not directly proportional to C'1 concentration, as would be predicted from the kinetic equation for second order autocatalysis. Thus, a 4-fold increase in C'1 concentration, from 240 to 960 units per ml., resulted in only 2.3-fold increase in rate; a 2-fold increase in C'1 concentration, from 960 to 1920 units per ml., resulted in only 1.7-fold increase in rate. This smaller than predicted effect of initial proenzyme concentration on reaction rate has also been observed in the autocatalytic activation of pepsinogen (19).

The effect of initial esterase concentration was also studied at 20°C., pH 7.1, ionic strength 0.15, and an initial concentration of C'1 of 2000 units per ml. Activated C'1 was first prepared by adjusting partially purified C'1 to pH 7.4, ionic strength 0.15 by dilut-

ing with an equal volume of water and adding 0.5 N NaOH. The resulting solution, diluted about 2-fold with respect to the original C'1, was incubated at 37°C. for 15 minutes to assure activation and then stored in the cold. Reaction mixtures were then prepared at 20°C. by mixing one part of partially purified C'1 at pH 5.5, ionic strength 0.30; 2 parts of pH 7.4 phosphate buffer, ionic strength 0.075; and 2 parts of an appropriate dilution of preformed esterase in 0.15 M NaCl. Aliquots were withdrawn at frequent intervals and titrated for C'1.

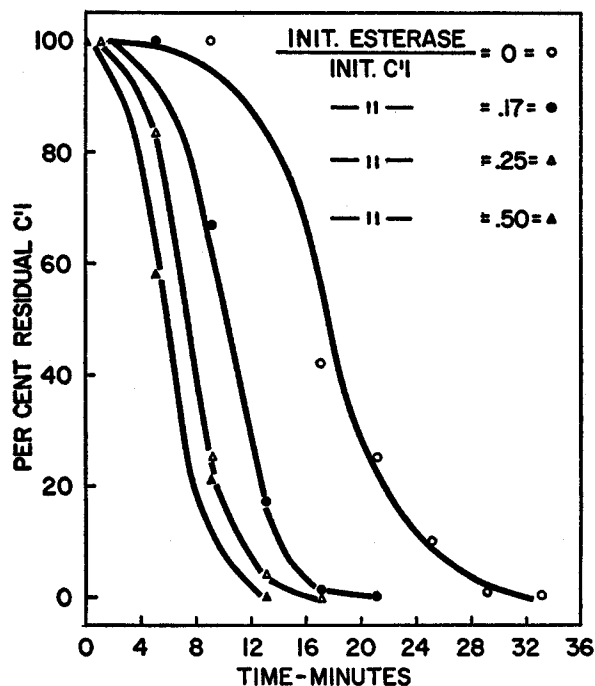


FIG. 7. The rate of disappearance of hemolytically active, partially purified C'1 as a function of initial esterase concentration, at 20°C., pH 7.1, ionic strength 0.15, initial concentration of C'1 of 2000 units per ml.

The data are presented in Fig. 7, in which the initial concentration of esterase is expressed as a ratio of initial esterase to initial C'1 concentrations, on an equivalent volume basis. Thus, if one part of partially purified C'1 were mixed with a buffer diluent containing 0.5 part of preformed esterase (diluted 2-fold with respect to the original C'1), the ratio of initial esterase to initial C'1 concentrations was taken as $\frac{0.5/2}{1} = 0.25$. It will be noted in Fig. 7 that the rate of disappearance of C'1 increased with increasing initial concentration of esterase in accordance with prediction for an autocatalytic reaction. However, as in the case of the experiments on initial C'1 concentration, the rate was not directly proportional to initial esterase concentration, a phenomenon also noted for the autocatalytic activation of pepsinogen (19).

6. *Inhibition of Activation of Partially Purified C'1 by Human Serum and Serum Reagents.*—

It was observed earlier (4) that fresh human serum or R1, the serum reagent deficient in the first component of complement, inhibited the activation of C'1 to esterase. This observation has been of great technical aid as a means of preventing activation during the titration of partially purified C'1 and of stopping the activation reaction in the kinetic studies described in this report. Preliminary investigation of the nature of the serum inhibitor will be described here with particular reference to its possible relation to components of complement.

At 20°C., one part of C'1 at pH 5.5, ionic strength 0.30 was mixed with one part of distilled water and four parts of a solution consisting of various ratios of Michaelis' acetate-barbital-saline buffer of pH 7.7, ionic strength 0.15 and of the serum reagent to be tested for inhibitory activity. These reaction mixtures, at pH 7.4-7.5, ionic strength 0.15, and an initial concentration of C'1 of 2500 units per ml., were incubated at 20°C. for 40 minutes. C'1 titers were obtained at the start and end of incubation. The results (Table III) were expressed in relative inhibition units, arbitrarily defined as twice the reciprocal of the volume of undiluted serum reagent required to effect 50 per cent inhibition of C'1 disappearance under the conditions described.

TABLE III

Some Characteristics of the Factor(s) in Human Serum Inhibiting Spontaneous Activation of Partially Purified C'1

Serum reagent	C'1	C'2	C'3	C'4	Relative inhibition*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Fresh serum	100	100	100	100	100
48°—30 min.	100	70	70	75	100
52°—30 "	5	0	40	75	40
56°—30 "	0	0	25	50	10
R1‡	0	70	15	40	65
R2‡	50	0	50	25	5
R3‡	75	70	0	45	100
R4‡	75	70	80	0	65
CF‡	5	0	50	0	75
SK‡	5	0	60	0	5

pH 7.4-7.5, ionic strength 0.15, initial concentration of C'1 of 2500 units per ml., 20°C. for 40 minutes.

* For definition, see text.

‡ R1, R2, R3, and R4 refer to serum reagents deficient in the first, second, third, and fourth components of complement, respectively. CF and SK refer to serum treated with antigen-antibody aggregates or streptokinase, respectively.

The serum inhibitor (or inhibitors) were found to withstand incubation at 48°C. for 30 minutes. However, about 60 per cent of the inhibitory activity of serum was destroyed at 52°C. for 30 minutes and about 90 per cent at 56°C. for 30 minutes. Thus, the inhibitor was relatively heat-labile. Among the specifically inactivated complement reagents, R1, R2, R3, and R4, only R2 (the serum fraction deficient in the second component of complement) was markedly deficient in the inhibitor. However, C'2 activity is completely destroyed at 50–52°C. for 30 minutes and is therefore more heat-labile than the serum inhibitor. Furthermore, C'2 is completely inactivated under suitable conditions by treatment of serum with antigen-antibody aggregates (complement-fixed serum) or with plasmin (streptokinase-treated serum) (23). Nevertheless, complement-fixed serum retained most of its inhibitory activity, while streptokinase-treated serum was almost entirely lacking in inhibitor. The susceptibility of the inhibitor to inactivation by streptokinase was not explained. Thus, the factor (or factors) in human serum responsible for inhibition of activation of C'1 resembled C'2 in some of its properties but could be distinguished from it.

These findings on the properties of the serum inhibitor of the activation of C'1 are in complete accord with those found for the serum inhibitor of esterase activity against synthetic amino acid esters (5). This agreement would be expected for an autocatalytic reaction, in which inhibition of the active enzyme would effect inhibition of activation of the proenzyme.

7. Activation of Partially Purified C'1 to Esterase by Streptokinase-Activated Plasmin.—

The inhibition of complement activity by plasmin has been attributed to reactions involving at least two steps (1). It was suggested that plasmin converted C'1 to an enzyme which in turn inactivated C'2 and C'4. In the experiments to be described, evidence is presented confirming the presence of plasminogen in partially purified preparations of C'1, and demonstrating that plasmin activates the esterase. These experiments take advantage of several earlier observations: (a) C'1 and the esterase are inactivated at 53°C. (5), a temperature at which plasminogen and plasmin are stable; (b) the esterase digests both *p*-toluenesulfonyl-L-arginine methyl ester and *N*-acetyl-L-tyrosine ethyl ester (3, 4), whereas plasmin digests only the former substrate (7); (c) soy bean trypsin inhibitor inhibits the action of plasmin, but not of the esterase (5).

A preparation of C'1 was divided into two portions, one of which was incubated at 53°C. or 30 minutes. Aliquots of 0.25 ml. of each were then incubated at 37°C. for 15 minutes with 0.75 ml. of a solution of streptokinase (10,000 units per ml.) in 0.3 M sodium chloride. Each preparation was then tested for its ability to hydrolyze *N*-acetyl-L-tyrosine ethyl ester or *p*-toluenesulfonyl-L-arginine methyl ester, maintaining ionic strength at 0.3 by the addition of hypertonic sodium chloride solution. In control experiments, either 0.3 M or 0.15 M sodium chloride solution was substituted for the solution of streptokinase in the initial step.

The addition of streptokinase to unheated C'1 resulted in hydrolysis of both *N*-acetyl-L-tyrosine ethyl ester and *p*-toluenesulfonyl-L-arginine methyl ester. On the other hand, the addition of streptokinase to heated C'1 resulted in hydrolysis of the *p*-toluenesulfonyl-L-arginine methyl ester alone (Table IV). The same result was obtained by activating C'1 by lowering the ionic strength to 0.15, although the digestion of *p*-toluenesulfonyl-L-arginine methyl ester was much less impressive in the absence of streptokinase.

TABLE IV
The Effect of Streptokinase on Heated and Unheated C'1

Activating agent	Ionic strength during activation	Esterase activity microequivalents of acid liberated in 15 min.	
		vs. <i>N</i> -ALTEc*	vs. TAMe†
I. Unheated C'1			
Streptokinase	0.3	2.5	7.7
Sodium chloride solution	0.3	0.3	0.8
Sodium chloride solution	0.15	6.2	1.8
II. C'1 heated at 53°C. 30 min.			
Streptokinase	0.3	0.2	3.2
Sodium chloride solution	0.3	0	0.9
Sodium chloride solution	0.15	0.1	1.1

* Versus *N*-acetyl-L-tyrosine ethyl ester at ionic strength 0.30.

† Versus *p*-toluenesulfonyl-L-arginine methyl ester at ionic strength 0.30.

These experiments confirm the view that partially purified preparations of C'1 contain plasminogen and that the addition of streptokinase to C'1 resulted in the appearance of esterase activity. The following experiments demonstrated that the appearance of esterase activity could be prevented by inhibiting the action of plasmin.

A volume of 0.3 ml. of streptokinase (10,000 units per ml.) in 0.3 M sodium chloride solution was incubated for 30 minutes at 20°C. with 0.3 ml. of C'1 and 0.3 ml. of phosphate buffer or of a solution containing 3 mg. of soy bean inhibitor per ml. of phosphate buffer. An aliquot was then removed and assayed for its ability to hydrolyze *N*-acetyl-L-tyrosine ethyl ester at ionic strength 0.3 and in the presence of soy bean inhibitor in a final concentration of 1 mg. per ml. Control experiments were performed in which either 0.3 M or 0.15 M sodium chloride was substituted for the solution of streptokinase.

In this experiment, the addition of soy bean inhibitor prevented the activation of esterase by streptokinase, but was without effect on the activation of esterase by the adjustment of ionic strength to 0.15 (Table V). This observation supports the hypothesis that the activation of the esterase by streptokinase proceeds in two stages: (a) the activation of plasmin by streptokinase, and (b) the conversion of C'1 to esterase by plasmin. On the other hand, the activation

TABLE V

The Effect of Soy Bean Inhibitor on Activation of C'1 by Streptokinase and by Adjustment of Ionic Strength

Activating agent	Ionic strength during activation	Esterase activity* microequivalents of acid liberated in 60 min.
Streptokinase + phosphate buffer	0.25	5.3
Streptokinase + soy bean inhibitor	0.25	2.5
Sodium chloride + phosphate buffer	0.25	2.0
Sodium chloride + soy bean inhibitor	0.25	2.4
Sodium chloride + phosphate buffer	0.15	16.9
Sodium chloride + soy bean inhibitor	0.15	17.0

* *Versus N-acetyl-L-tyrosine ethyl ester at ionic strength 0.30 with a concentration of 1 mg. per ml. of soy bean inhibitor in the assay mixture.*

of C'1 by adjustment of ionic strength did not seem to require the presence of plasmin since it was not blocked by soy bean inhibitor.

8. Attempted Demonstration of a Gross Alteration of C'1 Following Activation to Esterase.—

It was apparent that the activation of C'1 to esterase was similar in many respects to the activation of pepsinogen to pepsin (19) and trypsinogen to trypsin (20). Accordingly, it was of interest to attempt to demonstrate a chemical alteration of C'1 following activation to esterase. In the case of pepsinogen, autocatalytic activation is accompanied by release of a pepsin inhibitor, a polypeptide fragment with a molecular weight of 5,000 and soluble in trichloroacetic acid (24), while activation of trypsinogen to trypsin does not result in a gross chemical change (25).

Activation of C'1 to esterase was not accompanied by release of trichloroacetic acid-soluble nitrogen. Neither partially purified C'1 nor esterase contained any nitrogen soluble in 2.5 per cent final concentration of trichloroacetic acid. At lower concentrations of trichloroacetic acid, no significant differences were found between the soluble nitrogen in the partially purified C'1 and in the esterase derived from it.

Similarly, no significant differences were observed between the electrophoretic patterns of partially purified C'1 and of esterase. A three component system of indistinguishable distribution and mobility was obtained for both samples. Determinations were made in a Perkin-Elmer electrophoresis apparatus² at pH 5.5, ionic strength 0.30 at a protein concentration of 0.5 per cent.

² We are indebted to Dr. Howard Bensusan of the Departments of Biochemistry and Medicine, Western Reserve University, School of Medicine, for the use of his electrophoresis apparatus and for his interest and advice in this phase of the investigation.

The activation of partially purified C'1 to esterase appeared to be irreversible. Adjustment of activated esterase to pH 5.5, ionic strength 0.30, at 0°C. for time intervals up to 40 hours failed to result in the reappearance of C'1 activity.

DISCUSSION

It has been shown that under a wide range of conditions of pH, ionic strength, time, temperature, and inhibitor concentration, a positive correlation exists between the rate of disappearance of hemolytically active, partially purified first component of human complement, and the rate of activation of an esterase hydrolyzing *N*-acetyl-L-tyrosine ethyl ester and several other synthetic amino acid esters. Such a correlation may be predicted from the hypothesis proposed earlier (1-4) that the first component of complement exists in serum as a proenzyme which may be activated by an antigen-antibody reaction, by plasmin, or, in the partially purified state, by adjustment of physico-chemical conditions. The experimental realization of this prediction adds important evidence in favor of the role of the first component as a proenzyme.

Furthermore, the kinetic characteristics of the disappearance of first component activity and of the appearance of esterase activity were similar to the kinetics of activation of certain intensively studied enzyme systems. Thus, the appearance of esterase activity, correlating with disappearance of first component activity, followed the kinetics for second order autocatalysis. In accordance with this observation, the rate of the activation reaction was increased by increasing either the initial concentration of proenzyme (first component) or of esterase. The rate of activation was also influenced sharply by pH and ionic strength. These are characteristics also of the activation of trypsinogen to trypsin (20) and of pepsinogen to pepsin (19). Certain other characteristics of the activation of first component to esterase were particularly referable to the trypsinogen-trypsin system. As with trypsin, esterase activation was irreversible, was not associated with the liberation of a detectable molecular fragment, and occurred either autocatalytically or catalytically. The catalytic activation of first component to esterase by plasmin appeared analogous in principle to the activation of trypsinogen to trypsin by enterokinase (26) and by mold kinase (27).

The proenzymatic nature of the first component of complement and its activation by antigen-antibody has also been suggested by Becker (28, 29), employing an entirely different approach. Extending an observation of Levine (30), Becker has shown that diisopropylfluorophosphate (DFP), a known esterase inhibitor, inhibits the hemolytic activity of guinea pig complement by acting on the first component. He has shown further that sensitized sheep cells containing the activities of the first, second, and fourth components of guinea

pig complement are capable of hydrolyzing *p*-toluenesulfonyl-L-arginine methyl ester, and that this esterase activity correlates quantitatively with the activity of first component on the cells. We had shown previously that *p*-toluenesulfonyl-L-arginine methyl ester is a substrate for the esterase derived either from partially purified first component of human complement or from eluates of antigen-antibody aggregates treated with serum reagents containing first component (3, 4). Following the work of Levine and of Becker, we have been able to show an inhibiting effect of DFP on the activity of partially purified esterase, although only in relatively high concentrations (5). Thus, an increasing body of evidence is accumulating that the first component of complement is a pro-esterase. Ultimate proof will require much more highly purified preparations of first component than are now available.

TABLE VI

A Comparison of the Activation of First Component to Esterase with the Hydrolysis of N-Acetyl-L-Tyrosine Ethyl Ester by the Activated Esterase

	Activation of esterase	Esterase activity vs. <i>N</i> -acetyl-L-tyrosine ethyl ester (5)
Order of reaction	Second order autocatalysis	Zero order
Energy of activation (calories per mol)	31,000	12,000
pH optimum	7.3-7.7	7.5-8.2
Effect of ionic strength (μ)	Sharply inhibited at $\mu > 0.15$	Gradually inhibited at $\mu > 0.25$
Effect of EDTA	Inhibited by 5×10^{-5} M EDTA	No effect
Serum inhibitor	Heat-labile; unrelated to components of complement	Heat-labile; unrelated to components of complement
Effect of streptokinase	Activates esterase by activation of plasminogen to plasmin	No effect

A comparison of the reaction leading to activation of esterase with the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by the activated esterase is summarized in Table VI. It will be noted that the two reactions differ with respect to order of reaction, energy of activation, pH optimum, and the effect of ionic strength. Such differences are to be expected for fundamentally different reactions involving entirely different substrates. On the other hand, both reactions are inhibited by a serum factor (or factors) with similar properties. This observation is consistent with an autocatalytic activation reaction in which inhibition of the active enzyme would inhibit activation of the proenzyme. The nature and mode of action of the serum inhibitor appears of great interest and is

under investigation. The existence of such an inhibitor would be demanded by teleologic argument to explain the existence in normal blood of the proenzyme (first component) rather than the active enzyme (esterase).

The mechanism of inhibition of the activation reaction by ethylenediamine-tetraacetic acid (EDTA) remains unexplained at the present time. Unlike the serum inhibitor, EDTA does not affect esterase. Hence, if inhibition by EDTA is to be explained by chelation of a necessary cation, it is necessary to invoke the assumption that the cation is required only for the activation reaction. Furthermore, it is necessary to assume that the cation is tightly bound to protein, since exhaustive dialysis of partially purified C'1 or cation-exchanging with amberlite IRC-50 in the sodium cycle is without effect. It appears more probable that EDTA inhibits the activation reaction by binding directly to the first component. The available observations would then be explained by assuming that the dissociation constant for the protein-EDTA complex is greater than for the calcium-EDTA complex and sufficiently large to permit reversibility by dialysis.

Preparations of esterase derived either from partially purified first component or from eluates of antigen-antibody aggregates treated with serum reagents containing first component have the ability to inactivate complement (3, 4). The complement-inactivating activity of these preparations correlates with their esterase activity and is directed primarily against the fourth component and, to a lesser extent, against the second component. "Fixation" of human complement by aggregates of soluble antigen and rabbit antibody is known to lead to disappearance of the hemolytic activities of the first, second, and fourth components (1, 31). Activation of first component by an antigen-antibody reaction, with resulting inactivation of second and fourth components, has therefore been proposed as a possible mechanism of complement-"fixation" (1-4). Accordingly, it is of interest to compare the characteristics of the complement-"fixation" reaction with those presented in this paper for the activation of first component to esterase.

It was demonstrated previously (2) that complement-"fixation" could be separated into at least two distinct reaction stages. The first, designated the activation stage, involved the reaction of the antigen-antibody aggregate with first component and led to the formation of an activated aggregate. In the second stage, designated the transfer stage, the activated aggregate or a product derived from it inactivated the second and fourth components. A summary of the kinetic characteristics of these reaction stages, along with those of the activation of first component to esterase as described in this paper, is presented in Table VII. It is apparent that the characteristics of the reaction leading to activation of first component to esterase parallel closely those for the transfer stage, rather than the activation stage.

TABLE VII
A Comparison of the Activation and Transfer Stages of Complement-"Fixation" with the Activation of First Component to Esterase

Activation stage (2)	Transfer stage (2)	Activation of esterase
Optimal at low temperature (0°C.) Instantaneous at 0°C.	Optimal at higher temperatures ($\geq 37^\circ\text{C}.$) Occurs slowly at 0°C.; more rapidly at higher temperatures	Optimal at higher temperatures ($\geq 37^\circ\text{C}.$) Occurs very slowly at 0°C.; more rapidly at higher temperatures
Independent of pH between pH 6.5-9.5 Slight dependency on ionic strength	pH optimum at or near pH 7 Sharp dependency on ionic strength, with optimum at 0.15	pH optimum 7.3-7.7 Sharp dependency on ionic strength, with increasing inhibition above ionic strength 0.15
No effect of Ca^{++}	Potentiated by Ca^{++}	Inhibited by EDTA but mechanism does not necessarily involve Ca^{++}

TABLE VIII
Proposed Reaction Stages in Complement-"Fixation"

Proposed reaction	Previous nomenclature (2)
1. Antigen-antibody + first component	Activation stage
2. Activation of first component to esterase 3. Inactivation of second and fourth components in presence of activated esterase	Transfer stage

The apparent paradox in this correlation is primarily a result of the nomenclature adopted earlier (2). On the basis of the present work, it is suggested that complement-"fixation" is at least a *three* stage reaction. As pictured in Table VIII, the first reaction, corresponding to the previously designated activation stage, remains as the reaction between the antigen-antibody aggregate and first component. The previously designated transfer stage consists of at least two reactions: the activation to esterase of first component which has reacted with antigen-antibody, and the subsequent inactivation of the second and fourth components in the presence of esterase. It may be pictured tentatively that antigen-antibody in Reaction 1 (Table VIII) serves to separate first component from the serum inhibitor which normally prevents activation. It would be of importance that little of the inhibitor is taken up by antigen-antibody aggregates (Table III). In the effective absence of inhibitor, Reaction 2 would then proceed by autocatalysis, presumably on the surface of the antigen-antibody aggregate. The esterase so activated would inactivate the second and

fourth components in Reaction 3. Reaction 1 has already been studied in some detail (2), while Reaction 2 is described in this report. The kinetic characteristics of Reaction 3, however, are incompletely determined and remain for continuing investigation.

SUMMARY

It has been found that under a wide range of physico-chemical conditions a positive correlation exists between the rate of disappearance of hemolytically active, partially purified first component of human complement and the rate of activation of an esterase hydrolyzing *N*-acetyl-L-tyrosine ethyl ester. Both reactions follow the kinetic equation for second order autocatalysis, with an apparent energy of activation of 31,000 calories per mol. They occur optimally at pH 7.3–7.7 and are inhibited by ionic strengths greater than 0.15, by 5×10^{-6} M ethylenediaminetetraacetic acid, and by a heat-labile serum inhibitor which appears unrelated to any component of complement. The activation of first component to esterase resembles closely the activation of trypsinogen to trypsin. Partially purified first component, containing plasminogen, may also be activated to esterase by addition of streptokinase.

The significance of these data with respect to the postulated existence of first component as a proesterase and its possible role in complement-“fixation” is discussed.

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