THE CONVERSION OF C'1S TO C'1 ESTERASE BY PLASMIN AND TRYPSIN*

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Normal human plasma contains the precursor of a potent proteolytic enzyme of broad specificity, called plasmin or fibrinolysin. One action of plasmin, measurable in the test tube, is the inactivation of human complement (C') (1–3). This effect of plasmin appears to involve two steps. Plasmin activates an enzyme, C'1 esterase, from its precursor, the first component of complement (C'1) (4, 5). Once activated, C'1 esterase inactivates the hemolytic properties of the second and fourth components of complement, C'2 and C'4 (4).

C'1, as it exists in human serum, is a macromolecule containing at least three subcomponents, C'1q, C'1r, and C'1s (6, 7). The precursor of C'1 esterase has been identified with the C'1s moiety (7), while C'1q and C'1r are required for the "spontaneous" generation of C'1 esterase from C'1s (7). In the experiments to be described, plasmin converted C'1s into C'1 esterase in the absence of C'1q and C'1r. The concentration of plasmin which was effective was comparable to that which can be generated in vitro from normal human plasma.

The esterase-inducing properties of plasmin were demonstrated in preparations which had been activated either with streptokinase or chloroform. Laurell and her associates (3) were unable to confirm these observations. They suggested that the inactivation of complement described in our earlier studies was due to complement fixation by a complex formed between a component of the streptokinase preparation and specific antibody. In the present study, purified human plasminogen, the precursor of plasmin, was activated by urokinase, an agent in human urine thought to be nonantigenic (8, 9). Such urokinase-activated plasmin converted partially purified preparations of C'1s into C'1 esterase, as demonstrated by the appearance of esterolytic activity and the inactivation of C'2 and C'4. The identity of the C'1 esterase was supported by the observation that it was inactivated by the C'1 esterase inhibitor of human serum, but not by soybean trypsin inhibitor.

These various observations demonstrate that the activation of C'1s may take place through a proteolytic process. In agreement with this, trypsin was

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found to generate C'1 esterase from partially purified C'1s. Once C'1 esterase formed, however, its esterolytic properties were diminished by further incubation with plasmin or trypsin. The demonstration that C'1s can be changed to C'1 esterase by two proteases raises the possibility that the activation of C'1 esterase during complement fixation is enzymatic in nature.

Materials

Partially purified human C'1q, C'1r, and C'1s were prepared by chromatography upon columns of diethylaminoethyl cellulose (DEAE) in the presence of 0.001 M trisodium ethylenediaminetetraacetic acid (Na_3HEDTA) as described previously (7). Pools of these three subcomponents of C'1 were dialyzed separately against triethanolamine-buffered saline (TBS)[1] containing 0.001 M Na_3HEDTA at 1°C for 16 hr, reducing the ionic strength in each to 0.15. Aliquots were stored at −70°C for periods up to 2 months or maintained at 4°C for one wk. For purposes of expressing the concentrations of the subcomponents, these dialyzed pools were described as "undiluted."

Reagents deficient in C'1, C'2, and C'4, designated R1, R2, and R4 respectively were prepared from human serum essentially as described by Mayer (10) with modifications summarized by Wedgwood (11).

Sensitized sheep erythrocytes (EA) were prepared from sheep blood, collected monthly in an equal volume of Alsever's solution and stored at 4°C. The red cells were washed, standardized spectrophotometrically, and sensitized with an equal volume of anti-Forssman rabbit serum, as described previously (10). Following sensitization, the cells were washed twice with equal volumes of TBS-gelatin buffer and restandardized to the desired concentration.

Human serum was obtained by collecting human blood without an anti-coagulant. The blood was allowed to clot at room temperature, stored overnight at 4°C, and separated by centrifugation at 1°C. Pools from 5 to 20 donors were frozen in aliquots and stored at −70°C.

C'1 esterase, purified from human serum by the method of Haines and Lepow (12) and dissolved in TBS, was obtained from Dr. I. H. Lepow. The preparation used contained 52 units per ml, as tested upon a substrate of *N*-acetyl-L-tyrosine ethyl ester (ALTEE) by the method described in the present study, one unit being that amount which leads to the release of 0.5 microequivalent of acid in 15 min under the conditions used (13).

Partially purified plasminogen was prepared by adaptation of methods of Robbins and Summaria (14), Cole (15), and Hagan (16) from Cohn fraction III_4 of human plasma,[2] using a recently published method (17). In different batches, approximately 10–30% of such "plasminogen" was in the form of plasmin, measured by the capacity to digest casein without further activation. It contained trace amounts of clot-promoting activities which shortened the abnormally long clotting times of plasmas deficient in Hageman factor (factor XII), plasma thromboplastin antecedent (PTA, factor XI), Christmas factor (factor IX), factor VII, and Stuart factor (factor X). No measurable antihemophilic factor (factor VIII), proaccelerin (factor V), prothrombin (factor II), C'1 esterase, or C'1 subcomponents was present. The

[1] The following unusual abbreviations are used in this paper: ALTEE (also ATEE), *N*-acetyl-L-tyrosine ethyl ester; BAEE, *N*-benzoyl-L-arginine ethyl ester; CTA, Committee on Thrombolytic Agents, United States Public Health Service; EA, sensitized sheep erythrocytes; EI, human C'1 esterase inhibitor; PTA, plasma thromboplastin antecedent; PTCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; SBTI, soybean trypsin inhibitor; TAME, *P*-toluenesulfonyl-L-arginine methyl ester; TBS, triethanolamine-buffered saline; TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone.

[2] Cohn fraction III_4 was furnished by the Cutter Laboratories, Berkeley, Calif.

plasminogen preparations used in the present experiments, dissolved at concentrations of 4–8 Remmert and Cohen (18) units per ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.1 M sodium chloride, and 0.02 M lysine at pH 9.0, were dialyzed overnight at 4°C against approximately 100 volumes of TBS, with or without 0.001 M Na_3HEDTA. In some experiments, sufficient 0.15 M calcium chloride was added to provide a concentration of 0.001 M Ca^{2+} in the plasminogen solution. More concentrated plasminogen preparations were obtained by lyophilization in the presence of the Tris–sodium chloride–lysine buffer, reconstituting the residue with an appropriate volume of water and dialyzing the solutions against TBS; no loss was incurred by this procedure.

Purified human *urokinase*,[3] dissolved in TBS containing 0.001 M Na_3HEDTA or 0.001 M Ca^{2+} at a concentration of 500 "CTA" units per ml, was used to convert plasminogen to plasmin. The preparation of urokinase used contained 175,000 units per mg of nitrogen. The same results were obtained with a second preparation[4] containing 275,000 units per mg of nitrogen. The concentration of urokinase was one-fourth that which by itself hydrolyzed *N*-acetyl-L-tyrosine ethyl ester in the assay used.

Bovine trypsin, twice crystallized, dialyzed salt-free and lyophilized, was obtained from Worthington Biochemical Corporation, Freehold, N.J. According to the manufacturer, the preparation contained 180 esterolytic units per mg. The trypsin was dissolved in TBS. In some experiments the TBS contained either 0.001 M Na_3HEDTA or 0.001 M Ca^{2+}. A second preparation of bovine trypsin, described as "TPCK-treated" (Gallard-Schlesinger Chemical Manufacturing Corporation, New York) to reduce contamination with chymotrypsin, was also used. The lyophilized preparation, said to contain "8300 BAEE units" of trypsin and "40 ATEE units" of chymotrypsin per mg was dissolved in TBS containing 0.001 M Ca^{2+}.

Alpha chymotrypsin, three times crystallized (Worthington Biochemical Corporation) was dissolved at various concentrations in TBS containing 0.001 M Ca^{2+}.

Bovine thrombin ("topical thrombin," Parke Davis & Company, Detroit) was dissolved at a concentration of 1000 NIH units per ml of TBS containing 0.001 M Ca^{2+}. This preparation is known to be contaminated with plasminogen, Stuart factor, and proaccelerin, as well as other plasma components.

A suspension of *papain* (Worthington Biochemical Corporation), said by the manufacturer to contain 12.6 mg of protein per ml and 13.5 units per mg of protein, was diluted suitably in TBS containing 0.001 M Na_3HEDTA and 0.01 M cysteine, adjusted to pH 7.4.

L-1-tosylamido-2-phenylethylchloromethyl ketone (*PTCK*), B grade (Calbiochem, Los Angeles), an inhibitor of chymotrypsin, was dissolved at a concentration of 0.002 M in Methyl Cellosolve.

Soybean trypsin inhibitor (*SBTI*), three times crystallized and lyophilized (Worthington Biochemical Corporation), was suspended at suitable concentrations in TBS containing either 0.001 M Ca^{2+} or 0.001 M Na_3HEDTA.

Human C'1 esterase inhibitor (*EI*), partially purified from serum by chromatography upon Dowex 2 resin and DEAE,[5] was kindly provided by Dr. J. Pensky. It was dissolved at a concentration of 25 units per ml in TBS; 1 unit of EI, in the context of this paper, is the amount which neutralizes the esterolytic properties of 10 units of C'1 esterase, as assayed by the method described herein. This unitage does not correspond exactly to that used elsewhere, because the assay for C'1 esterase was slightly modified from that of Levy and Lepow (13).

N-acetyl-L-tyrosine ethyl ester (*ALTEE*), prepared in the laboratories of the Department of

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[3] Human urokinase was furnished by Abbott Laboratories, North Chicago, Ill.

[4] Human urokinase was provided by Sterling-Winthrop Research Institute, Rensselaer, New York.

[5] Pensky, J. Personal communication.

Chemistry, Western Reserve University, was dissolved at a concentration of 1 M in 2-methoxyethanol (Methyl Cellosolve, Eastman Organic Chemicals, Inc., Rochester, N.Y.). *P-toluenesulfonyl-L-arginine methyl ester* (*TAME*) (Nutritional Biochemicals Corporation, Cleveland, Ohio) was dissolved at concentration of 0.16 M in sodium phosphate buffer, ionic stregth 1.07, pH 7.4.

The *buffers* used were triethanolamine-buffered saline (TBS), at pH 7.4, ionic strength 0.15 (19), TBS-gelatin buffer, prepared by dissolving gelatin (Knox Gelatine Protein Products, Inc., Camden, N.J.) at a concentration of 0.05% in TBS, TBS containing Na_3HEDTA, calcium chloride, or magnesium sulfate at suitable concentrations, and sodium phosphate buffers of appropriate pH and ionic strength.

Salts of ethylenediaminetetraacetic acid (*EDTA*) were prepared in the following way. Reagent grade Na_2H_2EDTA was titrated to pH 7.4 at a stock concentration of 0.15 M, giving a solution largely in the form of Na_3HEDTA. $Na_2MgEDTA$ (Geigy Chemical Corporation, Ardsley, N.Y.) was titrated to pH 7.4 at a stock concentration of 0.15 M.

Methods

Measurement of Esterolytic Activity. The effect of plasmin upon subcomponents of C'1 was tested by mixing various combinations of 0.5 ml each of plasminogen, urokinase, C'1q or C'1r or 1.0 ml of C'1s, adjusting the volume to 2.0 ml with TBS. The mixtures, and suitable control mixtures, were incubated in disposable glass tubes (internal diameter 11 mm) at 37°C for 30 min, and then transferred to a bath of melting ice. The TBS used to prepare and dilute the reagents contained either 0.001 M Ca^{2+} or 0.001 M Na_3HEDTA.

The hydrolytic activity of the various mixtures was measured upon a substrate of ALTEE. Immediately after the tubes were placed in the ice bath, 0.375 ml of phosphate buffer (pH 7.4, ionic strength 1.07) was pipetted into each tube to inhibit spontaneous activation of C'1 esterase and to increase the buffering capacity of the assay mixture. In succession, 0.125 ml of 1 M ALTEE was added to each tube, which was incubated at 37°C for 15 min. Just after the addition of ALTEE and at the end of the 15 min period, 1 ml aliquots of the mixtures were added to 1 ml of neutralized 38% formaldehyde in the titration cup of a Radiometer model ABU 1 automatic burette (Cophenhagen, Denmark). The titratable acidity in each aliquot was measured by adding sufficient 0.05 M sodium hydroxide with the automatic burette to bring the hydrogen ion concentration of the mixture to a predetermined end point, usually pH 7.4. The titrations were performed with a Radiometer model TTT 11 titrator, controlled by a Radiometer model 25 pH meter.

The effects of trypsin, alpha chymotrypsin, thrombin, and papain were tested in the same manner, 1 ml of each enzyme, dissolved in TBS containing 0.001 M Ca^{2+} or 0.001 M Na_3-HEDTA, replacing plasminogen and urokinase in the enzyme-substrate mixture. Alpha chymotrypsin, thrombin, and papain were tested only for their effect upon C'1s, while trypsin was incubated with C'1q, C'1r, or C'1s. After alpha chymotrypsin had been incubated with C'1s for 30 min, in the manner described, 0.05 ml of 0.002 M PTCK was added in an unsuccessful attempt to block the action of this enzyme upon ALTEE. The TBS used to dissolve papain contained 0.001 M Na_3HEDTA and 0.01 M cysteine, adjusted to pH 7.4.

The effect of mixtures of plasmin and subcomponents of C'1 upon TAME was determined by essentially the same method. 2 ml of 0.16 M TAME was added to 2.0 ml of the mixture of plasminogen, urokinase, and C'1 subcomponents, and the mixture incubated at 37°C for 60 min. Aliquots of 1 ml were withdrawn before and after the hour of incubation and mixed with 1 ml formaldehyde solution, and the volume of 0.05 N sodium hydroxide needed to bring the pH of this mixture to 7.4 measured.

The effect of SBTI upon the development of esterolytic activity was determined by adding 0.1 ml of this inhibitor, at a concentration of 25 mg per ml, to the 2.0 ml mixture containing

plasmin or trypsin and components of C'1. By adding the SBTI before or after the enzyme–C'1 component mixtures were incubated, it was possible to determine whether this inhibitor influenced the activation of C'1 esterase, or the effect of the esterase upon ALTEE or TAME.

The influence of proteases upon the esterolytic activity of C'1 esterase was assessed by incubating 1.0 ml of C'1 esterase (25 units per ml) and either 1.0 ml of trypsin (6 μg per ml) or 0.5 ml each of plasminogen (16 units per ml) and urokinase (500 units per ml) for 30 min at 37°C. The reaction was then stopped by transferring the tubes to an ice water bath and adding 0.375 ml of sodium phosphate buffer (pH 7.4, ionic strength 1.07) containing 2.5 mg SBTI. Esterolytic activity was then measured upon the addition of 0.125 ml of 1 M ALTEE.

The effect of the serum inhibitor of C'1 esterase (EI) upon the enzymatic activity evolved by the interaction of plasmin and C'1s was determined by adding 0.0125–0.05 ml of a solution of EI containing 25 units per ml to a mixture of plasmin and C'1s which had been incubated at 37°C for 30 min. This mixture was incubated for an additional 30 min and then its esterolytic activity measured. SBTI, sufficient to provide a concentration during the esterolytic assay of 1 mg per ml, was added to the plasmin-C'1s mixture in a volume of 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07) just before the addition of EI, to halt further action of plasmin.

Measurement of Hemolytic Activity.—EAC'1, an intermediate complex possessing the functional activity of C'1, was measured as described earlier (7) by using the subcomponents of C'1 (C'1q, C'1r, and C'1s). The diluent was TBS-gelatin buffer containing 0.001 M Ca^{2+}. The activity of the complex EAC'1 was quantified by measuring the extent of hemolysis produced by a 1/100 dilution of human serum containing 0.016 M $Na_2MgEDTA$. Since this salt prevents further action of C'1 by chelating calcium ions, while permitting other components of complement to react, its addition provides a way to measure C'1 in unknown preparations.

C'1q, C'1r, and C'1s were measured by their ability to form the complex EAC'1, as described before (6, 7). In these experiments, a mixture containing 0.5 ml of a 1/100 dilution of each subcomponent was incubated with a button containing 5×10^8 sensitized erythrocytes (EA) for 10 min at 37°C to form the complex EAC'1. *The inactivation of C'4 in solution by C'1 esterase* was measured, as described by Haines and Lepow (20), by adding 0.4 ml of R1 to each of a series of 12 × 75 mm glass tubes containing 0.2 ml of serial 2-fold dilutions of the sample to be tested. These mixtures were incubated for 30 min at 0°C and a 1/8 dilution of each was made. An 0.2 ml aliquot of each dilution was tested for residual C'4 activity, using 0.1 ml R4, 0.2 ml buffer, and 1.0 ml EA (2.5×10^8 sensitized erythrocytes). TBS-gelatin buffer, containing 0.0005 M Mg^{2+} and 0.00015 M Ca^{2+}, was used as the diluent in this series of experiments. After incubation for 30 min at 37°C, the extent of hemolysis was estimated by visual comparison with a standard representing 50% hemolysis. The end point was taken as that dilution of the original test sample which resulted in 50% hemolysis after 1/8 dilution in the final incubation mixture.

The procedure for estimating the inactivation of C'2 by C'1 esterase was the same as that for C'4 inactivation except that all incubations were performed at 37°C and residual C'2 activity was assayed by using 0.05 ml of R2.

Sucrose Density Gradient Ultracentrifugation.—In these experiments, approximate linear gradients of 5–20% (w/v) sucrose in TBS buffer containing 0.001 M Na_3HEDTA were prepared as described previouly (6). Mixtures containing 1.5 ml of undiluted C'1s and 1.5 ml of either trypsin (6 μg per ml) or buffer were incubated for 30 min at 37°C, and 0.56 ml of SBTI (6.7 mg per ml) was added. 0.1 ml of each preparation and 0.1 ml of bovine serum albumin (10 mg per ml) (Armour Laboratories, Chicago) were layered upon separate sucrose gradients. After centrifugation, the C'1s fractions were tested for C'1s activity while the optical density of the albumin marker fractions was read at 210 mμ, using a Zeiss PMQII spectrophotometer.

RESULTS

I. The Effect of Plasmin upon the Hydrolysis of ALTEE by Subcomponents of C'1.—In agreement with previous studies, a mixture of C'1q, C'1r, and C'1s acquired the capacity to hydrolyze ALTEE, while each of the subcomponents alone or any combination of two lacked this property (Table I, Nos. 1–8).

TABLE I

Esterolytic Properties of Plasminogen, Urokinase, and the First Component of Complement (C'1)

Mixture*	Esterase activity	
	ALTEE	TAME
	μEq of acid liberated in 15 min	*μEq of acid liberated in 1 hr*
1. Buffer	0	0
2. C'1q	0	1.6
3. C'1r	0	1.1
4. C'1s	0	1.7
5. C'1q + C'1r	0	1.7
6. C'1q + C'1s	0	0
7. C'1r + C'1s	0.2	0.3
8. C'1q + C'1r + C'1s	7.6	3.9
9. Plasminogen	0.1	2.9
10. Urokinase	0.7	0
11. Plasminogen + urokinase	0.4	16.5
12. Plasminogen + C'1s	0.4	3.2
13. Urokinase + C'1s	0.1	0
14. Plasminogen + urokinase + C'1q	0	18.5
15. Plasminogen + urokinase + C'1r	0	14.5
16. Plasminogen + urokinase + C'1q + C'1r	0	13.3
17. Plasminogen + urokinase + C'1s	2.7	16.7
18. Plasminogen + urokinase + C'1s + SBTI (added before generation of esterase activity)	0.2	0.3
19. Plasminogen + urokinase + C'1s + SBTI (added after generation of esterase activity)	2.5	0.1
20. C'1q + C'1r + C'1s + SBTI (added before generation of esterase activity)	7.2	1.7
21. C'1q + C'1r + C'1s + SBTI (added after generation of esterase activity)	8.7	3.4

* Suitable combinations of 0.5 ml each of plasminogen (4 units per ml), urokinase (500 units per ml), "undiluted" C'1q and C'1r, 1.0 ml of "undiluted" C'1s, and sufficient TBS to bring the volume to 2.0 ml were incubated at 37°C for 30 min. Esterolytic activity for ALTEE was determined by adding 0.375 ml of sodium phosphate (pH 7.4, ionic strength 1.07) and 0.125 ml of 1 M ALTEE, and incubating this mixture for 15 min. Esterolytic activity for TAME was measured by adding 2 ml of 0.16 M TAME to the 2.0 ml of the original mixture and incubating for 60 min at 37°C. Additions of 0.1 ml SBTI (25 mg per ml) were made before or after the first period of incubation. In this experiment, all reagents were in TBS containing 0.001 M calcium chloride.

The preparations of plasminogen tested did not of themselves hydrolyze ALTEE (Table I, No. 9). They invariably contained some plasmin, as determined by tests for caseinolytic activity, but plasmin does not digest ALTEE measurably (17). Urokinase, in the concentrations used, lacked significant hydrolytic activity against ALTEE (Table I, No. 10). Similarly, a mixture of plasminogen and urokinase was without esterolytic activity directed against this substrate (Table I, No. 11).

When plasminogen and urokinase were incubated with a preparation of C'1s, significant esterolytic activity against ALTEE evolved. No such activity appeared when plasminogen and urokinase were incubated with either C'1q or C'1r or a combination of these two subcomponents (Table I, Nos. 12–17). The

TABLE II

Effect of Plasmin and Trypsin upon C'1 Esterase

Mixture*	Esterase activity
	μEq of acid liberated in 15 min
Plasminogen + urokinase	0.1
Trypsin	0.1
C'1 esterase	13.1
C'1 esterase + plasminogen + urokinase	10.4
C'1 esterase + trypsin	4.6

* Combinations of 0.5 ml plasminogen (16 units per ml), 0.5 ml urokinase (500 units per ml), 1.0 ml purified C'1 esterase (25 units per ml), and sufficient TBS to bring the volume to 2.0 ml were incubated at 37°C for 30 min. Esterolytic activity was measured by adding 0.375 ml of sodium phosphate buffer (pH 7.4, ionic strength 1.07) containing 2.5 mg SBTI and 0.125 ml 1 M ALTEE, and incubating this mixture for 15 min.

esterolytic activity which could be measured in mixtures of plasminogen, urokinase, and C'1s was always less than that which developed in mixtures of C'1q, C'1r, and C'1s. Increasing the concentration of plasminogen fourfold increased the titer of esterase activity only from 2.6 to 3.9 microequivalents of acid liberated in 15 min. This suggested that plasmin not only activated C'1s but also inhibited C'1 esterase. Indeed, a mixture of plasminogen, urokinases and purified C'1 esterase had less esterolytic activity than C'1 esterase alone (Table II). Thus, the degree of esterolytic activity measured in mixtures of plasminogen, urokinase, and C'1s may have been limited by this inhibitory property of plasmin.

In summary, a mixture of plasminogen, urokinase, and C'1s evolved the capacity to digest ALTEE, a property not shared by the mixture of plasminogen and urokinase or C'1s alone. These data are compatible with the view that urokinase-activated plasmin can convert C'1s to C'1 esterase in the absence of C'1q and C'1r.

II. The Effect of Plasmin upon the Hydrolysis of TAME by Subcomponents of C'1.—In contrast to ALTEE, TAME is a relatively poor substrate of C'1 esterase, but an excellent substrate of plasmin (5). Thus, a mixture of C'1q, C'1r, and C'1s evolved slight hydrolytic activity towards TAME, while each subcomponent alone or any combination of two had a lesser effect upon this substrate (Table I, Nos. 1–8). As would be expected, the preparation of plasminogen tested had some esterolytic activity that was greatly enhanced by incubation with urokinase (Table I, Nos. 9–11). Under the conditions of the assay, no further activity against TAME evolved upon incubation of urokinase and plasminogen with C'1s, or, indeed, with any subcomponent of C'1 (Table I, Nos. 12–17).

These experiments sharply separate the esterolytic properties of plasmin and C'1 esterase, in conformity with our expectations.

III. The Effect of Inhibitors upon the Esterolytic Properties of Plasmin and C'1 Esterase.—SBTI is an effective inhibitor of plasmin (21), but does not inhibit the hydrolysis of ALTEE by C'1 esterase. In the present study, the addition of SBTI to a mixture of plasminogen and urokinase inhibited the evolution of esterolytic activity against both ALTEE and TAME (Table I, Nos. 17, 18). After plasminogen, urokinase, and C'1s had been incubated together for 30 min, the addition of SBTI blocked hydrolysis of TAME, but was without effect upon hydrolysis of ALTEE (Table I, No. 19). It did not prevent the evolution of hydrolytic activity against ALTEE in a mixture of C'1q, C'1r, and C'1s although it may have had a minimal effect (Table I, Nos. 20, 21).

Serum possesses inhibitory activity directed against C'1 esterase. EI, a purified fraction of serum, rich in this inhibitory activity, interfered with the digestion of ALTEE by the agent which developed in mixtures of plasminogen, urokinase, and C'1s (Table III). The degree of inhibition was essentially that predicted from the action of EI upon purified C'1 esterase. This observation is in agreement with the view that the enzyme derived from C'1s upon treatment with plasmin is C'1 esterase.

IV. The Effect of Plasmin upon the Hemolytic Activity of the Subcomponents of C'1.—The experiments thus far described imply that plasmin changes C'1s to C'1 esterase. These experiments do not exclude the possibility that the preparations of plasminogen and urokinase might have been contaminated with C'1 or its subcomponents. Earlier, such contaminants could not be demonstrated in plasminogen prepared by the technique used in the present study (17). This possibility was reexamined because of its crucial importance in the interpretation of the current experiments.

The hemolytic activity of C'1q, C'1r, and C'1s was measured by determining the extent of the formation of the intermediate complex EAC'1. In these experiments, formation of this intermediate complex was assayed by pre-

incubating the subcomponents of C'1 with EA, i.e. sensitized erythrocytes, for 10 min at 37°C and measuring the extent of hemolysis after the addition of human serum containing $Na_2MgEDTA$ (7). As shown previously, a mixture of C'1q, C'1r, and C'1s was effective in forming the complex EAC'1, while the complex did not form when combinations of any two subcomponents were tested (Table IV).

The effect of urokinase and plasminogen upon formation of the complex EAC'1 was studied in a similar way. As shown in Table IV, preparations of

TABLE III

Effect of Serum Inhibitor of C'1 Esterase (EI) upon Esterolytic Properties of Mixtures of Plasminogen, Urokinase, and C'1s

EI added to test mixture*	Esterase activity*	Inhibitory activity‡	
		Experimental	Predicted
units	*μEq of acid liberated in 15 min*		
0	3.7	0	0
0.31	2.5	1.2	1.6
0.62	1.1	2.6	3.1
1.25	0.3	3.4	3.7

* Mixtures of 0.5 ml plasminogen (16 units per ml), 0.5 ml urokinase (500 units per ml), and 1.0 ml C'1s were incubated at 37°C for 30 min, and then transferred to an ice water bath. A volume of 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07) containing 2.5 mg SBTI, and 0 to 0.05 ml of a solution of EI (25 units per ml) were added to each tube and the mixtures reincubated at 37°C for 30 min. The esterolytic activity of the mixtures was then determined by incubating each with 0.125 ml 1 M ALTEE for 15 min.

‡ *Experimentally* determined inhibitory activity was obtained by subtracting the number of microequivalents of acid liberated in 15 min in the presence of EI from that liberated in its absence. The *predicted* inhibitory activity was derived from the inhibitory titer of the preparation of EI, as tested against purified C'1 esterase by the same esterolytic method.

urokinase or plasminogen, alone or in combination, were not capable of forming this intermediate complex. In addition, mixtures containing urokinase and plasminogen and combinations of any two subcomponents of C'1 were unable to form the complex EAC'1. The addition of urokinase and plasminogen to mixtures containing the three subcomponents of C'1 did not appreciably affect the extent of formation of this complex, while SBTI may have decreased the yield of EAC'1 slightly when added before or after the preincubation of subcomponents of C'1 with EA.

These experiments show that neither urokinase nor the plasminogen preparations used contained significant amounts of C'1q, C'1r, or C'1s. Nor did urokinase-activated plasmin function as a subcomponent of C'1 in the formation of EAC'1. These experiments indicate, therefore, that the conversion of C'1s

to C'1 esterase by plasmin cannot be attributed to its contamination with C'1q and C'1r.

V. The Inactivation of C'2 and C'4 by Plasmin-Activated C'1 Esterase.— Previous experiments by Haines and Lepow (20) have demonstrated that solutions of C'2 and C'4 are inactivated by C'1 esterase. If plasmin truly

TABLE IV

Effect of Plasmin on Formation of the Complex EAC'1

C'1q*	C'1r*	C'1s*	Urokinase*	Plasminogen*	SBTI	OD‡
+	+	+	−	−	−	0.862
+	+	−	−	−	−	0.000
+	−	+	−	−	−	0.000
−	+	+	−	−	−	0.000
+	+	−	+	−	−	0.000
+	−	+	+	−	−	0.000
−	+	+	+	−	−	0.000
+	+	−	−	+	−	0.000
+	−	+	−	+	−	0.000
−	+	+	−	+	−	0.000
−	−	−	−	+	−	0.016
+	+	−	+	+	−	0.005
+	−	+	+	+	−	0.000
−	+	+	+	+	−	0.000
−	−	−	+	+	−	0.000
+	+	+	+	+	−	0.787
+	+	+	+	+	+§	0.707
+	+	+	+	+	+‖	0.757
−	−	−	+	−	−	0.007

* These preparations were such that the concentration of each was a 1/100 dilution of the same reagent used for studying the generation of C'1 esterase. The total volume in all cases was 1.5 ml.

‡ Optical density at 541 mμ, corrected for EA control.

§ 0.1 ml SBTI, containing 25 mg/ml added to reaction mixture before formation of the complex EAC'1.

‖ 0.1 ml SBTI containing 25 mg/ml added to reaction mixture after formation of the complex EAC'1.

changed C'1s to C'1 esterase, one would expect that the product of the interaction of plasminogen, urokinase, and C'1s would inactivate C'2 and C'4. To investigate this, aliquots of the mixtures used to test the generation of esterolytic activity were incubated at 37°C for 30 min and maintained in an ice bath until used.

When a mixture of C'1q, C'1r, and C'1s or a mixture of plasminogen, urokinase, and C'1s was incubated with R1, a significant amount of C'4 was inactivated, as shown in Table V. For example, the mixture containing un-

diluted C'1q, C'1r, and C'1s inactivated C'4 in the R1 preparation. Only when the mixture of C'1q, C'1r, and C'1s was diluted 64-fold before mixing with R1 was its capacity to inactivate C'4 decreased to the point at which as much as 50% hemolysis was noted when the R1 preparation was assayed with R4. In contrast, mixtures of plasminogen and urokinase alone or plasminogen and C'1s alone did not inactivate significant amounts of C'4. In all cases, samples capable of inactivating C'4 contained esterolytic activity, as measured by the hydrolysis of ALTEE. Mixtures incapable of inactivating C'4 did not hydrolyze this substrate. In a companion series of experiments, using the same reaction mixtures, C'2 was also inactivated only by those mixtures possessing the capacity to hydrolyze ALTEE.

These experiments demonstrated that C'1s, incubated in the presence of either a mixture of C'1q and C'1r or urokinase and plasminogen acquired the

TABLE V

Inactivation of C'4 by C'1 Esterase Generated from Mixtures Containing Plasminogen, Urokinase, and C'1s

C'1q	C'1r	C'1s	Urokinase	Plasminogen	Hemolytic titer, dilution of test sample*
+	+	+	−	−	1/64
−	−	−	+	+	1/2
−	−	+	−	+	1/8
−	−	+	+	+	1/32

* Dilution of test sample required for 50% hemolysis.

ability to inactivate C'4 and C'2 in the R1 fraction of serum. This inhibitory property paralleled the esterolytic activity of these mixtures. Since the inactivation of C'4 and C'2 in solution is a function of C'1 esterase (20), these data support the view that plasmin can convert C'1s to C'1 esterase in the absence of C'1q and C'1r.

VI. The Effect of Trypsin upon the Esterolytic Properties of C'1.—The activation of C'1s by plasmin suggested that other proteases might effect this transformation. To test this, crystalline trypsin was incubated with C'1q, C'1r, or C'1s for 30 min at 37°C and the reaction was then halted by the addition of soybean trypsin inhibitor (Table VI). Under these conditions, even more esterolytic activity evolved in mixtures of trypsin and C'1s than from the interaction of C'1q, C'1r, and C'1s. The reaction proceeded optimally when the concentration of trypsin was 3 μg per ml, less esterolytic activity evolving under these circumstances at higher or lesser concentrations of the enzyme. Presumably, excessive trypsin inhibited either the formation of C'1 esterase or its activity. In agreement with this, less esterolytic activity than anticipated

evolved when the C'1s was diluted serially (Table VI). Moreover, when trypsin was incubated for 30 min with purified C'1 esterase, significant loss of esterolytic activity occurred (Table II). Thus trypsin, like plasmin, appeared to inhibit C'1 esterase, as well as to bring about its activation.

The evolution of C'1 esterase did not take place if SBTI was added to trypsin before its incubation with C'1s (Table VII). Activation was slowed by incubation of trypsin with C'1s at 0°C, but even at this temperature, significant esterolytic activity developed within as brief a time as 15 sec (Table VIII).

TABLE VI

The Esterolytic Properties of Trypsin and the First Component of Complement

Mixture*	Esterase activity
	μEq of acid liberated in 15 min
Buffer	0
C'1q	0
C'1r	0
C'1s	0
C'1q + C'1r + C'1s	4.1
Trypsin	0
Trypsin + C'1q	0.5
Trypsin + C'1r	1.5
Trypsin + C'1s	6.8
Trypsin + C'1s (diluted 1/2)	2.4
Trypsin + C'1s (diluted 1/4)	0.7

* Suitable combinations of 1.0 ml trypsin (6.25 μg per ml), 0.5 ml C'1q, 0.5 ml C'1r, and 1.0 ml C'1s, and TBS were incubated in a volume of 2 ml. All reagents had been dissolved in or dialyzed against TBS containing 0.001 M trisodium EDTA. After 30 min at 37°C, 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07) containing 25 mg SBTI and 0.125 ml 1 M ALTEE were added, and the amount of acid liberated during incubation at 37°C for 15 min was measured by the method described.

TABLE VII

The Effect of Soybean Trypsin Inhibitor on the Activation of C'1s by Trypsin

Mixture*	Esterase activity
	μEq of acid liberated in 15 min
SBTI + trypsin + C'1s incubated 30 min	0.5
Trypsin + C'1s incubated 30 min, then SBTI added	8.9

* 1.90 ml C'1s and 0.10 ml trypsin (62 μg per ml) were incubated at 37°C for 30 min. Before or after the period of incubation, 0.10 ml of SBTI (25 mg per ml) was added to the mixture. Esterase activity was then determined after the addition of 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07) and 0.125 ml 1 M ALTEE. The trypsin, C'1s, and SBTI were dissolved in TBS containing 0.001 M trisodium EDTA.

That the enzyme derived from the interaction of trypsin and C'1s was in fact C'1 esterase was supported by its quantitative inhibition by purified C'1 esterase inhibitor (Table IX).

VII. The Effect of Trypsin upon the Hemolytic Activity of the Subcomponents of C'1.—As the preceding data show, trypsin seems to convert C'1s to C'1

TABLE VIII

The Effect of Temperature on the Evolution of Esterase Activity in Mixtures of Trypsin and C'1s

Time of incubation	Esterase activity*	
	0°C	37°C
sec	*μEq of acid liberated in 15 min*	
10	1.8	2.3
30	2.4	3.5
60	3.3	4.5
180	4.7	4.9
300	5.8	5.2

* Mixtures of 6 ml trypsin (6 μg/ml) and 6 ml C'1s were incubated at 0°C or 37°C. At the intervals noted, 1.9 ml aliquots were transferred to tubes containing 0.1 ml SBTI (25 mg per ml) and 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07), and the esterase activity measured at 37°C upon the addition of 0.125 ml 1 M ALTEE. The trypsin, C'1s, and SBTI were dissolved in TBS containing 0.001 M trisodium EDTA.

TABLE IX

Effect of Serum Inhibitor of C'1 Esterase (EI) upon Esterolytic Properties of Mixtures of Trypsin and C'1s

EI added to test mixture*	Esterase activity*	Inhibitory activity‡	
		Experimental	Predicted
units	*μEq of acid liberated in 15 min*		
0	7.0	0	0
0.31	5.3	1.7	1.6
0.62	3.9	3.1	3.1
1.25	1.5	5.5	6.3

* Mixtures of 1.0 ml trypsin (6 μg per ml) and 1.0 ml C'1s were incubated at 37°C for 30 min and then transferred to an ice water bath. A volume of 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07) containing 2.5 mg SBTI and 0 to 0.05 ml of a solution of EI (25 units per ml) were added to each tube and the mixtures reincubated at 37°C for 30 min. The esterolytic activity of the mixtures was then determined by incubating each with 0.125 ml 1 M ALTEE for 15 min.

‡ *Experimentally* determined inhibitory activity was obtained by subtracting the number of microequivalents of acid liberated in 15 min in the presence of EI from that liberated in its absence. The *predicted* inhibitory activity was derived from the inhibitory titer of the preparation of EI, as tested against purified C'1 esterase by the same esterolytic method.

esterase. The question arose whether it would substitute for C'1q and C'1r in the formation of the complex EAC'1. As shown in Table X, trypsin alone, or in combination with any two subcomponents of C'1, was incapable of forming the complex EAC'1. When 0.5 ml aliquots of C'1q, C'1r, and C'1s, each at a 1/100 dilution, were incubated with EA for 10 min in the presence of 0.03 μg of trypsin, formation of the complex EAC'1 was reduced by approximately 80%. However, when SBTI was added to the reaction mixture before the 10 min incubation period, formation of the complex EAC'1 was only reduced by

TABLE X

Effect of Trypsin on Formation of the Complex EAC'1

C'1q, 0.5 ml	C'1r, 0.5 ml	C'1s, 0.5 ml	Trypsin,* 0.5 ml	SBTI, 0.1 ml	OD‡
+	+	+	−	−	0.940
+	+	−	−	−	0.020
+	−	+	−	−	0.025
−	+	+	−	−	0.035
+	+	−	+	−	0.010
+	−	+	+	−	0.012
−	+	+	+	−	0.020
−	−	−	+	−	0.015
+	+	+	+	−	0.170
+	+	+	+	+§	0.690
+	+	+	+	+‖	0.150

* Final trypsin concentration of 0.03 μg per ml.

‡ Optical density at 541 mμ, corrected for EA control.

§ 0.1 ml of SBTI containing 2.5 mg per ml added to reaction mixture before formation of the complex EAC'1.

‖ 0.1 ml of SBTI containing 2.5 mg per ml added to reaction mixture after formation of the complex EAC'1.

approximately 25%. The addition of SBTI after 10 min at 37°C was without effect, i.e., formation of the complex EAC'1 was again reduced by about 80%.

These experiments demonstrate that although C'1s could be converted to C'1 esterase by trypsin, the hemolytic activity of C'1 was markedly reduced by this enzyme. Among other possibilities, this reduction of hemolytic activity may have been due to changes induced in C'1q, C'1r, or C'1s, other than the formation of C'1 esterase, which may have occurred during tryptic digestion. This possibility was investigated by preincubating each subcomponent of C'1 with trypsin for 10 min at 37°C. Tryptic activity was inhibited by the addition of SBTI to the reaction mixture before or after the preincubation period. Each subcomponent was then assayed for hemolytic activity by determining the extent of formation of the complex EAC'1 when incubated in the presence of EA and the remaining two subcomponents of C'1.

When 0.05 μg of trypsin was preincubated with C'1q, the hemolytic activity of this subcomponent of C'1 was markedly reduced, while the hemolytic activity of C'1r and C'1s was only moderately decreased (Table XI). Additional experiments revealed that the effect of trypsin was dependent upon its concentration, since 5.0 μg inactivated more than 90% of the hemolytic activity of either C'1q, C'1r, or C'1s. But trypsin preincubated for 10 min at 37°C with a 500-fold excess of SBTI still inhibited the hemolytic activity of C'1q and, to a lesser extent, that of C'1r and C'1s (Table XI). These observations raised the possibility that SBTI itself inhibited complementary activity, a suggestion which will be examined in subsequent paragraphs.

TABLE XI

Effect of Trypsin on the Hemolytic Activity of C'1q, C'1r, and C'1s

C'1q	C'1r	C'1s	Trypsin	SBTI	OD*
+	+	+	−	−	0.900
+	+	−	−	−	0.002
+	−	+	−	−	0.010
−	+	+	−	−	0.011
+‡	+	+	+	+	0.300
+	+‡	+	+	+	0.708
+	+	+‡	+	+	0.769
+§	+	+	+	+	0.390
+	+§	+	+	+	0.800
+	+	+§	+	+	0.839

* Optical density at 541 mμ, corrected for EA control.

‡ Preincubation of either C'1q, C'1r, or C'1s with trypsin (0.05 μg per ml) before the addition of SBTI (0.025 mg per ml).

§ Preincubation of either C'1q, C'1r, or C'1s with trypsin (0.05 μg per ml) after the addition of SBTI (0.025 mg per ml).

These experiments with trypsin were compromised by the possibility that the preparation used was contaminated with chymotrypsin, which would not have been inhibited by SBTI, even though the trypsin preparation used did not measurably digest ALTEE. But trypsin treated to reduce contamination with chymotrypsin decreased the hemolytic activity of C'1q, C'1r, and C'1s in the same way. Possible quantitative differences in tryptic activity among the trypsin preparations tested were not investigated. Notably, the hemolytic activity of C'1q was partially inhibited by incubation with chymotrypsin-poor trypsin which had first been incubated with SBTI, while the hemolytic activity of C'1r and C'1s was only slightly depressed.

These experiments make it unlikely that the loss of hemolytic C'1 activity, observed when C'1q, C'1r, or C'1s were incubated with trypsin, was due to contamination by chymotrypsin. Furthermore, the inhibitory effect of chymo-

trypsin-poor trypsin preincubated with SBTI was unexplained. Since SBTI completely inhibited the conversion of C'1s to C'1 esterase by trypsin, it seemed unlikely that the decrease in hemolytic activity could be related to tryptic activity which was not inhibited by SBTI. These experiments could be explained were SBTI capable of inhibiting one or more of the subcomponents of C'1.

To test this hypothesis, SBTI was incubated for 10 min at 37°C with either C'1q, C'1r, or C'1s, and the hemolytic activity of each subcomponent was then measured as described above. The hemolytic activity of C'1q was partially inhibited while the decrease in C'1r and C'1s activity was less marked (Table XII).

TABLE XII

Effect of SBTI on the Hemolytic Activity of C'1q, C'1r, and C'1s

C'1q	C'1r	C'1s	SBTI	OD
+	+	+	−	0.900
+	+	−	−	0.010
+	−	+	−	0.013
−	+	+	−	0.020
+*	+	+	+	0.500
+	+*	+	+	0.770
+	+	+*	+	0.752
+‡	+	+	+	0.720
+	+‡	+	+	0.828
+	+	+‡	+	0.860
+§	+	+	−	0.890
+	+§	+	−	0.875
+	+	+§	−	0.878

* Preincubation of either C'1q, C'1r, or C'1s with SBTI (5.0 mg/ml).
‡ Preincubation of either C'1q, C'1r, or C'1s with SBTI (0.5 mg/ml).
§ Preincubation of either C'1q, C'1r, or C'1s in the absence of SBTI.

The inhibitory effect of SBTI appeared to be directed primarily toward C'1q, but its presence during the assay of each subcomponent made this uncertain. The available data suggest, however, that the apparent inhibition observed when SBTI was preincubated with C'1r and C'1s may be due to the interaction of C'1q and SBTI during the formation of the complex EAC'1.

VIII. The Inactivation of C'2 and C'4 in Solution by C'1 Esterase Generated from C'1s and Trypsin.—The preceding experiments imply that trypsin, like plasmin, could convert C'1s to C'1 esterase in the absence of C'1q and C'1r. Were this the case, trypsin-activated C'1 esterase should inactivate C'2 and C'4, as in the case of plasmin-activated C'1s. To investigate this, 0.2 ml aliquots of mixtures prepared for examining the effect of trypsin upon the esterolytic properties of C'1 were used. Tryptic activity was terminated by the addition of SBTI, and the mixtures were maintained in an ice bath until used.

The results from these experiments are shown in Table XIII. Mixtures containing C'1s and trypsin inactivated C'4, while those containing C'1s or trypsin alone lacked this property. The capacity to inactivate C'4 paralleled esterolytic activity, as measured by the hydrolysis of ALTEE. A companion series of experiments demonstrated that mixtures containing trypsin and C'1s inactivated C'2 in solution, while C'1s or trypsin alone did not inactivate this component of complement.

These experiments, therefore, show that C'1s incubated in the presence of trypsin acquired the capacity to inactivate C'4 and C'2 in the R1 fraction of serum. Since the ability to inactivate these two components of complement was associated with the capacity to hydrolyze ALTEE, these data provide additional evidence that trypsin can convert C'1s to C'1 esterase in the absence of the other subcomponents of C'1.

TABLE XIII

Inactivation of C'4 by C'1 Esterase Generated from Mixtures Containing Trypsin and C'1s

C'1s	Trypsin	TBS	Hemolytic titer, dilution of test sample*
+	−	+	1/2
−	+	+	1/2
+	+	−	1/32
−	−	+	1/2

* Dilution of test sample required for 50% hemolysis.

IX. Ultracentrifugal Studies on C'1s Activated to C'1 Esterase by Trypsin.—One possible mechanism for the conversion of C'1s to C'1 esterase by trypsin was provided by the hypothesis that the activation of C'1s was related to cleavage of a peptide fragment from C'1s by trypsin. The cleavage of a peptide fragment during the activation of C'1s might be associated with a change in the sedimentation rate of this protein. The sedimentation behavior of C'1s, incubated in the presence and absence of trypsin, was therefore investigated. No such change was observed upon sucrose gradient ultracentrifugation of C'1 esterase obtained by incubating C'1s with trypsin.

These experiments, therefore, suggest that the conversion of C'1s to C'1 esterase by trypsin does not appreciably alter the size or shape of C'1s. Previous experiments (6, 12) have shown that C'1 esterase (activated by C'1q and C'1r) and C'1s both had a sedimentation coefficient of about 4S, and that the activation of C'1 to C'1 esterase was not accompanied by release of trichloracetic acid-soluble nitrogen or by electrophoretic changes (5). Thus, the conversion of C'1s to C'1 esterase by trypsin may be similar to the activation of C'1s to C'1 esterase by the interaction of the three subcomponents of C'1.

X. The Effect of Other Proteases upon C'1s.—The incubation of 1.0 ml of

C'1s and 1.0 ml of chymotrypsin, at concentrations of 1.25 to 10 μg per ml, for 30 min at 37°C did not result in esterolytic activity, measured upon a substrate of ALTEE, beyond that which could be attributed to the chymotrypsin alone. Nor did incubation of 1.0 ml of C'1s with 1.0 ml of thrombin, at concentrations ranging from 31 to 500 NIH. units per ml, or with 1.0 ml of papain, at concentrations of 0.08–1.25 mg per ml, induce esterolytic activity.

DISCUSSION

The first component of complement, C'1, behaves as if it were the precursor of a specific hydrolytic enzyme, C'1 esterase (22–25). Partially purified preparations of C'1 lose C'1 activity, as measured in certain hemolytic systems, and gain esterolytic properties, as tested upon synthetic substrates, when the ionic strength is lowered from 0.30 to 0.15 (5, 22). This "spontaneous" formation of C'1 esterase takes place only when three separable subcomponents of C'1 (C'1q, C'1r, and C'1s) are present (7). In serum, these three subcomponents appear to be loosely bound together in the form of a macromolecule (6). Esterolytic activity will not evolve even at low ionic strengths in solutions of any of the three subcomponents alone, but only when all three are incubated together. In this reaction, C'1q and C'1r behave as if they were in some way responsible for the activation of an esterase derived from C'1s (7).

The formation of C'1 esterase from preparations of C'1 can also be brought about by exposure to antigen-antibody aggregates (23). How antigen-antibody aggregates bring about the generation of C'1 esterase is not understood. The experiments presently described point to one possible mechanism, that the activation of C'1 esterase is through a proteolytic process. This view has been proposed by Taylor and Fudenberg (26), who reported that epsilon aminocaproic acid inhibited the formation of C'1 esterase by sensitized red blood cells. That proteases can convert the first component of complement to C'1 esterase had been suggested by the observation that this process could be brought about by plasmin. Incubation of human serum at 37°C with streptokinase, an activator of plasminogen, resulted in the inactivation of complement (1). The decrease in complementary activity appeared to be due to inactivation of the first, second, and fourth components of complement, C'1, C'2, and C'4. Bovine plasmin, activated by chloroform, had little effect upon whole serum, but readily inactivated C'1 and C'4 in a euglobulin fraction of serum, R2 (1).

Further experiments seemed to indicate that the inactivation of components of complement occurred at least in part as the result of a sequence of reactions. C'2 and C'4 were inactivated by streptokinase or bovine plasmin only in the presence of calcium ions and only if C'1 was present (27). The sequence of events appeared to be the conversion of C'1 to an esterase by plasmin, and then the inactivation of C'2 and C'4 by C'1 esterase.

Recently, Laurell and her associates (3) challenged the assumption that

plasmin converts C'1 into C'1 esterase. They were unable to demonstrate significant inactivation of complement by a highly purified preparation of streptokinase, Kabikinase (AB KABI, Stockholm, Sweden), or that treatment of serum with urokinase or human plasmin would decrease the titer of C'2 or C'4. If the serum was first diluted, both of these components were inactivated by human plasmin, but inactivation occurred in the absence of C'1. These experiments were interpreted to mean that plasmin did not convert C'1 into C'1 esterase, but was capable of direct proteolytic destruction of C'2 and C'4. The effect of Varidase, the streptokinase preparation used in our earlier studies, was thought to be due to complement fixation by a complex between components of the streptokinase preparation and specific antibodies in the serums tested. Since Kabikinase did not decrease complementary activity, these specific antibodies were presumably not directed towards streptokinase itself.

The present studies did not touch upon the question whether complement fixation may have explained our earlier results. Rather they demonstrated that partially purified preparations of plasmin might have converted C'1s into C'1 esterase. The preparations of C'1s tested were only partially purified, but the formation of C'1 esterase took place in the absence of C'1q and C'1r, making it unlikely that complement fixation was responsible. Moreover, the effect of plasmin was blocked by soybean trypsin inhibitor, a potent antagonist of plasmin. The agent which evolved when C'1s was incubated with plasmin was presumably C'1 esterase, since it hydrolyzed ALTEE, inactivated C'2 and C'4, and was quantitatively inhibited by purified esterase inhibitor derived from human serum, while it was not blocked by soybean trypsin inhibitor. In these experiments, urokinase was used to activate plasminogen. This agent, derived from human urine, is believed to be nonantigenic (8, 9). In any case, the activation of C'1s was also accomplished in the absence of urokinase by that fraction of the purified plasminogen preparation which was already in the form of plasmin.

These experiments do not bear upon Laurell's (3) interpretation of the effect of Varidase upon complement. Nonetheless, we have been unable to confirm the supposed inertness of Kabikinase towards complement. This agent, incubated with human serum at a concentration of 500 units per ml, reduced the titer of complement in human serum from 32.3 to 4.5 $C'H_{50}$ units per ml, while the same amount of Varidase brought the titer to 8.8 units.[6] The reasons for the discrepancies between our experiments and those of Laurell and her associates are not clear. In their experiments, they assessed the degree of available plasmin-like activity by applying the material to be tested to heated fibrin plates. Areas of fibrinolysis were measured after a period of 18 hr. This technique has the inherent drawback that plasminogen, which may not have been activated at the start of the assay, might have changed into plasmin during the incubation

[6] Lepow, I. H., and O. D. Ratnoff. Unpublished observations.

period. Moreover, plasminogen is readily adsorbed onto fibrin, and in this condition appears to be activated more readily by streptokinase than it is in unclotted plasma (28, 29). Thus the fibrin plate assay may have made it seem as if more fibrinolytic activity was available to act upon complement than was actually the case. Perhaps the differences between Laurell's findings and ours might be explained by an overestimation of the amount of plasmin-like activity in her experiments.

Our studies are in agreement with the view that plasmin can convert the first component of complement into C'1 esterase. Since the concentration of plasmin used was comparable to that which can generate in human serum, it is possible that, given the proper setting, plasmin may activate C'1 in the body. Although human serum contains potent inhibitors against plasmin, perhaps local concentrations of the active enzyme may be of physiological significance.

The observation that C'1s was changed to C'1 esterase by plasmin led to an examination of the effect of other proteolytic enzymes. Among those tested, only trypsin had the capacity to induce the formation of C'1 esterase, but in the test tube it was more effective than either plasmin or a mixture of C'1q and C'1r in the absence of calcium ions. That the enzyme evolved was C'1 esterase was confirmed by demonstrating its inhibition by a purified preparation of its specific inhibitor EI. Moreover, the C'1 esterase which evolved appeared capable of inactivating C'2 and C'4. Suitable experiments demonstrated that the property of activating C'1 esterase resided in the trypsin molecule itself and not in a contaminant of the preparation. Whatever the alteration induced in C'1s by trypsin, it could not be detected by a change in its sedimentation characteristics, as determined by sucrose gradient ultracentrifugation.

The effects of plasmin and trypsin were not limited to the activation of C'1 esterase. Evidence was obtained that both of these enzymes diminished the esterolytic properties of C'1 esterase itself. Thus both C'1s and C'1 esterase appeared to serve as substrates for these enzymes.

The various experiments described lead directly to the possibility that the activation of C'1 esterase from C'1s is brought about by the proteolytic action of C'1q, C'1r, or a combination of these subcomponents of C'1. In the present study, evidence is reported that soybean trypsin inhibitor may block the *hemolytic* properties of C'1q. In other experiments, not yet published,[7] TAME and phenylmethylsulfonylfluoride, which inhibit the action of some proteases, appear to block the action of C'1r upon C'1s. Perhaps, then, C'1r, in its active form, is a protease which is able to convert C'1s, a zymogen, into C'1 esterase, a second protease. Such a sequence is reminiscent of the "waterfall" or "cascade" hypothesis which attempts to explain the steps that lead to the formation of a clot as a series of reactions in each of which an enzyme forms which catalyzes the next step.

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[7] Naff, G. B., and O. D. Ratnoff. Unpublished observations.

SUMMARY

The formation of C'1 esterase from C'1, the first component of complement, may be brought about by the action of plasmin or trypsin upon C'1s, a subcomponent of C'1. These enzymes also decrease the esterolytic activity of C'1 esterase. The formation of C'1 esterase was demonstrated by measuring the appearance of an agent or agents with esterolytic properties and the capacity to inactivate C'2 and C'4, attributes of C'1 esterase. The activity of the agent which evolved was blocked by serum inhibitor of C'1 esterase. The implications of these observations, that the formation of C'1 esterase during complement fixation is mediated by proteolytic processes, are under study. The possible inhibition of C'1q by soybean trypsin inhibitor is in agreement with this hypothesis.

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