THE ENZYMATIC NATURE OF C'1r

Conversion of C'1s to C'1 Esterase and Digestion of Amino Acid Esters by C'1r*

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The mechanisms through which antigen-antibody complexes initiate the events attributed to the action of complement (C') are incompletely understood. When antigen-antibody aggregates are incubated with partially purified preparations of the first component of complement (C'1), esterolytic activity is generated (1–3). This property has been related to a subunit of C'1 designated as C'1s, which can be converted to a specific enzyme, C'1 esterase if two other subunits, C'1q and C'1r are present (4, 5). Once C'1 esterase forms, it enables the other components of C', of which at least eight have been distinguished, to participate in such reactions as immune hemolysis.

The way in which C'1q and C'1r bring about the formation of C'1 esterase has not been investigated. Recently, we demonstrated that trypsin and plasmin, two proteases, can change C'1s to C'1 esterase in the absence of C'1q and C'1r (6). This observation led us to speculate that C'1r might act enzymatically to transform C'1s to C'1 esterase.

In the studies to be described, preparations of C'1r, which appeared to be in an activated form, converted C'1s to C'1 esterase by enzymatic means. Such preparations contained an agent with specific esterolytic properties. The esterolytic enzyme and the agent responsible for the activation of C'1s behaved as if identical. Our experiments are consistent with the view that the formation of C'1 esterase by C'1r is a proteolytic process.

Materials

Human serum was obtained by collecting blood from normal donors without an anticoagulant. The blood was allowed to clot at room temperature, stored overnight at 4°C, and

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separated by centrifugation at 1°C. Pools from 10 to 20 donors were frozen in aliquots and stored at −70°C.

Crude fractions containing human C'1q, C'1r, and C'1s were separated from the euglobulin fraction of human serum by chromatography upon columns of diethylaminoethyl cellulose in the presence of 0.001 M trisodium ethylenediaminetetraacetic acid (Na_3HEDTA) as described previously (4). Pools of these subcomponents of C'1 were dialyzed separately against buffers suitable for the experiments contemplated. Unless otherwise stated, this buffer was sodium phosphate; in experiments on the effect of pH, the fractions were dialyzed against 0.15 M sodium chloride solution, while in those testing the action of different cations, they were dialyzed against barbital-saline buffer. For the purposes of expressing the concentrations of the subcomponents, dialyzed pools were described as "undiluted." All dialyses were performed at 4°C in cellophane tubing.

Sensitized sheep erythrocytes (EA) were prepared from blood collected monthly in an equal volume of Alsever's solution, and stored at 4°C. The cells were washed, standardized spectrophotometrically, and sensitized with anti-Forssman rabbit serum, as described previously (7). After sensitization, the cells were washed twice with triethanolamine-buffered saline (TBS)–gelatin buffer and restandardized.

C'1 esterase, purified by a modification of the method of Haines and Lepow (8), was obtained from Dr. J. Pensky, Case Western Reserve University. The preparations contained 550 units of activity per milligram of protein (9). To test its activity upon various substrates, it was dissolved in phosphate-saline buffer at a concentration of 5 units/ml.

Bovine trypsin, twice crystallized, containing 50% magnesium sulfate (Worthington Biochemical Corp., Freehold, N. J.) was dissolved at a concentration of 0.2 mg/ml in 0.001 N hydrochloric acid, and diluted to 2 μg/ml with phosphate-saline buffer to test its esterolytic properties. Presumably, the concentration of trypsin in this solution was 1 μg/ml.

Bovine thrombin, used in esterolytic studies, was prepared from Topical Thrombin (Parke, Davis and Company, Detroit, Mich.) by a modification of Rasmussen's (10) method which minimized contamination with Stuart factor (factor X) (11), and diluted just before use in phosphate-saline buffer to contain 2.8 NIH units/ml. *Plasminogen-poor thrombin*, for determination of fibrinolytic activity, was prepared by the method of Markus and Ambrus (12).

Urokinase, the gift of Abbott Laboratories, North Chicago, Ill., contained 2200 C.T.A.[1] units/mg of dry weight; the powder was dissolved in phosphate-saline buffer at an initial concentration of 500 units/ml for tests of its esterolytic activity.

Purified human plasma kallikrein, provided through the kindness of Dr. Robert Colman, the Massachusetts General Hospital, was diluted 5-fold in phosphate-saline buffer before testing its esterolytic activity (13).

Bovine α-chymotrypsin, three times crystallized, inactivated with diisopropyl phosphofluoridate, dialyzed, and lyophilized, was obtained from Worthington Biochemical Corp. and dissolved at a concentration of 1 mg/ml in phosphate-saline buffer to test its esterolytic properties.

Partially purified *activated plasma thromboplastin antecedent (PTA,[2] factor XI)* was prepared

[1] Committee on Thrombolytic Agents.

[2] The following unusual abbreviations are used in this paper: AAME, *N*-acetyl-L-arginine methyl ester hydrochloride; AGLME, *N*-α-acetylglycyl-L-lysine methyl ester acetate; AGME, *N*-acetylglycyl methyl ester; ALME, *N*-α-acetyl-L-lysine methyl ester hydrochloride; ALTEE, *N*-acetyl-L-tyrosine ethyl ester; AME, L-arginine methyl ester dihydrochloride; BAPA, benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; CBZAME, *N*-α-carbobenzoxy-L-arginine methyl ester hydrochloride; CBZLME, *N*-α-carbobenzoxy-L-lysine methyl ester hydrochloride; LME, L-lysine methyl ester dihydrochloride; PMSF, phenyl methylsulfonyl fluoride; PTA, plasma thromboplastin antecedent; TAME, *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride; TLME, α-toluenesulfonyl-L-lysine methyl ester hydrochloride.

by a previously described method (14); the material was approximately 50–60-fold purified compared with the serum from which it was separated. It was dissolved at a concentration of 1 mg/ml of phosphate-saline buffer to test its esterolytic properties.

Streptokinase (Varidase, American Cyanamid Co., Wayne, N. J.) was dissolved in phosphate-saline buffer at a concentration of 2000 Christensen units per ml. The preparation contained 25,000 units of streptococcal deoxyribonuclease for every 100,000 units of streptokinase.

Human plasmin was prepared from partially purified plasminogen, separated by a technique described earlier (15). The sample used contained approximately 10 Remmert and Cohen units/ml of solution, and 8 units/mg of protein. The preparation was diluted 64-fold in phosphate-saline buffer and converted to plasmin by the addition of one-fifth volume of streptokinase solution before assay of its esterolytic properties.

Chromatographically homogenous, five times crystallized *chymotrypsinogen A* (Worthington Biochemical Corp.) was dissolved at a concentration of 1.5 mg/ml in Tris buffer (tris[hydroxymethyl]aminomethane) (0.05 M, pH 7.4) containing 0.0035 M calcium chloride.

Synthetic amino acid esters were, for the most part, obtained from commercial sources. *N*-acetyl-L-tyrosine ethyl ester (ALTEE), synthesized by the Department of Chemistry, Case Western Reserve University, or by Dr. F. M. Bumpus, The Cleveland Clinic, was dissolved at a concentration of 1 M in 2-methyoxyethanol (Methyl Cellosolve). Other esters were dissolved at appropriate concentrations in sodium phosphate or phosphate-saline buffers. The pH of solutions of L-lysine methyl ester dihydrochloride (LME) was adjusted to 6.5, and that of the other esters to 7.4 with 0.15 M sodium hydroxide solution. *N*-acetyl-L-arginine methyl ester hydrochloride (AAME) was purchased as an oil from Cyclo Chemical Corp., Los Angeles, Calif. or synthesized by Dr. Bumpus. The commercial material was washed exhaustively with ether, dissolved in water, shell frozen, and lyophylized; the residue was a glass-like white mass which could be stored only in a vacuum because of its hygroscopic nature. The ester prepared by Dr. Bumpus was lyophylized without extraction or solution. The results obtained with both preparations were the same, but different lots contained variable amounts of material reacting like methyl alcohol in chromatropic acid assays. *N*-acetyl-L-arginine methyl ester-*p*-toluene sulfonic acid, synthesized by Dr. Bumpus, was used in some experiments, and was more satisfactory because it was not hygroscopic and had less chromatropic acid–reacting material than the hydrochloride. *N*-acetylglycyl methyl ester (AGME), *N*-α-acetylglycyl-L-lysine methyl ester acetate (AGLME), *N*-α-acetyl-L-lysine methyl ester hydrochloride (ALME), *N*-α-carbobenzoxy-L-arginine methyl ester hydrochloride (CBZAME), and *N*-α-carbobenzoxy-L-lysine methyl ester hydrochloride (CBZLME) were obtained from Cyclo Chemical Corp.; L-arginine methyl ester dihydrochloride (AME) and *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAME) from Nutritional Biochemicals Corp., Cleveland, Ohio; lysine methyl ester (LME) from Mann Research Laboratories, New York City; and α-toluenesulfonyl-L-lysine methyl ester hydrochloride (TLME) from Calbiochem, Los Angeles, Calif.

Benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA),Nutritional Biochemicals Corp., was dissolved at a concentration of 0.001 M in 1% dimethylsulfoxide in Tris buffer (pH 8.25, 0.05 M) containing 0.02 M calcium chloride (16).

Denatured hemoglobin was prepared as a 2.2% solution from bovine hemoglobin (Difco Laboratories, Detroit, Mich.) by the method of Northrop, Kunitz, and Herriott (17). *Casein* was prepared as a 3% solution from "Hammersten quality" material (Nutritional Biochemicals Corp.), by the method of Kline and Fishman (18). Crystalline *egg albumen* (Nutritional Biochemicals Corp.) was dissolved at a concentration of 1% in phosphate buffer. Lyophilized *bovine fibrinogen* (Warner-Chilcott, Morris Plains, N. J.) was reconstituted with water to a concentration of 3 mg/ml in 0.85% sodium chloride solution.

Inhibitors: Soybean trypsin inhibitor (three times crystallized), pancreatic trypsin inhibitor,

and lima bean trypsin inhibitor (Worthington Biochemical Corp.), heparin (156 Toronto units/mg, a gift of the Upjohn Co., Kalamazoo, Mich.), hirudin (grade II, Sigma Chemical Co., St. Louis, Mo.), hexadimethrine bromide (Polybrene, the gift of Abbott Laboratories), Trasylol kallikrein inhibitor, lyophylized, the gift of FBA Pharmaceuticals Inc., New York, N.Y sodium polyanetholsulfonate (Liquoid, the gift of Hoffman-LaRoche, Inc., Nutley, N. J.), and ovalbumin trypsin inhibitor (Nutritional Biochemical Corp.) were dissolved at appropriate concentrations in phosphate buffer at ionic strengths 0.15 and 1.07 to test their inhibitory activities. Epsilon aminocaproic acid (Mann Research Laboratories) was dissolved at a concentration of 1 M in water.

Formaldehyde (38%, Fisher Scientific Co., Pittsburgh, Pa.) was rendered slightly alkaline to phenolphthalein by the addition of 1 M sodium hydroxide solution just before use.

The buffers used were sodium phosphate, pH 7.4, at ionic strengths 0.15 or 1.07, sodium phosphate buffered saline solution (0.067 M sodium phosphate, pH 7.4 in 0.6% sodium chloride solution), triethanolamine-buffered saline solution containing 0.05% gelatin (Knox Gelatine, Protein Products, Inc., Camden, N. J.) (19), barbital-saline (0.025 M barbital in 0.125 M sodium chloride) pH 7.5, Tris 0.05 M, pH 8.25, containing 0.02 M calcium chloride solution, and Michaelis's acetate-barbital saline buffers, at ionic strength 0.15 and various pHs (20). The term phosphate buffer refers to sodium phosphate pH 7.4 at ionic strength 0.15, and high ionic strength phosphate buffer to sodium phosphate buffer at ionic strength 1.07, pH 7.4.

Methods

The evolution of C'1 esterase activity was measured by an adaptation of methods used in earlier studies (6). In most experiments, 0.5 ml C'1r, 0.5 ml phosphate buffer, and 1.0 ml C'1s were incubated at 37°C in disposable glass tubes (internal diameter 11 mm) for 30 min; the volumes used, the proportions of C'1r and C'1s, and the duration of incubation were varied as appropriate for the purpose of the experiment. A volume of 0.375 ml sodium phosphate buffer (pH 7.4, ionic strength 1.07) was then added, followed by 0.125 ml of 1 M ALTEE. Unless its presence would vitiate the purpose of the experiment, the phosphate buffer at ionic strength 1.07 contained 66 μg/ml of Liquoid to retard the continued action of C'1r upon C'1s; in some experiments, a higher concentration of Liquoid was used. Aliquots of 1.0 ml were withdrawn as soon as the ALTEE was added and after 15 min, and transferred to the reaction cups of a Radiometer, model ABU 1, automatic burette (Copenhagen, Denmark), containing 1.0 ml of 38% formaldehyde. The titratable acid was then determined by the addition of enough 0.05 N sodium hydroxide to bring the hydrogen ion concentration of the mixture to pH 7.4, using the automatic burette. The titrations were performed with a Radiometer, model TTT 11, titrator controlled by a Radiometer, model 25, pH meter.

The relative *esterolytic activity* of the various enzymes tested was measured by a minor modification of Siegelman's (21) method. A volume of 0.25 ml of the enzyme to be tested, in phosphate-saline buffer, was added to 2.50 ml of substrate, dissolved in the same buffer at a concentration of 0.015 M, and neutralized with 0.15 N NaOH if necessary. All assays were performed at pH 7.4 except those in which lysine methyl ester was the substrate; this substance was tested at pH 6.5. 1 ml aliquots of the enzyme-substrate mixture were withdrawn after incubation at 37°C for 1 min and 61 min, and transferred to disposable glass tubes (internal diameter 14 mm) containing 0.5 ml 0.75 M perchloric acid, as suggested by the Committee on Thrombolytic Agents of the National Institutes of Health. In some early experiments, the aliquots were added instead to 0.5 ml of 15% (w/v) trichloroacetic acid, in accordance with Siegelman's directions. Without centrifugation, 0.1 ml of a 2% aqueous potassium permanganate solution, 0.1 ml of a 10% aqueous sodium bisulfite solution, and 4.0 ml of chromatropic acid "working reagent" were then added in the manner described by Siegelman (21). The reagents were freshly prepared. The color evolved after heating in a boiling water

bath for 15 min was measured in 10 mm cuvettes at 580 mμ in a Beckman DU spectrophotometer with a Gilford absorbance meter. The color was compared with a freshly prepared standard of methanol in phosphate-saline buffer, assayed in the same way. Because only small amounts of plasma kallikrein were available, this enzyme was incubated with the substrates for 2 hr. Since the solvent, Methyl Cellosolve, was chromogenic in the Siegelman assay, the hydrolytic effect of the various enzymes for ALTEE was tested by the method used for assaying C'1 esterase activity.

The chromatropic acid assay was difficult to use and somewhat erratic, the methyl alcohol standard varying from experiment to experiment. In addition, as already noted, some preparations of AAME could not be freed of material reacting like methyl alcohol. For this reason, in all other experiments with AAME, microformol titration was used. 0.5 ml C'1r, 1.0 ml phosphate buffer, and 1.0 ml 0.15 M AAME hydrochloride were incubated at 37°C for 60 min in disposable glass tubes (internal diameter 11 mm); in some experiments, the concentration of AAME was varied as noted. At the start and after 60 min, 1.0 ml aliquots were transferred to the reaction cups of a Radiometer automatic burette containing 1.0 ml of 38% formaldehyde, and the titratable acid determined by the addition of 0.05 N sodium hydroxide with the Radiometer titrator, as in the assay for C'1 esterase. The end point of the titration was usually pH 7.45; when the reagents were dissolved in barbital-saline buffer, the end point was pH 7.75.

The *hydrolysis of BAPA* by C'1r was tested by mixing in duplicate 1.0 ml of C'1r in triethanolamine-buffered saline solution with 1.0 ml BAPA reagent in disposable glass test tubes (internal diameter 14 mm). 1 ml of 30% acetic acid was added to one tube at the start and to the other after incubation at 37°C for 120 min. The color evolved during incubation was measured in 10 mm cuvettes at 410 mμ in a Beckman model B spectrophotometer.

Proteolysis of protein substrates by C'1r was tested by incubating 6.0 ml of this enzyme in phosphate buffer with 6.0 ml of the substrates at 37°C. 2 ml aliquots were removed at the start and after 16 hr and added to 3.0 ml of 10% (w/v) trichloroacetic acid. The absorbance of the material soluble in trichloroacetic acid was measured in 10 mm cuvettes at 280 mμ in a Beckman spectrophotometer. The substrates tested were denatured hemoglobin, casein, and egg albumen. The *fibrinolytic properties* of C'1r were tested by incubating 0.4 ml C'1r in phosphate buffer with 0.1 ml of plasminogen-poor thrombin (10 NIH units/ml) and 0.5 ml bovine fibrinogen at 37°C for 24 hr in disposable glass tubes (internal diameter 8 mm).

The effect of C'1r upon chymotrypsinogen A was tested by incubating 2 ml of a solution of chymotrypsinogen with 1 ml of C'1r in 0.15 M sodium chloride. At the start and after 120 min, 0.75 ml aliquots were mixed with 1.625 ml of the same buffer and 0.125 ml 1 M ALTEE. The hydrogen ion released during 15 min of incubation at 37°C was measured by continuous titration with the Radiometer automatic titrator, adding 0.025 M sodium hydroxide to maintain a pH of 7.4.

The *influence of ionic strength* upon the reaction between C'1r and C'1s was tested by substituting sodium chloride solutions of varying concentration for the 0.5 ml of phosphate buffer during the first period of incubation. After the addition of high ionic strength phosphate buffer (containing Liquoid), an additional 1.0 ml of sodium chloride solution was added to each tube such that the ionic strength of all mixtures was equalized. The influence of ionic strength upon the reaction between C'1r and AAME was measured by substituting 1.0 ml of saline solution of varying concentration for phosphate buffer, in a total volume of 2.5 ml.

The *influence of pH* was tested with these reagents prepared in 0.15 M sodium chloride solution. The pH was adjusted by the addition of Michaelis buffers and the mixtures (2 ml in volume) incubated at 37°C for 30 min. The reaction was then inhibited by the addition of 0.375 ml Liquoid (133 μg/ml) in phosphate buffer (pH 7.4, ionic strength 1.07). The pH of each mixture was then readjusted to 7.4 by the addition of Michaelis buffer and the volume

in all instances brought to 3.375 ml. An aliquot of 2.375 ml was then withdrawn, 0.125 ml 1 M ALTEE added and the C'1 esterase activity measured as described.

The *influence of pH upon the reaction between C'1r and AAME* was measured by incubating 0.25 ml C'1r in 0.15 M sodium chloride, 2.05 ml of Michaelis buffer of varying pH, and 0.20 ml of 0.375 M AAME for 90 min at 37°C. The aliquots withdrawn at 0 and 90 min were added to a mixture of 1.0 ml of high ionic strength phosphate buffer and 1.0 ml of 38% formaldehyde, and then titrated to an end point of pH 7.5 in the manner described.

The *effect of heat upon preparations of C'1r* was tested by incubating this reagent in disposable glass test tubes at 56°C, the temperature being that of the solution. Samples were withdrawn before heating and after 5, 10, 20, and 30 min, and transferred to tubes which were kept in an ice water bath until tested.

The effect of cations upon the reaction between C'1r and C'1s and between C'1r and AAME was tested by preparing 0.05 M aqueous solutions of the chloride salts of divalent cations and 0.15 M aqueous potassium and sodium chloride solutions. All solutions were then diluted to the concentrations needed in 0.15 M sodium chloride. C'1r and C'1s were dialyzed against barbital-saline buffer before use. To study the effect of salts upon the evolution of C'1 esterase, a mixture of 0.25 ml of C'1r, 0.55 ml of barbital-saline buffer, and 0.20 ml of the cation to be tested was incubated at room temperature for 10 min, and 1.0 ml of C'1s was then added. After 30 min at 37°C, 0.325 ml of Liquoid (66 μg/ml in high ionic strength phosphate buffer) and 0.125 ml 1 M ALTEE were added and esterolytic activity was then assayed by microformol titration. Suitable control mixtures were tested to be certain that the effects observed were not artefacts of the production of phosphate salts during the esterolytic assay. The effect of cations upon the reaction between C'1r and AAME was tested in a similar way, incubating 1.0 ml of C'1r, 0.25 ml of barbital-saline buffer, 0.25 ml of the salt to be tested and 1.0 ml of 0.075 M AAME in barbital-saline buffer, neutralized to pH 7.4. The mixture was incubated at 37°C for 1 hr, and esterolytic activity measured by microformol titration.

The *effect of inhibitors* upon the interaction of C'1r and C'1s was tested by incubating 0.5 ml C'1r in phosphate buffer and 0.5 ml of the inhibitor under test in the same buffer at 37°C for 20 min. To each tube was then added 0.375 ml of sodium phosphate buffer (pH 7.4, ionic strength 1.07) and 1.0 ml C'1s. This mixture was incubated at 37°C for an additional 30 min and its C'1 esterase activity then tested upon the addition of 0.125 ml of 1 M ALTEE. Agents which were inhibitory by this technique were further studied to localize their site of action. C'1r was incubated with C'1s under the conditions just described, and the substance to be tested was added just before the addition of ALTEE. If the agent studied was inhibitory in the first test, but not the second, the assumption was made that it blocked the activation of C'1s but not the action of C'1 esterase. The concentrations at which the various agents were tested at either stage, after *all* dilutions, were soybean trypsin inhibitor, pancreatic trypsin inhibitor, lima bean trypsin inhibitor, and ovalbumin (1 mg/ml), Trasylol and hirudin (200 μg/ml), hexadimethrine bromide and Liquoid (20 μg/ml), heparin (10 Toronto units/ml), and epsilon aminocaproic acid (EACA), 0.04 M. Liquoid, because of its special interest, was also tested at lesser concentrations and without the preliminary 20 min period of incubation.

The *inhibition of esterolytic activity* was tested upon a substrate of 0.15 M AAME hydrochloride (pH 7.6). A volume of 0.25 ml of C'1r was incubated at 37°C for 20 min with 1.50 ml of phosphate-saline buffer alone or the same buffer containing the agent to be tested. Then 0.25 ml of AAME was added to each tube and the incubation continued for an additional 30 min. Aliquots of 1 ml were removed at 0 and 30 min and tested by the chromatropic acid technique. The concentration of the inhibitors in the enzyme-substrate mixture was the same as those used in tests of their effect upon the interaction of C'1r and C'1s.

PMSF-treated C'1r was prepared by incubating 1.8 ml of C'1r at 25°C for 30 min with 0.2 ml of 0.05 M phenyl methylsulfonyl fluoride (PMSF) (B grade, Calbiochem) dissolved in

isopropyl alcohol. As controls, 1.8 ml of C'1r was incubated with 0.2 ml of isopropyl alcohol or phosphate buffer. The three preparations were dialyzed separately overnight against 2 liters of phosphate buffer and tested for their ability to convert C'1s to C'1 esterase, to digest AAME, and to participate in the formation of the complex EAC'1,4.

Measurement of Hemolytic Activity.—C'1r was measured by its ability to form the complex EAC'1, or by reforming the complex EAC'1,4 from EAC'4, when incubated with C'1q and C'1s. This method has been described in detail elsewhere (4, 5). In these experiments, pellets of EA containing 5×10^8 cells were suspended in 0.5 ml of the sample to be tested for C'1r activity. Aliquots of 0.5 ml of $\frac{1}{100}$ dilutions of the other two subcomponents were then added. The diluent used was TBS-gelatin buffer containing 0.001 M Ca^{2+}. After incubation at 37°C for 10 min, formation of the complex EAC'1 was quantified by measuring the extent of hemolysis produced by the addition of a $\frac{1}{100}$ dilution of human serum in TBS-gelatin buffer

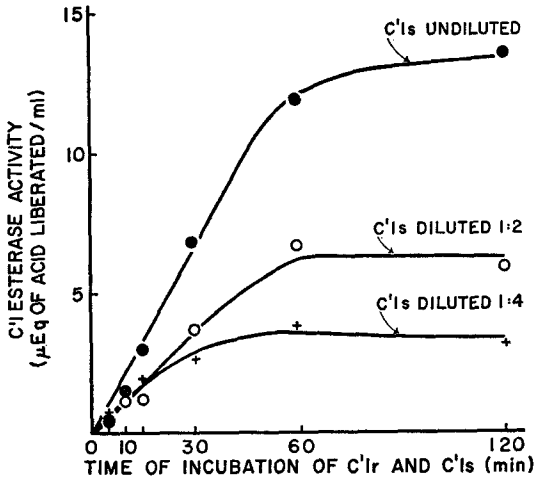

FIG. 1. The influence of the concentration of C'1s upon the evolution of C'1 esterase activity in mixtures containing a fixed amount of C'1r.

containing 0.016 M $Na_2MgEDTA$. The results are recorded in terms of optical density units such that complete hemolysis gives an optical density of 1.31, and lesser degrees of hemolysis proportionately smaller indices. All optical densities were measured with a Zeiss PMQ II spectrophotometer at 541 mμ.

Experiments were performed in a similar manner when formation of the complex EAC'1,4 was used to measure C'1r, except that EAC'4 was used rather than EA. EAC'4 was prepared as described before (4).

In all cases, the intermediate complex was washed with warm TBS-gelatin buffer containing 0.001 M Ca^{2+} before the addition of buffer containing human serum and $Na_2MgEDTA$.

RESULTS

The Conversion of C'1s to C'1 Esterase by Preparations of C'1r.—In earlier studies, the incubation of mixtures of C'1r and C'1s did not result in the evolution of appreciable esterolytic activity directed against ALTEE unless C'1q

was also present. In contrast, in the present experiments, such esterolytic activity gradually generated in the absence of C'1q. The ultimate titer of esterolytic activity toward ALTEE was proportional to the concentration of C'1s in the initial mixture (Fig. 1). The yield of C'1 esterase was, however, almost independent of the concentration of C'1r which determined, instead, the rate at which the esterase formed (Fig. 2). The rate at which C'1s was

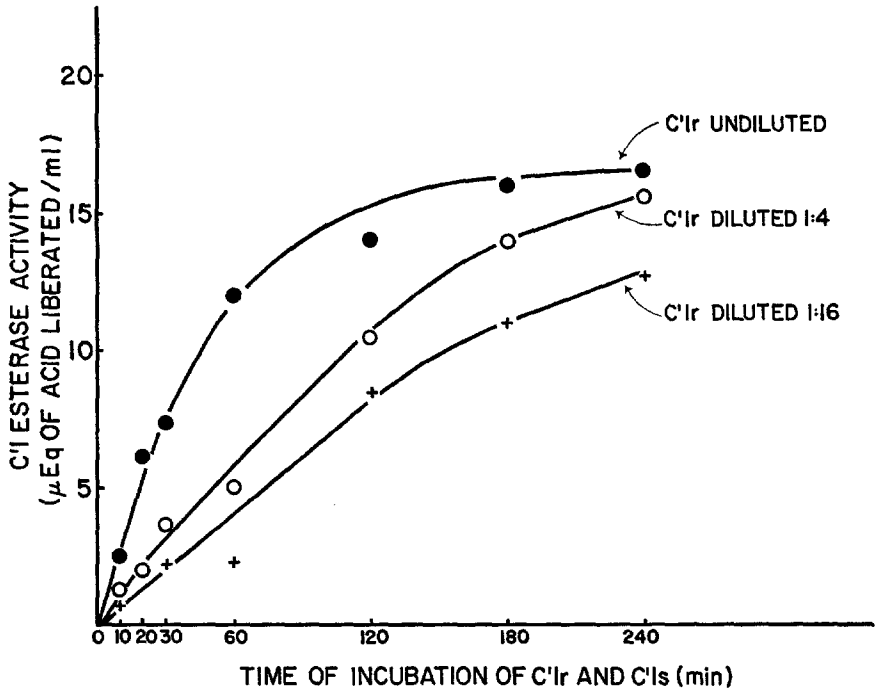

FIG. 2. The influence of the concentration of C'1r upon the evolution of C'1 esterase activity in mixtures containing a fixed amount of C'1s.

converted to C'1 esterase was also related to its initial concentration; the reciprocal of the velocity of formation of C'1 esterase was a linear function of the reciprocal of the initial concentration of C'1s (Fig. 3).

Over a span of temperatures, the rate at which C'1 esterase activity developed in a mixture of C'1r and C'1s was a function of the temperature at which these subunits were incubated. An Arrhenius plot relating the reciprocal of the absolute temperature of incubation with the logarithm of the yield of C'1 esterase within a given time was linear between 9° and 37°C; the apparent energy of activation required for the interaction of C'1r and C'1s was 23,000 cal/mole (Fig. 4). Above 37°C, increasing the temperature of the reaction

mixture caused a proportionately smaller increase in the yield of C'1 esterase, and at 50°C, the yield dropped sharply. This result might have been anticipated from the known heat lability of C'1 esterase (8, 22).

The optimal pH of the reaction between C'1r and C'1s was broad, approximately the same esterolytic activity evolving over a range extending from pH 7.0 to 9.0 (Fig. 5). The evolution of C'1 esterase was inhibited slightly by doubling the ionic strength of the reaction mixture from 0.113 to 0.225 (Table I). The formation of C'1 esterase proceeded at the same rate in the presence

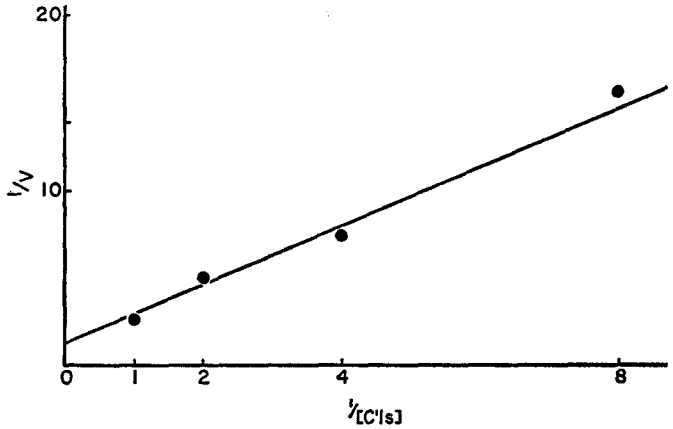

FIG. 3. C'1 esterase formation as a function of substrate concentration. In this experiment, 1.0 ml of C'1s (concentrated four-fold and then diluted serially in phosphate buffer) was incubated with 0.5 ml phosphate buffer and 0.5 ml C'1r for 20 min. The reaction was then stopped by the addition of 0.375 ml Liquoid (66 μg in high ionic strength phosphate buffer) and the C'1 esterase activity evolved was measured. Suitable control experiments were performed to show that Liquoid inhibited substantially all C'1r activity. In the figure, the vertical axis represents the reciprocal of the milliliters of 0.5 N NaOH used in the titration of liberated H^+ and the horizontal axis the reciprocal of the C'1s concentration with that of the most concentrated preparation designated as one.

or absence of 0.001 M Na_2HEDTA. But calcium ions at a concentration of 0.001 M, prevented the interaction of C'1r and C'1s. Manganese and strontium ions tested only at a concentration of 0.001 M were also inhibitory, while magnesium and potassium ions were not.

The formation of C'1 esterase in mixtures of C'1r and C'1s was strongly inhibited by small amounts of Liquoid. The concentration of Liquoid which was effective was a direct function of the initial concentration of C'1r. In many experiments, as little as 10 μg/ml completely blocked the activation of C'1s by C'1r (Table II). In contrast, this agent had no effect upon C'1 esterase, once this had evolved. When Liquoid was added before full activation of C'1s had taken place, it inhibited its further activation, demonstrating that under

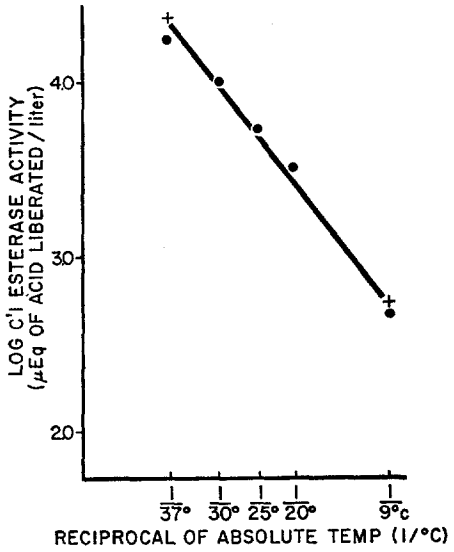

FIG. 4. The relationship between temperature and the rate of formation of C'1 esterase. The temperature is plotted as the reciprocal of the absolute temperature, but the legend appears in terms of the centigrade scale for convenience. In this experiment, 0.5 ml C'1r, 0.5 ml phosphate buffer, and 1.0 ml C'1s were incubated for 30 min at varying temperatures, after which 0.375 ml Liquoid (66 μg in high ionic strength phosphate buffer) was added to each tube, and the C'1 esterase activity evolved was then measured at 37°C. The graph is drawn between two calculated points indicated by the symbol X, using the method of least mean squares.

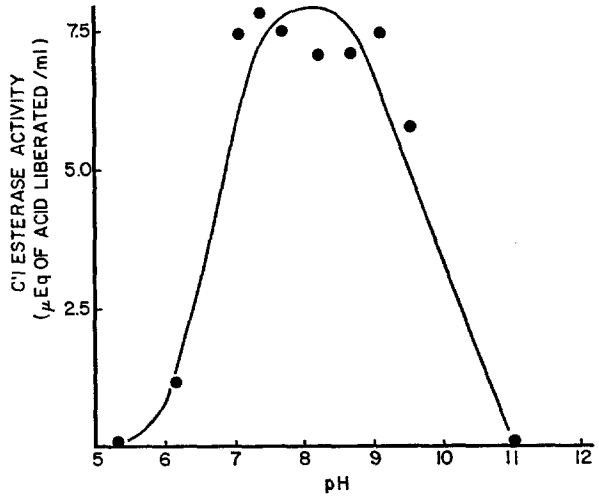

FIG. 5. The effect of pH upon the evolution of C'1 esterase activity in mixtures of C'1r and C'1s (see Methods).

these conditions autocatalysis of C'1s by C'1 esterase did not take place (Fig. 6).

Pretreatment of C'1r with phenyl methylsulfonyl fluoride, at a concentration of 0.005 M, partially inhibited its capacity to activate C'1s (Table III).

TABLE I

The Effect of Ionic Strength upon the Action of C'1r

Ionic strength	C'1 esterase activity evolved	Hydrolysis of AAME
	*μEq of acid liberated in 15 min**	*μEq of acid liberated in 60 min**
0.225	5.2	5.7
0.175	5.5	6.7
0.150	6.2	7.0
0.113	6.5	—
0.100	—	6.8

* See Methods; the hydrolysis of AAME was determined by microformol titration.

TABLE II

The Effect of Liquoid upon the Action of C'1r

Concentration of Liquoid	C'1 esterase activity evolved	Hydrolysis of AAME
μg/ml	*μEq of acid liberated in 15 min**	*μEq of acid liberated in 60 min** ‡
20	1.0	0.7
10	1.9	1.6
5	2.7	2.9
3.75	3.3	3.4
2.5	8.0	3.6
1.25	9.1	3.9
0	9.5	3.5
0	9.6§	—

* In the usual assay for the evolution of C'1 esterase activity, 0.5 ml C'1r, 1.0 ml of Liquoid in phosphate buffer and 0.5 ml C'1s were incubated for 30 min. The concentrations of Liquoid in this table refer to those in this first step of the assay.

‡ Aliquots of 1.0 ml of C'1r, 0.5 ml of Liquoid in phosphate buffer, and 1.0 ml of 0.15 M AAME were incubated for 60 min. The μEq of acid liberated were determined by microformol assay.

§ In this tube, the high ionic strength phosphate buffer added after the first incubation period contained sufficient Liquoid to bring the final concentration of this agent to 16 μg/ml, the same as that in the 20 μg tube.

On the other hand, at the concentrations tested, soybean, lima bean, pancreatic and ovalbumin trypsin inhibitors, hirudin, Trasylol, heparin, hexadimethrine bromide, and EACA were without effect, distinguishing C'1r from other known plasma proteases.

The capacity of C'1r preparations to induce the formation of C'1 esterase

was decreased by about two-thirds by incubation at 56°C for 30 min (Table IV).

The experiments that have been described demonstrate that the preparations

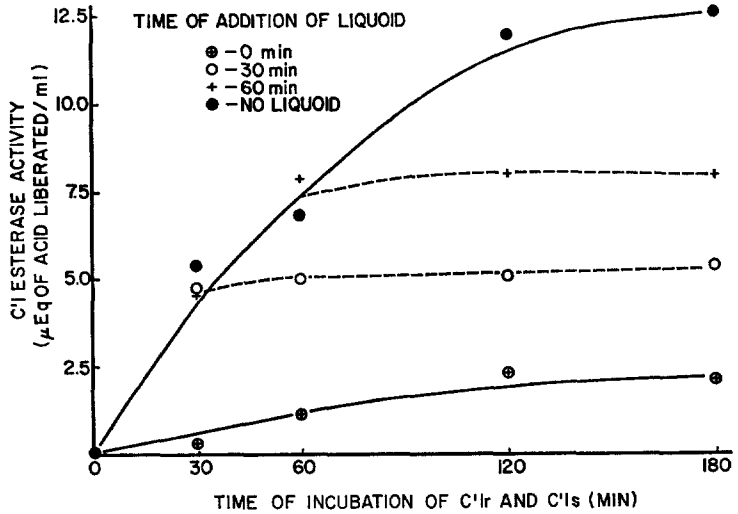

FIG. 6. The effect of Liquoid upon the reaction between C'1r and C'1s. In this experiment, C'1r and C'1s were incubated in quadruplicate for 3 hr. At intervals, C'1 esterase activity of aliquots of the mixtures was determined. At the start and at 30 and 60 min, the reaction between C'1r and C'1s was halted by the addition of sufficient Liquoid to give a final concentration of 10 μg/ml in the final reaction mixture.

TABLE III

The Effect of Phenyl Methylsulfonyl Fluoride upon the Action of C'1r

Preparations of C'1r	C'1 esterase activity evolved	Hydrolysis of AAME	Formation of EAC'1; extent of hemolysis in OD*
	*μEq of acid liberated in 15 min**	*μEq of acid liberated in 60 min**	
Buffer-treated	6.4	3.6	0.733
Isopropyl alchohol-treated	7.1	3.4	0.282
PMSF-treated	1.8	0.9	0.439

* See Methods; hydrolysis of AAME was determined by microformol titration.

of C'1r tested contained a heat-labile agent which could convert C'1s to C'1 esterase, a property blocked partially by PMSF and exquisitely by Liquoid.

The Esterolytic and Proteolytic Properties of Preparations of C'1r.—In experiments reported earlier, C'1r, alone or in combination with C'1q, did not hydrolyze ALTEE, a substrate of C'1 esterase and chymotrypsin (4, 6). The

preparations used in the present study were equally ineffective in hydrolyzing ALTEE. Nor did they digest BAPA, a substrate of trypsin, plasmin, and thrombin but not of PTA.

TABLE IV

The Effect of Temperature upon the Action of C'1r

Duration of incubation C'1r at 56°C	C'1 esterase activity evolved	Hydrolysis of AAME	Formation of EAC'1; extent of hemolysis in OD*
min	*μEq of acid liberated in 15 min**	*μEq of acid liberated in 60 min**	
0	7.2	12.5	0.480
5	6.5	9.9	0.015
10	6.1	8.6	0
20	4.1	4.8	0
30	2.7	3.4	0

* See Methods; hydrolysis of AAME was determined by microformol titration.

TABLE V

*Hydrolysis of Synthetic Amino Acid Esters**

Substrate	Enzyme‡							
	C'1r (undilute)	C'1 esterase	Plasmin	PTA	Thrombin	Trypsin	Urokinase	Kallikrein§
		5 units/ml	*0.16 units/ml*	*1 mg/ml*	*3 units/ml*	*1 μg/ml*	*500 units/ml*	
AME	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.32 (0.2)	0.00 (0.0)	0.22 (0.06)	0.00 (0.0)	0.18 (0.6)
AAME‖	1.27 (1.6)	0.88 (0.2)	0.63 (0.4)	1.40 (1.0)	0.70 (1.8)	0.94 (0.13)	0.28 (0.3)	0.54 (1.9)
BAME	0.19 (0.2)	0.18 (0.04)	0.26 (0.2)	0.63 (0.5)	0.23 (0.5)	0.26 (0.07)	0.02 (0.02)	0.47 (1.7)
CBZAME	0.00 (0.0)	0.44 (0.1)	0.34 (0.2)	2.02 (1.5)	0.86 (2.1)	1.30 (0.4)	0.10 (0.10)	1.07 (4.0)
TAME	0.05 (0.06)	0.30 (0.07)	0.27 (0.2)	1.36 (1.0)	0.40 (1.0)	1.12 (0.3)	0.04 (0.04)	0.44 (1.6)
LME	0.00 (0.0)	0.00 (0.0)	0.36 (0.3)	0.00 (0.0)	0.00 (0.0)	0.22 (0.06)	0.00 (0.0)	0.07 (0.2)
ALME	0.08 (0.1)	2.44 (0.6)	0.22 (0.2)	0.60 (0.4)	0.24 (0.6)	0.88 (0.2)	0.50 (0.5)	0.18 (0.6)
AGLME	0.78 (1.0)	4.34 (1.0)	1.41 (1.0)	1.38 (1.0)	0.40 (1.0)	3.60 (1.0)	0.96 (1.0)	0.28 (1.0)
CBZLME	0.00 (0.0)	1.92 (0.4)	0.25 (0.2)	0.61 (0.5)	0.46 (1.2)	1.45 (0.4)	0.17 (0.2)	0.24 (0.9)
TLME	0.00 (0.0)	0.48 (0.1)	0.18 (0.1)	0.48 (0.3)	0.60 (1.5)	1.13 (0.3)	0.06 (0.06)	0.08 (0.3)
AGME	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.00)	—

* μM of methyl alcohol liberated in 1 hr/ml of enzyme-substrate mixture. The number in parentheses is the rate of hydrolysis of the ester relative to that of N-α-acetylglycyl-L-lysine methyl ester.

‡ The concentration of the enzymes is recorded as that *before* dilution in the assay. The concentration of trypsin is corrected for its concentration of magnesium sulfate.

§ μM of methyl alcohol liberated in 2 instead of 1 hr. The preparation of kallikrein was said to release sufficient methyl alcohol from 10 volumes of 0.05 M TAME to give an optical density in the chromatropic acid assay of 0.600 (Colman, R. W., personal communication).

‖ *N*-acetyl-L-arginine methyl ester *p*-toluene sulfonic acid.

Preparations of C'1r were incubated with a number of substituted arginine, lysine, and glycine methyl esters. The effect of C'1r upon these substrates was compared with that of plasmin, trypsin, thrombin, C'1 esterase, urokinase, kallikrein, and PTA (Table V). In comparing different enzymes, pre-

vious investigators have usually recorded the digestion of different substrates relative to their effectiveness upon TAME (23). This proved impractical here. Some preparations of C'1r hydrolyzed TAME slightly, while others were ineffective. The relative activity of the different enzymes studied was therefore related instead to the hydrolysis of AGLME, which was digested by all the enzymes tested. Relative to the capacity of the various enzymes to hydrolyze AGLME, C'1r, thrombin, and kallikrein hydrolyzed AAME with especial avidity. C'1r was, however, readily distinguished from thrombin, for it did not digest CBZAME, TAME, CBZLME, or TLME appreciably, and from kallikrein, which hydrolyzed CBZAME particularly rapidly. Notably, α-chymotrypsin, at a concentration of 0.1 mg/ml, did not digest any of the substrates listed in Table V.

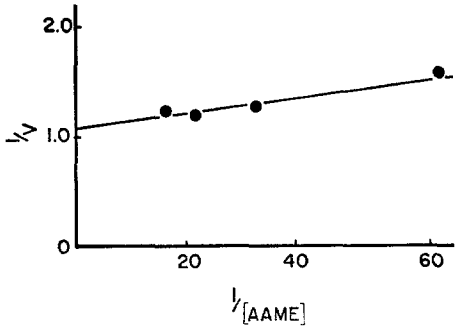

FIG. 7. Hydrolysis of AAME by C'1r as a function of substrate concentration. In this experiment, 1.0 ml of C'1r was incubated with 4.0 ml of AAME (diluted in phosphate buffer) and the H^+ liberated during incubation at 37°C for 60 min was determined by microformol titration. The velocity (vertical axis) is expressed as microequivalents of H^+ liberated per hour and the concentration of AAME is expressed in moles per liter (horizontal axis).

The digestion of AAME by preparations of C'1r was dependent upon the concentration of the substrate (Fig. 7), but the relationship was not clear. The reaction appeared to lag at low concentrations of substrate during the first 30 min of incubation. Thereafter, the reciprocal of the net hydrolysis between 30 and 60 min (or 0 to 60 min) was a linear function of the reciprocal of the concentration of AAME. These results are such that the calculation of the Michaelis constant seems inappropriate. The reaction was temperature dependent (Fig. 8); the apparent energy of activation in the range of temperatures between 9° and 40.5°C was 12,000 cal/mole. Above 40.5°C, increments of temperature increased the enzymatic activity of C'1r progressively less, and its hydrolytic activity decreased above 50°C. Preliminary incubation of C'1r at 56°C for 30 min before the addition of substrate reduced its activitv to about one-third of the unheated preparation (Table IV).

The hydrolysis of AAME by preparations of C'1r was maximal over a

broad range of pH (Fig. 9). The lack of a sharp dependency upon pH was noted in assays for the hydrolysis of this substrate in which the release of either methyl alcohol or of hydrogen ions was measured. Although the relationship between pH and hydrolysis is shown as a continuum in Fig. 9, a

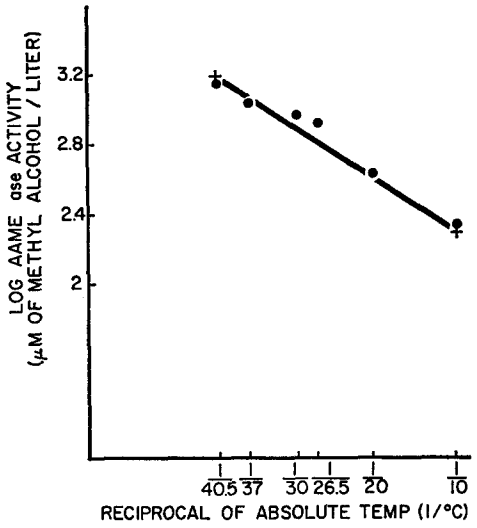

FIG. 8. The relationship between temperature and the rate of hydrolysis of AAME. The temperature is plotted as the reciprocal of the absolute temperature, but the legend appears in terms of the centigrade scale for convenience. In this experiment, 0.25 ml of C'1r and 2.0 ml of phosphate buffer were incubated at the indicated temperatures with 0.25 ml of 0.15 M AAME for 30 min. The micromoles of methyl alcohol liberated per milliliter of enzyme-substrate mixture were determined by the chromatropic acid assay. The graph is drawn between two calculated points indicated by the symbol X, using the method of least mean squares.

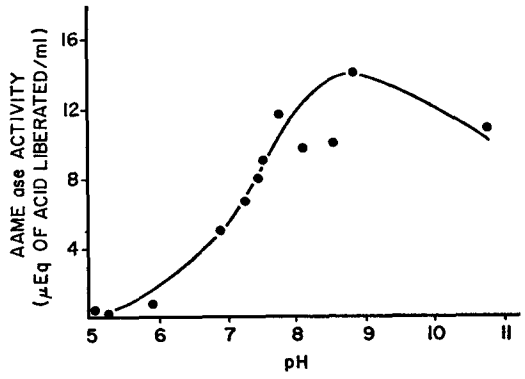

FIG. 9. The effect of pH upon the hydrolysis of AAME by C'1r (see Methods).

slight dip in activity at approximately pH 8.2 was observed on several occasions. Doubling the ionic strength of a mixture of C'1r and AAME inhibited hydrolysis only slightly (Table I). The addition of Na_3HEDTA or the chlorides of calcium, barium, strontium, manganese, magnesium, or potassium at a concentration of 0.001 M did not affect the hydrolysis of AAME by C'1r.

The digestion of AAME was blocked by as little as 5 μg of Liquoid/ml (Table II), and was partially inhibited by preliminary incubation of C'1r with 0.005 M PMSF (Table III). Soybean, lima bean, pancreatic and ovomucoid trypsin inhibitors, heparin, hirudin, Trasylol, and hexadimethrine bromide, on the other hand, were without effect.

Under the conditions used, the preparations of C'1r did not digest BAPA, casein, denatured hemoglobin, egg albumen, or bovine fibrin.

TABLE VI

Inhibition of C'1r by N-Acetyl-L-Arginine Methyl Ester

Mixture*	C'1 Esterase Activity Evolved
	μEq of acid liberated in 15 min
C'1r + sodium chloride solution	8.4
C'1r + AAME	3.5
C'1r + sodium chloride solution‡	7.7

* 0.5 ml C'1r, 0.5 ml 0.15 M sodium chloride solution, or 0.5 ml 0.15 M AAME, and 0.75 ml C'1s were incubated for 30 min, after which 0.375 ml high ionic strength phosphate buffer (containing 66 μg Liquoid/ml), and 0.5 ml 0.15 M sodium chloride solution were added and the esterolytic activity then determined upon the addition of 0.125 ml 1 M ALTEE.

‡ In this tube, 0.5 ml of 0.15 M AAME was substituted for the 0.5 ml 0.15 M sodium chloride solution added after the first 30 min period of incubation.

The observations described thus far suggest that the same agent in C'1r preparations which digested AAME was responsible for the conversion of C'1s to C'1 esterase. Were this the case, one would anticipate that AAME would block the formation of C'1 esterase. This proved to be the case (Table VI); 0.043 M AAME inhibited the activation of C'1s by C'1r significantly. The effect of AAME was due neither to a detectable change in pH nor of ionic strength. TAME, a substituted arginine ester not appreciably digested by C'1r, was even more effective as an inhibitor of the evolution of C'1 esterase, blocking the reaction at a concentration of 0.006 M.

Among the enzymes tested, only C'1 esterase hydrolyzed ALTEE. This substrate is also digested by chymotrypsin. Since C'1r was capable of converting C'1s to C'1 esterase, the question arose whether it might not also activate chymotrypsinogen to form chymotrypsin. Under the conditions used, however, C'1r did not activate this enzyme.

The Effect of C'1r Preparations upon Immune Hemolysis.—The preceding

experiments show that preparations of C'1r contain an agent which has the properties of converting C'1s to C'1 esterase and digesting certain synthetic amino acid esters. Both properties were affected in a similar manner by changes in pH, ionic strength, or temperature, and inhibited by Liquoid or PMSF but not by other known inhibitors of proteolytic enzymes, indicating that these

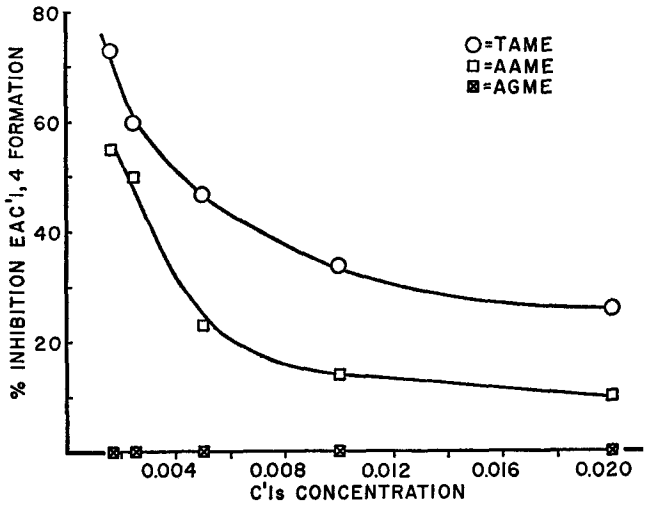

FIG. 10. The effect of TAME, AAME, and AGME upon the formation of the intermediate complex EAC'1,4. In this experiment, either AAME, TAME, or AGME was mixed with C'1r diluted $\frac{1}{200}$ and 0.5 ml of this mixture was added to tubes containing 0.5 ml C'1q diluted $\frac{1}{100}$, 0.5 ml C'1s diluted serially in TBS-gelatin buffer, and EAC'4 (5×10^8/ml). The substrate concentration in the final reaction mixture was 0.0067 M. After incubation at 37°C for 10 min, each sample was centrifuged, washed, and 4 ml of buffer containing $Na_2MgEDTA$ and serum diluted $\frac{1}{100}$ was added. Formation of the complex EAC' 1,4 was quantified by measuring the extent of hemolysis after an additional incubation at 37°C for 60 min. In control experiments, saline was added to C'1r at the same concentration as the substrate under test. The per cent inhibition of EAC'1,4 formation was obtained from a comparison of the extent of formation of the complex EAC'1,4 in the presence and absence of each substrate tested.

properties are functions of a single agent in C'1r preparations. Since C'1r is known to participate in immune hemolysis, the effect of various procedures upon the hemolytic activity of C'1r was investigated to further establish its identity as the agent responsible for the conversion of C'1s to C'1 esterase and the hydrolysis of AAME.

In the reactions leading to immune hemolysis, C'1r is thought to act in conjunction with C'1q and C'1s in the formation of the intermediate complex EAC'1. Our preparations of C'1r digested AAME and brought about the evolution of C'1 esterase in the absence of C'1q. Nonetheless, these prepara-

tions did not support hemolysis unless C'1q was also present. These observations suggest that C'1r possesses at least two functional sites. A comparison of the effect of various procedures upon the enzymatic and hemolytic functions of C'1r seems to confirm this view.

As tested by their effect upon C'1s and upon AAME, the preparations of C'1r were partially inactivated by incubation at 56°C for 30 min. In contrast, virtually all hemolytic activity was lost within 5 min at this temperature, suggesting that the portion of C'1r which may react with C'1q is more heat labile than the catalytic site (Table IV).

TABLE VII

The Effect of Liquoid upon the Hemolytic Action of C'1r

Concentration of Liquoid	Formation of EAC'1,4*
μg/ml	*OD*‡
16.66	0.000
3.33	0.000
1.66	0.006
0.33	0.020
0.16	0.250
0.03	1.020

* In this experiment, 0.5 ml C'1q and 0.5 ml C'1s at $\frac{1}{100}$ dilutions, and 0.5 ml C'1r at $\frac{1}{200}$ dilutions were mixed with a button of EAC'4 (5×10^8/ml) in an ice bath. The Liquoid was mixed with C'1r just before addition to the C'1q, C'1s, EAC'4 mixture; the concentration of Liquoid recorded is that in the final reaction mixture. After incubation at 37°C for 10 min, each sample was centrifuged, washed, and 4.0 ml of buffer containing $Na_2MgEDTA$ and serum diluted $\frac{1}{100}$ was added. The extent of formation of the complex EAC'1,4 was determined by the degree of hemolysis after an additional incubation at 37°C for 60 min.

‡ Optical density at 541 mμ, corrected for EAC'4 control.

As demonstrated in earlier paragraphs, PMSF inhibits the activity of C'1r as measured upon C'1s or AAME. The results of studies on the action of PMSF-treated C'1r as measured by formation of the complex EAC'1,4 were paradoxical. Although the PMSF-treated C'1r was less effective than untreated C'1r, isopropyl alcohol–treated C'1r was even less active (Table III). The inhibition of C'1r activity by treatment with isopropyl alcohol, like the inhibitory effect of heating, suggests that this subcomponent of C'1 has at least two active sites. The PMSF was dissolved in isopropyl alcohol, yet it was less inhibitory than the alcohol alone. The apparently protective effect of PMSF against inactivation of C'1r by alcohol is unexplained.

If the properties of C'1r preparations which we have described were attributable to this component of C', one would expect that AAME would inhibit the formation of the intermediate complex EAC'1,4. That this was true is demonstrated in Fig. 10. At a concentration of 0.0067 M, AAME blocked the

formation of this intermediate complex, as reflected by inhibition of hemolysis. This inhibitory effect was not due to alteration of pH or ionic strength and the effect of AAME was progressively greater the lower the concentration of C'1s in the mixture. TAME, a substrate not readily hydrolyzed by C'1r, and as noted above, an inhibitor of the conversion of C'1s to C'1 esterase by C'1r, was also an effective inhibitor of the formation of the complex EAC'1,4 (Fig. 10). In contrast, AGME, a substrate which was not digested by C'1r, was without effect. These experiments do not localize the stage in the formation of EAC'1,4 at which inhibition occurs.

Finally, since Liquoid inhibited the digestion of AAME and the conversion of C'1s to C'1 esterase by C'1r, this inhibitor should block the formation of the complex EAC'1,4. This, indeed, proved to be the case as shown in Table VII. These experiments do not localize the inhibitory effect to a reaction between C'1r and Liquoid, but since earlier experiments demonstrated that Liquoid inhibits the activation of C'1s by C'1r, this seems to be the most likely possibility. Further experiments have shown that inhibition of the formation of the complex EAC'1,4 is not due to an effect of Liquoid upon EAC'4, since incubation of Liquoid and EAC'4, followed by washing, did not inhibit subsequent formation of the complex EAC'1,4.

DISCUSSION

The experiments which we have presented demonstrate that preparations of C'1r have the three properties of participating in immune hemolysis, converting C'1s to C'1 esterase, and of hydrolyzing various synthetic amino acid esters, of which AAME appeared to be particularly susceptible. Our data suggest that these properties can be attributed to a single entity, C'1r, but not necessarily to the same portion of its molecular structure. The C'1r preparation used was by no means pure, containing approximately six protein bands identifiable by disc electrophoresis, but it represented about a 20-fold increase in purity compared to the euglobulin fraction from which it was separated.

With two exceptions, the functions attributed to C'1r were affected in the same way by various manipulations. All three functions were blocked by small amounts of Liquoid, a known anticomplementary agent previously thought to act upon a unique component of C' designated as $C'1_x$ (24, 25). Further, in unpublished experiments with Dr. J. Pensky and Dr. D. Ogston, C'1 esterase inhibitor, an agent in human plasma which blocks the activity of C'1 esterase, has been found to inhibit hydrolysis of AAME and the activation of C'1s by preparations of C'1r, adding additional force to the view that these two properties of C'1r preparations are due to the same moiety. In contrast, soybean, lima bean, pancreatic and ovalbumin trypsin inhibitors, hirudin, Trasylol, heparin, and hexadimethrine bromide were without effect on the hydrolysis of AAME or the activation of C'1s by C'r preparations. Additional support for

the identity of the various properties ascribed to C'1r was found in the inhibition of the formation of C'1 esterase and of the intermediate complex EAC'1,4 by AAME. Suitable experiments to determine whether inhibition of this arginine ester was competitive were not performed.

Less easily understood are the effects of phenyl methylsulfonyl fluoride and of heating upon the properties of C'1r. Pretreatment of C'1r with PMSF, an agent which inhibits some hydrolytic enzymes, partially blocked its capacity to hydrolyze AAME and to convert C'1s to C'1 esterase. The effect of PMSF upon the hemolytic function of C'1r was not clear since its solvent, isopropyl alcohol, was even more inhibitory. Heating the preparations of C'1r to 56°C for half an hour decreased its activity toward AAME and C'1s by about two-thirds, but after only 5 min at this temperature, it was unable to function in the formation of the complex EAC'1.

The energy of activation of C'1r, in its reaction with C'1s, was 23,000 cal/mole. This value is essentially the same as that previously found for the interaction of C'1q, C'1r, and C'1s in experiments in which the product of these reactions was measured in a hemolytic system (5). This similarity is supportive evidence for the postulate that the formation of C'1 esterase by the reaction between C'1r and C'1s and the participation of the C'1 complex in immune hemolysis are facets of the same reaction. In its reaction with AAME, the energy of activation was considerably less, being 12,000 cal/mole. Interestingly, this is the same value obtained in studies of the reaction between C'1 esterase and ALTEE (8, 22).

The preparations of C'1r used in the present experiments did not function in the formation of the intermediate complexes EAC'1 and EAC'1,4 unless both C'1q and C'1s were present. They hydrolyzed AAME and converted C'1s to C'1 esterase, however, even though C'1q was not present. This observation was in contrast to that of earlier studies in which C'1q was needed for the activation of C'1s (4). Examination of the conditions under which the earlier and present experiments were performed suggests an explanation. In the current studies, the interaction of C'1r and C'1s proceeded in solutions devoid of calcium ions and was inhibited in their presence. In the experiments reported earlier, C'1r and C'1s had always been incubated together in the presence of calcium ions; C'1 esterase evolved only if C'1q was added to the mixture. Experiments to be published separately seem to rationalize the apparently contradictory observations just noted. C'1q appears to have among its functions the capacity to overcome the inhibitory effect of calcium upon the reaction between C'1r and C'1s. Thus, what we interpreted as a difference between the C'1r preparations studied, earlier and now, seems instead to be a difference in the ionic composition of the test systems used.

The dependence of the reaction between C'1r and C'1s upon the presence of C'1q suggests that C'1r has at least two functions, namely, the capacity to

react with C'1q and with C'1s. We have already noted that the hemolytic function of C'1r was much more heat labile than its function as a hydrolytic agent for AAME, or as an activator of C'1s, and that isopropyl alcohol inhibits the hemolytic activity of C'1r but not its other properties. Perhaps, then, the portion of the molecule which interacts with C'1q is more susceptible to heating and isopropyl alcohol than that portion which acts upon AAME and C'1s.

The question arises whether the enzymatic properties of preparations of C'1r might not be due to contamination with a known enzyme. Data concerning the synthetic substrates susceptible to the action of C'1r appear to separate this enzyme from trypsin, chymotrypsin, plasmin, urokinase, C'1 esterase, PTA, kallikrein, and thrombin. Additionally, the activity of C'1r was not blocked by soybean trypsin inhibitor, known to impede the action of trypsin, plasmin, kallikrein, plasma permeability factor, and activated Stuart factor (factor X). Nor was C'1r inhibited by hirudin, an antithrombic agent, or by heparin, which blocks the function of activated PTA and thrombin. Its failure to digest ALTEE, and its inhibition by Liquoid distinguished it from C'1 esterase itself. We do not wish to imply that the preparations of C'1r studied were pure; indeed, they are known to contain small amounts of plasminogen.

These observations are in agreement with the proposal we made in an earlier paper, that C'1r in its active form may be a protease capable of converting C'1s to C'1 esterase, a second hydrolytic enzyme (6). That one protease may activate another is, of course a commonplace. That two subunits of a macromolecule may have distinct but interrelated functional activities has been demonstrated in the tryptophane synthetase system of *Escherichia coli* (26). In this system, however, one enzymic subcomponent does not activate another. The behavior of bovine procarboxypeptidase A, however, bears a direct resemblance to that of C'1. Freisheim, Walsh, and Neurath (27) have disaggregated this zymogen by succinylation; one unit, designated succinyl fraction I, can be converted into active carboxypeptidase by treatment with bovine trypsin or by the subcomponent of procarboxypeptidase designated as fraction II, an endopeptidase. Another system in which such a relationship has been postulated is the prothrombin complex (28). Seegers believes that thrombin arises because of sequential reactions among subcomponents of the prothrombin molecule; this view is not universally accepted (28).

The studies we have presented furnish further support for the view that certain biological functions are carried out by a succession of proteolytic reactions. In this way, the early steps in the immune mechanism which require the participation of complement are similar to those already described for the coagulation of blood and for the formation of the biologically active polypeptide kinins. The evolutionary meaning of these processes remains to be investigated.

SUMMARY

Human C'1, a macromolecular complex composed of three subunits, is the zymogen for at least two distinct enzymes. Preparations of one subunit, C'1r, functioned as a protease which converted another subunit, C'1s, to C'1 esterase. The conversion of C'1s to C'1 esterase by C'1r was blocked by Liquoid, phenyl methylsulfonyl fluoride, and calcium ions, but not by soybean trypsin inhibitor, hirudin, or heparin.

Preparations of C'1r also possessed two additional functions, i.e., the ability to hydrolyze certain synthetic amino acid esters and to participate in immune hemolysis. Evidence was presented which indicates that these three functions are properties of a single entity, C'1r, but not of the same portion of its molecular structure. These observations suggest that C'1r has at least two active sites, one for its reaction with C'1q, an additional subunit of C'1, and one for its reaction with C'1s; together, the three subcomponents, C'1q, C'1r, and C'1s, form a single functional unit, the first component of complement.

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