THE INACTIVATION OF COMPLEMENT AND ITS COMPONENTS BY PLASMIN*

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Human serum contains an inactive proteolytic enzyme, plasminogen, which may be converted to its active form, plasmin, either spontaneously or by the addition of various agents. Plasmin digests fibrinogen, fibrin, prothrombin (1), and accelerator globulin (2) at or near neutral pH, but little or nothing is known about its activity on other serum proteins.

Human serum also contains a complex of substances, known collectively as complement, of which four components have been recognized (3, 4). Complement plays a significant role in certain immune reactions.

Streptokinase, a principle found in filtrates of cultures of hemolytic streptococci, converts plasminogen to plasmin (5–7). We have found that the addition of streptokinase to human serum also inactivates complement. This suggested that plasmin digested components of complement. It was further noted that complement components are destroyed by chloroform-activated plasmin. These observations and their biological implications are reported in this paper.

Nomenclature

Complement is designated C′ and its four recognized components are indicated by the symbols C′1, C′2, C′3, and C′4 (8). Serum fractions employed as reagents for the titration of the components of C′ are designated R1, R2, R3, and R4 (9). R1, for the titration of C′1, consists of the proteins soluble at pH 5.4 and ionic strength 0.02 at 0° (10). R2, for the titration of C′2, contains the proteins insoluble at pH 5.4 and ionic strength 0.02 at 0° (10). R3, the test reagent for C′3, is prepared by treatment of C′ with zymosan (10, 11). R4, for the titration of C′4, is prepared by the addition of hydrazine to C′ (10, 12). Detailed procedures for the preparation and characterization of these reagents have been published (4, 9, 10, 13).

The terms *plasmin*, *plasma proteolytic enzyme*, and *plasma protease* will be used interchangeably to designate the substance or substances in plasma or serum which can digest fibrinogen, fibrin, and certain other proteins *in vitro* at or near neutral pH. This enzyme,

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which has also been called fibrinolysin (14), is usually present in plasma or serum as an inactive *precursor*, sometimes designated *plasminogen.* The filterable principle of cultures of beta-hemolytic streptococci which activates the precursor will be called streptokinase (SK) (15). The *clot lysis time* is the length of time required for a visible clot to disappear. In all experiments, the clot lysis time was measured from the time that the reagents were mixed, rather than from the time that clotting took place. This was a matter of convenience, because the clotting time was usually short compared with the clot lysis time.

Materials

Complement.—Human blood from healthy donors was chilled shortly after being drawn. The serum was separated within 15 hours and centrifuged clear of red cells at 4000 R.P.M. at 1° in a refrigerated centrifuge. The clear serum was maintained at 1° until needed and any unused portion discarded after 24 hours. R1, R2, R3, and R4 were diluted with 0.15 M NaCl to 1.5 times the original serum volume, adjusted to pH 7.0 ± 0.2, and maintained at 1° until needed.

Sensitized Sheep Cells.—The red cells from fresh sheep blood collected in Alsever's solution (16) were washed 3 times with barbital buffer (17) containing 0.0015 M $CaCl_2$ and 0.0005 M $MgCl_2$ and then suspended to a 1.25 per cent suspension in buffer containing 4 units per ml. of rabbit hemolysin.

Streptokinase.—"Varidase,"[1] a lyophilized preparation of streptokinase, was dissolved in distilled water or barbital buffer. This preparation contained streptococcal desoxyribonuclease as well as streptokinase.

Trypsin and Pancreatic Desoxyribonuclease.—Each once recrystalized, and obtained from Worthington Biochemical Company.

Plasmin.—A crude solution of human plasmin, activated by chloroform, was prepared by a method previously described (18). Chloroform-activated bovine plasmin,[2] prepared by the method of Loomis, George, and Ryder (14) was also tested. The preparation used contained 0.82 Loomis unit per mg.

Bovine Thrombin[3].—The thrombin was dissolved in buffer to a concentration of 1000 National Institutes of Health (N.I.H.) units (19) per ml., stored at −20°, and diluted to 10 N.I.H. units per ml. with barbital buffer when needed.

Bovine Fibrinogen.[4]—Fibrinogen, prepared by the method of Ware, Guest, and Seegers (20), was dissolved in barbital buffer and the undissolved residue was separated by filtration through Whatman No. 1 filter paper. In all experiments with the fibrinolytic system, 100 mg. portions of dried fibrinogen, which contained sodium chloride in a ratio of 3.1 gm. of protein to 0.9 gm. of salt, were dissolved in 2.6 ml. of water and 17.4 ml. of barbital buffer. Assay of the filtrate demonstrated approximately 300 mg. of coagulable protein per 100 ml. of solution.

Barbital Buffer.—Buffer for use *in the fibrinolytic system* consisted of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5.

All other reagents were the best grade obtainable. Double distilled water was employed throughout. All glassware was cleaned with sodium dichromate-sulfuric acid solution and washed in distilled water until free of all traces of cleaning solution. International PR-1 refrigerated centrifuges were used for centrifugation. Constant temperature water baths, accurate to ± 0.5°, were employed for incubation. Hydrogen ion determinations were made with the Cambridge research model pH meter.

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[1] Kindly supplied by F. Ablondi of Lederle Laboratories Division, American Cyanamid Company.

[2] Kindly supplied by Dr. E. C. Loomis of Parke, Davis and Co.

[3] Kindly supplied by Dr. E. C. Loomis of Parke, Davis and Co.

[4] Kindly supplied by Dr. Walter Seegers.

Methods

Titration of C′ and Its Components (4, 9, 10, 13).—All titrations were carried out at the 50 or 100 per cent hemolytic end-points, or both. A unit of complement is defined here as the smallest amount of complement which will cause complete hemolysis of 1 ml. of a 1.25 per cent suspension of sensitized cells in a final volume of 1.5 ml. after 30 minutes at 37°. A 50 per cent unit represents the smallest amount of complement, or mixture of complement components and complement reagents, necessary to produce lysis of 50 per cent of the cells in this system.

A rough determination was made of the presence or absence of a component of complement in specific reagents and in serum after treatment with streptokinase or other agents. Two 100 per cent units of complement inactivated by different methods were added to an equal amount of test reagents. 1 ml. of sensitized cells was added and the volume brought up to 1.5 ml. with buffer. The mixture was incubated at 37° for 30 minutes and finally centrifuged at 1500 R.P.M. for 5 minutes. The clear hemoglobin supernatants were compared with a series of standard hemoglobin solutions and the degree of hemolysis was expressed as percentage of complete hemolysis.

The estimation of the actual titer of individual components was carried out with slight modification as described by Bier *et al.* (9) and by Kabat and Mayer (13) using a 50 per cent hemolytic end-point. For example, 2 to 3 units of test reagents were added to 0.2 ml. of varying dilutions of an inactivated C′; 1 ml. of sensitized cells was added, and the mixtures were incubated at 37° for 30 minutes. They were then centrifuged at 1500 R.P.M. for 5 minutes and the amount of the inactivated C′ which gave 50 per cent hemolysis in the presence of the test reagents was considered a unit. The dilution of sample multiplied by 5 gave the total number of component units per milliliter of sample. Untreated C′ was tested simultaneously and the results were expressed either in units or as per cent inactivation of each component compared to the untreated serum.

General Methods for Testing the Inactivation of C′ by Streptokinase, Plasmin, etc.—To x ml. of human serum of known C′ activity, y ml. of streptokinase containing a specified number of units, or other agents, was added with stirring at the desired temperature, ionic strength, hydrogen ion concentration, and serum dilution. The mixtures were then incubated at the desired temperature for 1 hour and immediately tested for C′ activity, C′ component activity, or both. In some experiments, samples were stored at 0° before testing because it was found that streptokinase did not destroy C′ in a few hours at this temperature. The ionic strength did not exceed 0.15 except in experiments in Tables VI, VII, and VIII.

The *spontaneous proteolytic activity* of serum was assayed in the following manner. The sample to be tested was diluted serially 256-fold with buffer. 0.3 ml. of each dilution was mixed with 0.3 ml. of bovine fibrinogen and the mixture was then clotted by the addition of 0.1 ml. of bovine thrombin. A drop of toluol was added to each tube and the tubes were stoppered, incubated at 37°, and observed frequently for evidence of fibrinolysis. Normally no fibrinolysis is observed by this technique within 48 hours.

The *inhibitory activity* of serum was tested against plasmin. The substance to be tested was diluted serially 256-fold with buffer. 0.3 ml. of each dilution was mixed with 0.3 ml. of bovine plasmin. The mixtures were incubated at room temperature for 10 minutes and then placed in an ice bath. 0.1 ml. of bovine thrombin and 0.3 ml. of bovine fibrinogen were added in succession. The mixtures were then incubated at 37° and observed for fibrinolysis. In each experiment the rate of fibrinolysis was compared with the rate of fibrinolysis by plasmin in the absence of the substance to be tested. In most experiments, the concentration of enzyme, after all dilutions, was 0.75 Loomis unit per ml.

The *total amount of plasminogen* which was available for activation by streptokinase cannot be estimated satisfactorily because serum contains substances which interfere with the activation of the enzyme from its precursor and with the active enzyme itself. Relative estimates

of the amount of plasminogen available were made in the following way. The serum was diluted to one-fourth its original concentration with buffer. 0.3 ml. of the diluted serum was mixed with 0.3 ml. of streptokinase solution which had been diluted serially with buffer. The mixtures were allowed to stand at room temperature for 10 minutes. Then 0.1 ml. of bovine thrombin and 0.3 ml. of bovine fibrinogen were added to each tube. The clot lysis time of the mixtures was determined at 37°. The concentration of streptokinase in the tube with the shortest clot lysis time was considered optimal. 1 ml. of the serum to be tested, diluted with buffer to one-fourth its original concentration, was then mixed with an equal volume of streptokinase solution of optimal concentration and allowed to stand at room temperature for 10 minutes. The mixture was then diluted serially 128-fold with buffer. 0.6 ml. of each dilution was then mixed with 0.1 ml. of bovine thrombin and 0.3 ml. of bovine fibrinogen. The rate of clot lysis was then observed at 37° and compared in the various samples tested.

RESULTS

1. The Effect of Streptokinase (SK) on C′ of Various Species.—Fresh pooled serums from human adult, human infant (age 2 to 3 months), monkey, rabbit, dog, and guinea pig were incubated with varying amounts of streptokinase at 37° for 1 hour. It will be seen in Table I that small amounts of SK inactivated human adult C′. 10 to 50 times more SK was required to inactivate infant C′ than adult C′. C′ from other animal serums was only slightly or not at all inactivated by enormous doses of SK.

TABLE I

The Effect on C′ of the Addition of Streptokinase to Serums of Various Animals

Serum	SK units/ml. serum required to inactivate C′
Human adult	75–150
Human infant	2000–5000
Monkey	>50,000 (*ca.* 50 per cent inactivation at 20,000)
Rabbit	>50,000 (*ca.* 50 per cent inactivation at 50,000)
Dog	>50,000*
Guinea pig	>50,000*

* Highest concentration tested was 50,000 per ml. which only slightly reduced the C′ titer.

Mixtures of human, monkey, and guinea pig C′ were treated with 500 units of SK and the C′ titer determined. These experiments are summarized in Table II which shows that the addition of SK to a mixture of an equal part of human serum and either monkey or guinea pig serum resulted in the inactivation of both the monkey or guinea pig C′ along with the human C′. Monkey alone, guinea pig alone, or mixtures of these two serums were not inactivated by SK. These results eliminated the possibility of a direct action of SK on C′ and indicated that the human plasmin activated by SK had the ability to destroy C′ of guinea pig and monkey serum. However, the experiment did not differentiate between the possibilities that either monkey and guinea pig serums were low in plasminogen or that the SK was specific for human plasminogen.

2. *The Effect of Desoxyribonuclease.*—"Varidase," which is a purified preparation of streptokinase, contains varying amounts of desoxyribonuclease. Streptokinase has not been prepared free of this enzyme and it seemed necessary to determine the effect of desoxyribonuclease on C′. Crystalline pancreatic desoxyribonuclease, in amounts varying from 0.1 to 10 mg., was added to 1 ml. of human serum, incubated for 1 hour at 37°, and then tested for C′.

TABLE II

The Effect on C′ of the Addition of Streptokinase to Mixtures of Monkey, Guinea Pig, and Human Serum

Human serum	Monkey serum	Guinea pig serum	SK	C′ activity
ml.	*ml.*	*ml.*	*units*	*hemolytic units/ml.*
0.5	0.5		500	0
0.5		0.5	500	0
0.5			500	0
	0.5	0.5	500	92
	0.5		500	20
		0.5	500	163
0.5	0.5		0	22
0.5		0.5	0	95
0.5			0	25
	0.5		0	20
		0.5	0	163

All mixtures made up to a final volume of 1.5 ml. with 0.9 per cent saline.

TABLE III

The Effect on C′ of the Addition of Streptokinase to Human Serum at 1° and 37°

Human serum	SK	Conditions	C′ activity
ml.	*units*		
(A)2	2000	1 hr. at 37°	0
(B)2	2000	24 hrs. at 1°	100
(C)	Serum B, reincubated for 1 hr. at 37°		0

No inactivation of C′ occurred and it appeared that pancreatic desoxyribonuclease did not destroy C′.

3. *The Possible Role of Complement Fixation.*—It is well known that human serum may contain varying amounts of antibodies to streptokinase because of previous streptococcal infections. It is also well known that antigen-antibody aggregates will inactivate C′. "Fixation" of C′ to immune aggregates occurs at 1° as well as at 37° (21, 22). If SK failed to inactivate C′ at 1° but did inactivate it at 37°, this would be additional evidence that "fixation" of C′ was not involved. The results of such an experiment are given in Table III which reveals that the addition of large amounts of SK to human serum failed

to inactivate C′ at 1° in 24 hours, while such amounts destroyed C′ readily at 37° in 1 hour. Reincubation of the serum which had been treated with SK at 1° for an additional hour at 37° inactivated C′. These results indicate that SK destroyed C′ indirectly by converting plasminogen to plasmin which in turn acted upon C′.

4. The Effect of Temperature.—The effect of temperature is portrayed in Fig. 1 which shows the effect on human C′ of the concentration of SK as a function of temperature and time. It will be observed that the inactivation of C′ was dependent both upon temperature and SK concentration, proceeding faster at higher temperatures and higher concentrations of SK. At tempera-

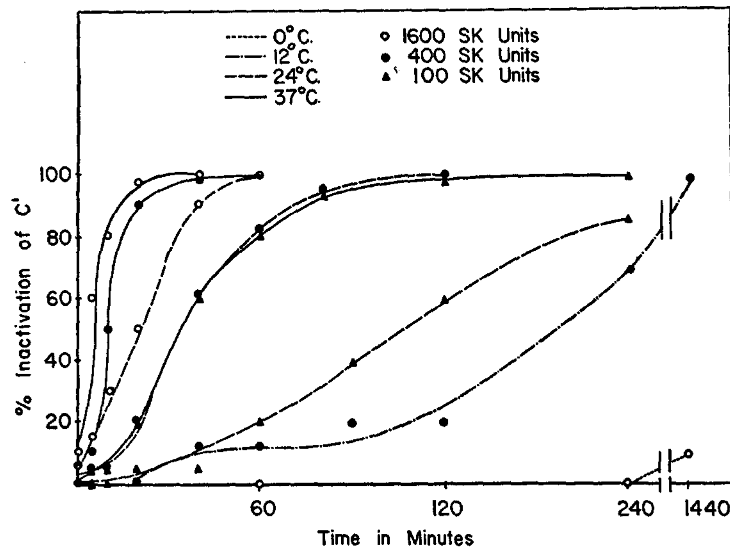

FIG. 1. The effect of temperature on the inactivation of C′ by streptokinase.

tures above 12° the same degree of inactivation of C′ could be achieved either by incubating at higher temperatures with a given amount of SK or at lower temperatures with larger amounts of SK. The degree of inactivation per unit time appeared to be more sensitive to temperature than to SK concentration.

5. The Influence of Hydrogen Ion Concentration.—Aliquots of a pool of fresh human serum were adjusted to various hydrogen ion concentrations with HCl or NaOH. Untreated serums and mixtures of these serums with streptokinase were incubated for 1 hour at 37° and then tested for C′ activity. The results of this experiment are summarized in Table IV which shows that (1) hydrogen ion concentrations greater than pH 7.0 inhibited the action of SK, and (2) SK operated optimally at neutral or alkaline pH. These results, with the exception of the increased inactivation of C′ by SK at pH 8.9, are similar

to those reported earlier (23) for the effect of pH on the digestion of casein by plasmin.

Table V further shows the effect of pH on the inactivation of the components of human C′ by SK. It will be observed that no inactivation of C′1 and only slight inactivation of C′3 occurred at pH 5.5 and that fully 80 per cent of C′2 and C′4 activity was destroyed. Upon increasing the alkalinity of the mixtures, C′1, C′2, and C′4 were progressively inactivated while C′3 maintained

TABLE IV

The Influence of pH on the Inactivation of C′ by Streptokinase-Activated Plasmin

Final pH	Untreated serum	Serum + 100 units SK/ml.	Serum + 500 units SK/ml.
	Original C′ activity remaining		
	per cent	*per cent*	*per cent*
5.5	95	80	30
6.6	100	60	0
8.0	100	20	0
8.5	100	35	0
8.9	95	<5	0

Serum and reagents adjusted to desired final pH with HCl or NaOH. Mixture of serum and streptokinase, and serum alone incubated for 1 hour at 37° and then tested for C′ activity.

TABLE V

The Influence of pH on the Inactivation of the Components of C′ by Streptokinase-Activated Plasmin

Serum + 150 SK units/ml.	Inactivation			
pH	C′1	C′2	C′3	C′4
	per cent	*per cent*	*per cent*	*per cent*
5.5	0	80	20	80
6.8	75	>95	50	>95
7.8	85	>95	50	>95
8.5	95	100	50	100

50 per cent of its activity even at pH 8.5. These results compare favorably with those shown in Table IV on the effect of pH on C′ titer and indicate that the immediate inactivation of C′ by plasmin was due to the destruction of C′2 and C′4.

6. The Effect of Ionic Strength.—Mixtures of different amounts of SK and aliquots of human serum adjusted to the desired final ionic strength with 2 M NaCl were incubated at 37° for 1 hour and tested for C′ activity. It will be seen in Table VI that the ionic strength of the medium had a profound influence on the action of SK. At ionic strengths over 0.15 (isotonic), the inac-

tivation of C′ by SK was inhibited, becoming most marked at 0.3 but recognizable at 0.2. Increasing the concentration of SK overcame this inhibition to some extent but did not entirely abolish it.

TABLE VI

The Influence of Ionic Strength on the Inactivation of C′ by Streptokinase

Ionic strength*	SK/ml. undiluted serum	C′ activity remaining
	units	*per cent*
0.075‡	100	5
0.15	100	5
0.20	100	45
0.25	100	80
0.3	100	100
0.075‡	200	0
0.15	200	0
0.20	200	5
0.25	200	40
0.30	200	80
0.075‡	1000	0
0.15	1000	0
0.3	1000	25
0.6	1000	70

* Human serum adjusted to desired *final ionic strength* with 2 M NaCl and made up to a final dilution of 1:2 with H_2O. Mixtures incubated for 1 hour at 37° and then tested for C′ activity.

‡ Diluted with an equal part of water.

TABLE VII

*The Influence of Ionic Strength on the Activation of Plasmin by Streptokinase**

Ionic strength	0.10	0.15	0.20	0.25	0.30
Clot lysis time, *min*	2½	3	4	9	11

* Clot lysis time of a mixture of 0.1 ml. thrombin, 0.1 ml. streptokinase (312 units per ml.), 0.3 ml. sodium chloride or water, 0.2 ml. fresh serum, and 0.3 ml. bovine fibrinogen.

The effect of hypertonic solutions may have been due to inhibition of the activation of plasminogen by SK, or to inhibition of the active enzyme, plasmin. Preliminary experiments make it seem likely that hypertonic solutions interfere with the activation of plasminogen by SK. Increasing the ionic strength inhibited fibrinolysis by mixtures of serum and suboptimal amounts of SK (Table VII). This inhibitory effect was noted even though increasing the ionic strength increased the rate of fibrinolysis by active bovine protease

(Table VIII). One may conclude tentatively that the inhibitory effect of ionic strength was on the activation process. In any event, caution should be observed in maintaining precise control of the salt concentration in testing SK. It would appear safer to work in hypotonic than in hypertonic media. It is possible that some of the many reported activators or inhibitors of plasmin may have exhibited their actions by virtue of a non-specific ionic strength effect.

TABLE VIII

*The Influence of Ionic Strength on the Activity of Bovine Plasmin**

Ionic strength	0.10	0.15	0.20	0.25	0.30
Clot lysis time, *min*	19‡	11	9	9	8

* Clot lysis time of a mixture of 0.1 ml. thrombin, 0.3 ml. bovine plasmin (2.5 Loomis units per ml.), 0.3 ml. sodium chloride in varying concentrations, and 0.3 ml. bovine fibrinogen.

‡ Poor end-point.

TABLE IX

The Inactivation of the Components of C′ by Streptokinase-Activated Plasmin

SK units/ml. of serum	Inactivation of complement component			
	C′1	C′2	C′3	C′4
	per cent	*per cent*	*per cent*	*per cent*
0	0	0	0	0
100	15	80	5	94
300	67	93	25	100
2000	75	96	25	100

It is interesting that hypertonic salt solution also inhibits the "fixation" of the components of C′ to immune aggregates (22) and the inactivation of C′3 by zymosan (24).

7. The Inactivation of C′ Components.—In the preceding sections, the effect of SK-activated plasmin was observed mainly on C′ activity. Studies were next undertaken to discover the effect of SK-activated plasmin on the individual components of C′.

Varying amounts of SK were added to human serum and the per cent inactivation of each C′ component measured. The results are given in Table IX which shows that addition of small amounts of SK to serum (100 units/ml. of serum) destroyed mainly C′4 and C′2 but had little effect on C′1 and C′3. The addition of increasing concentrations of SK (300 to 2000 units/ml. of serum) caused destruction also of C′1, with C′3 remaining most resistant to inactivation. The use of similar amounts of SK in the fibrinolytic assay re-

vealed that 100 SK units/ml. of serum failed to digest a fibrin clot completely in 24 hours, while 400 and 1600 units caused rapid clot lysis (Table X). It is of interest to note that C′4 is also rapidly and almost completely removed by immune aggregates (22) and is immediately involved in immune fixation and lysis. It is also of interest that C′3 which is poorly, if at all, fixed by immune systems (22), is most resistant to inactivation by plasmin.

8. The Inactivation of C′ Components by the Addition of SK to R1, R2, R3, and R4.—Experiments were designed to test the effect of the addition of SK to fractions of C′ lacking one or more components and fractions rich or poor in plasminogen or its inhibitors. The results of these experiments are given in Table XI which shows that:—

R2, the fraction of serum insoluble at pH 5.4 and ionic strength 0.02, which is rich in plasminogen and poor in its inhibitors (25, 26), required only 16 units of SK to destroy C′1, C′3, and C′4 while 25 to 100 times as much SK was required to destroy completely these components in whole serum.

TABLE X

*The Activation of Plasminogen by Streptokinase**

Final concentration of SK units/ml.......	0	100	400	1600
Clot lysis time, *min*.............	>1440	1440‡	3	5

* Clot lysis time of a mixture of 1 ml. of human serum, 0.05 ml. of streptokinase in varying concentrations, 0.05 ml. of bovine thrombin, and 0.4 ml. of bovine fibrinogen.

‡ Partial lysis only.

R1, the fraction of serum soluble at pH 5.4 and ionic strength 0.02, which is rich in protease inhibitors but low in plasminogen (25, 26), required amounts of SK similar to those required for whole serum to inactivate the small amounts of C′3 and C′4 present in R1, while C′2 appeared to be unaffected by large doses of SK. Apparently, the small amounts of available activated plasmin were not able to destroy C′2 in the presence of a large excess of inhibitor. The total C′3 and C′4 content in this R1 was about 25 per cent of that present in untreated serum and would, of course, be inactivated by smaller amounts of plasmin than those required for whole serum.

The effect of SK on the components of C′ present in fractions R3 and R4 was almost identical with its effect on the components in untreated serum. R3 and R4 are serums which have been treated with zymosan and hydrazine respectively. Fractions R3 and R4 contained the same amount of plasminogen as untreated serum. Furthermore, treatment of serum with zymosan or hydrazine did not result in abnormal spontaneous activation of plasminogen of the serum as measured by incubation at 37° for 48 hours in the fibrinolytic system. However, the inhibitory activity of serum against bovine plasmin was

decreased by incubation at 37° for 1 hour with one-fifth its volume of 0.16 M hydrazine of pH 7.8 (Table XII). This observation implies that hydrazine either destroys inhibitors of plasmin along with C′4, or that C′4 itself may be an inhibitor of plasmin. Zymosan was without effect on the protease inhibitory

TABLE XI

The Inactivation by SK-Activated Plasmin of Components of C′ in Fractions of C′

Fraction*	SK/ml. of fraction	Inactivation of each component‡			
		C′1	C′2	C′3	C′4
	units	*per cent*	*per cent*	*per cent*	*per cent*
R1	2,000		15	50	100
R1	400		0	50	90
R1	80		0	20	50
R1	16		0	0	30
R2	2,000	100		100	100
R2	400	100		95	100
R2	80	100		90	100
R2	16	100		90	100
R3	2,000	100	100		100
R3	400	95	80		100
R3	80	50	30		100
R3	16	20	20		30
R4	2,000	95	100	100	
R4	400	80	80	95	
R4	80	50	30	60	
R4	16	20	20	30	
Serum	2,000	75	95	50	100
Serum	400	60	90	50	100
Serum	80	15	80	5	95
Serum	16	0	20	0	30

* R1 = deficient in C′1; R2 = deficient in C′2; R3 = deficient in C′3; R4 = deficient in C′4.

‡ Per cent inactivation of components originally present in each fraction.

activity of serum. It should be pointed out that there is slight inactivation of all components of C′ in the preparation of these reagents. The results given in Table XI have not been corrected for this non-specific inactivation but are given as per cent inactivation of the components present in each fraction.

These results indicate that the addition of SK to fractions containing sufficient plasminogen resulted in the inactivation of the components of C′ even in the presence of plasmin inhibitors in serum. However, the amount of SK

required seemed to depend on the amount of inhibitory activity present. The ratio of plasminogen to inhibitors determined the extent of inactivation by SK of the complement components, especially C′1 and C′2. C′4 appeared to

TABLE XII

*The Effect of Hydrazine on the Protease Inhibitory Activity of Serum**

Final concentration of serum	Final concentration of plasmin	Clot lysis time		
		Untreated serum	Serum treated with hydrazine	Buffer‡
	units/ml.	*min.*	*min.*	*min.*
1:25	0.75	90	21	—
1:50	0.75	30	11	—
1:100	0.75	13	11	—
1:200	0.75	9	9	—
1:400	0.75	9	9	—
1:800	0.75	9	9	—
0	0.75	—	—	9
0	0.375	—	—	14

* Clot lysis time at 37° of a mixture of 0.3 ml. bovine plasmin, 0.3 ml. serum, serially diluted with buffer, 0.1 ml. bovine thrombin, and 0.3 ml. bovine fibrinogen. The enzyme and serum were incubated at 37° for 10 minutes before the addition of thrombin and fibrinogen. 1 ml. of serum was mixed with 0.2 ml. of 0.16 M hydrazine (pH 7.8) and incubated at 37° for 1 hour before its protease inhibitory activity was tested.

‡ Substituted for serum.

TABLE XIII

A Comparison of Effect of Bovine Plasmin, Trypsin, and Streptokinase-Activated Plasmin on C′ and Its Components

	C′ activity remaining	Component activity remaining			
		C′1	C′2	C′3	C′4
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
(A) 1 ml. human serum + 20 mg. (16 units) bovine plasmin.............	15	100	20	100	10
(B) 1 ml. human serum + 0.6 mg. of crystalline trypsin................	0	5	0	75	0
C) 1 ml. human serum + 400 units SK............................	0	10	0	50	0

be most susceptible to the action of plasminogen either in the presence or absence of inhibitors. C′3, except in the absence of inhibitors, was the most resistant component to inactivation.

A Comparison of the Effect of Plasmin, Crystalline Trypsin, and Streptokinase on C′ and Its Components.—The experiments which have been de-

scribed make it clear that streptokinase destroys C′ by activation of plasminogen to plasmin which in turn digests or inactivates the components of C′. Bovine and human preparations of plasmin, not activated by streptokinase, were less effective in destroying C′ in whole serum (Table XIII). However, when these enzymes were added to R2, the fraction of serum insoluble at pH 5.4 and low ionic strength, the remaining components of C′ were readily destroyed (Table XIV). Presumably the removal of plasmin inhibitors allowed the bovine or human plasmins to inactivate C′1 and C′3, which were partially protected in whole serum. Partially purified $CHCl_3$-activated plasmin failed to inactivate complement in whole serum and its activity could be shown only after the removal of serum inhibitors. It has also been shown that $CHCl_3$ destroys guinea pig complement (27). A specific inactivation of C′4 has been claimed by these authors but this was not confirmed here. All the

TABLE XIV

The Inactivation of C′ Components in R2 by Various Plasmins

	Activity remaining		
	C′1	C′3	C′4
	per cent	*per cent*	*per cent*
1 ml. R2 + 0.5 ml. of 3 times concentrated $CHCl_3$-human plasmin (partially purified)	0	<5	0
1 ml. R2 + 20 mg. bovine plasmin (16 units)	0	0	0
1 ml. R2 + 5 units SK	0	<5	0
R2	100	100	100

components were inactivated by $CHCl_3$. The effect of $CHCl_3$ on human C′ was reinvestigated during the course of this study and it was again noted that $CHCl_3$ inactivated all four components of C′. Moreover, the addition of adequate amounts of $CHCl_3$-treated serum to C′ inactivated all C′ components. The $CHCl_3$-activated human plasmin was inactivated at 37° in 30 minutes, either being unstable under these conditions or neutralized by serum inhibitors.

Crystalline trypsin also destroyed C′ in serum (Table XIII) and appeared to behave like SK-activated plasmin.

DISCUSSION

Evidence has been presented that C′ is inactivated by plasmin, the proteolytic enzyme of plasma or serum active at or near neutral pH. The activation of plasminogen, the precursor of this enzyme, by streptokinase resulted in the inactivation of C′ from adult human serum. The components C′2 and C′4 along with varying amounts of C′1 and C′3 were destroyed. C′3 was the

most resistant to inactivation. The addition of streptokinase to infant serum proved less effective in destroying C′. Boisvert (28) has demonstrated that infant serum is relatively deficient in plasminogen. Moreover, the addition of streptokinase to monkey, rabbit, dog, and guinea pig serum resulted in little or no inactivation of C′. It has been demonstrated by others that these species contain little or no plasminogen susceptible to activation by streptokinase (29, 30). In contrast, the addition of streptokinase to mixtures of human and monkey serums and to mixtures of human and guinea pig serums inactivated not only the C′ in human but in the animal serums as well. It was observed that the inactivation of human C′ by the addition of streptokinase was inhibited by high hydrogen ion concentrations, low temperature, and slight elevations in ionic strength.

Plasmin which had been activated by chloroform also inactivated C′ in systems which did not contain large amounts of plasmin inhibitors. The relatively greater efficiency of streptokinase-activated plasmin has been commented upon earlier (23, 31). Studies with fractions of C′ showed that the degree of inactivation of C′ and its components by the addition of streptokinase was dependent on the amount of plasminogen available and on the amount of plasmin inhibitor present in the fractions.

Studies on the nature and function of components of C′ (4, 22, 32–35) and the facts presented in this paper suggest a possible relationship between C′ and the plasmin system and common mechanisms in complement "fixation" and immune hemolysis. While there is no direct evidence that any component of C′ is identical with any component of the plasmin system, there are superficial similarities between the properties of C′1 and plasminogen and between those of C′2 and C′4 and plasmin inhibitors. A parallelism exists between the inactivation of the components of C′ by plasmin and by antigen-antibody aggregates (22). Both systems inactivate C′2 and C′4 and varying amounts of C′1 with C′3 being most resistant to destruction. Furthermore, the extent of inactivation of each component of C′ depends upon the quantity of either plasmin or antigen-antibody aggregate present in the mixtures. Moreover, the inactivation of the components of C′ by plasmin or by antigen-antibody aggregates (22) in special reagents, R1, R2, R3, and R4, is almost identical. In each case, the components of R2 were more susceptible to inactivation or "fixation" than those of R1. The components in R3 and R4 were inactivated or "fixed" in a manner similar to the components in untreated serum. Hydrazine destroys both C′4 and plasmin inhibitors. The fact that plasmin is activated by antigen-antibody reactions is also significant (36–38). The inhibition both of the "fixation" of the components of C′ to specific aggregates (22) and of the activation of plasmin in slightly hypertonic salt solutions or in high hydrogen ion concentrations is suggestive that similar mechanisms are involved. These results suggest that the plasmin system may be involved in

complement "fixation" and in immune hemolysis. The discovery that the components of C′ are inactivated by plasmin may also help to elucidate the roles of these agents in allergic states. Work is under way here to determine the exact role of the enzyme and its associated factors in immune phenomena.

The "spontaneous" inactivation of C′ and the marked instability of C′ and its components on standing have long been laboratory problems. Serum, on standing, decreases in C′ titer, or becomes anticomplementary, or both. Bacterial contamination or the addition of various bacteriostatic agents, especially organic solvents, causes a sharp fall in C′ titer with the development of anticomplementary properties in the serum. It is likely that part or all of such inactivation of C′ and the acquisition of anticomplementary properties are due to "spontaneous" activation of plasmin or to activation of this enzyme by bacterial products or other agents. Work is in progress here to find suitable plasmin inhibitors which are not anticomplementary *per se* and do not interfere in immune hemolysis, but which will stabilize C′. On the basis of the foregoing work, a revision of present concepts of the nature and mode of action of many anticomplementary agents may be necessary.

Purification of the components of C′ has been handicapped by instability during fractionation procedures. C′1, C′3, and C′4 are separated initially by conditions which also precipitate plasminogen (32, 33). It was noted during purification of human C′1 that C′3 and C′4 disappeared spontaneously and such samples often exhibited anticomplementary properties (32, 33). It has since been noticed here that C′1 titers also decrease. The rate of this inactivation can be increased greatly by the addition of minute amounts of streptokinase to the purified samples (Table X) or by increasing the alkalinity of the samples to pH 7.0 or greater. Such observations can now be explained on the basis that the activation of plasmin in these fractions results in the inactivation of the C′ components. Attempts are being made to separate plasminogen from C′1, C′3, and C′4, and to investigate the possibility of finding specific inhibitors for plasmin or for the conversion of plasminogen to plasmin. The actual isolation of native components of C′ will necessarily await the outcome of such studies.

Finally, the inactivation of the components of C′ by plasmin may serve as a method for the estimation of plasminogen, plasmin, and their activators and inhibitors. The test is very sensitive, especially when R2 is the substrate. One objection to this method is that substrate, precursor, enzyme, and other factors are present simultaneously. However, experiments are in progress to attempt to overcome this objection.

SUMMARY

Human complement is inactivated by plasmin, the proteolytic enzyme of plasma or serum active at or near neutrality.

The addition of streptokinase to human serum, which converts plasminogen to plasmin, also causes the inactivation of complement components C′2 and C′4 and varying amounts of C′1. C′3 is the most resistant to inactivation by plasmin. Chloroform-activated human plasmin and bovine plasmin also destroy these components of complement, but are less effective than the streptokinase-activated enzyme. The inactivation of complement by the addition of streptokinase to human serum is inhibited by high hydrogen ion concentrations, low temperature, and elevated ionic strength. The inactivation of the components of complement in various fractions of serum is influenced by the available plasminogen and the content of plasmin inhibitors in these fractions.

Certain similarities are pointed out between the components of complement and the factors in the plasmin system and between the inactivation of the components of complement by antigen-antibody reactions, by specific agents, and by plasmin.

The possible significance of these relationships in immune hemolysis and complement fixation, and the possible role of the plasmin system in the instability of complement and the development of anticomplementary properties in serum are discussed.

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