THE INFLUENCE OF CALCIUM IONS ON THE INACTIVATION OF HUMAN COMPLEMENT AND ITS COMPONENTS BY PLASMIN*

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It has been shown recently (1) that human complement (C′) is inactivated by streptokinase-activated plasmin and by chloroform-activated human or bovine plasmin. These observations are of particular interest because of similarities between components of C′ and factors in the plasmin system and similarities between the inactivation of components of C′ by plasmin and their "fixation" by antigen-antibody mixtures (1, 2).

Further investigation of the inactivation of human complement by plasmin has revealed that calcium ions play an important role in this process. Much higher concentrations of plasmin were required to inactivate C′ in the absence of Ca^{++} than in its presence. Furthermore, the susceptibility of certain components of complement to inactivation by plasmin was found to be different in the presence and absence of Ca^{++}. The present report is concerned with these observations and their possible significance.

Nomenclature

In accordance with previous usage (1, 3), complement is designated C′ and its four recognized components are indicated by the symbols C′1, C′2, C′3, and C′4. Serum fractions employed as reagents for the titration of the components of C′ are designated R1, R2, R3, and R4 (4). R1, for the titration of C′1, consisted of the non-dialyzable portion of human serum soluble at pH 5.4 and ionic strength 0.02 at 0°, fortified with C′3 and C′4 from guinea pig serum heated at 56° for 30 minutes (4, 5). R2, for the titration of C′2, contained the non-dialyzable portion of human serum insoluble at pH 5.4, and ionic strength 0.02 at 0°, fortified with C′3 and C′4 from guinea pig serum heated at 56° for 30 minutes (4, 5). R3, for the titration of C′3, was prepared by treatment of human serum with zymosan (5, 6). R4, for the titration of C′4, was prepared by the addition of hydrazine to either human or guinea pig

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serum (5, 7). Detailed procedures for the preparation and characterization of these reagents have been published (2, 4, 5, 8).

Serum treated with the cation exchange resin, amberlite IRC-50 (sodium cycle) is referred to as *resin-treated serum*. Serum not treated in this manner is designated *normal serum*.

The term *plasmin* is used to designate the substance or substances in serum which can digest fibrinogen, fibrin, and certain other proteins *in vitro* at or near neutral pH. The inactive precursor of this enzyme is designated *plasminogen*. The filterable principle of cultures of beta-hemolytic streptococci which activates plasminogen will be called streptokinase (SK) (9).

Materials

Complement.—Blood from healthy human donors was chilled 1 hour after being drawn. The serum was then separated by centrifugation at 4000 R.P.M. at 1° in a refrigerated centrifuge and maintained at 1° for use within a 12 hour period. Guinea pig blood was drawn by cardiac puncture and the serum separated as above. R1, R2, R3, and R4 were adjusted to neutral pH, diluted to 2 times the original serum volume with 0.15 M NaCl or barbital buffer of pH 7.4 and ionic strength 0.15, containing 1.5×10^{-3} M $CaCl_2$ and 5×10^{-4} M $MgCl_2$ (8, 10) and maintained at 1° until needed.

Sensitized Sheep Cells.—Sheep blood was collected weekly in an equal volume of Alsever's solution (11). The red cells were washed 2 times with 0.15 M NaCl, finally with barbital-Ca^{++}-Mg^{++} buffer, and then suspended to a 1.25 per cent suspension in buffer containing 4 units of rabbit hemolysin per ml.

Streptokinase.—"Varidase,"[1] a lyophilized preparation of streptokinase, was dissolved in distilled water.

Bovine Plasmin.—Chloroform-activated bovine plasmin,[2] prepared by the method of Loomis, George, and Ryder (12), was dissolved in barbital buffer of pH 7.4 and ionic strength 0.15. The preparation contained 0.4 Loomis unit per mg.

Cation Exchange Resin.—Amberlite IRC-50 in the hydrogen cycle was converted to the sodium cycle in the following manner. Approximately 750 gm. of the dry resin was suspended in 500 ml. of 0.3 M NaCl. 500 ml. of distilled water was added and the resulting suspension brought to pH 7.7–7.8 by gradual addition of about 500 ml. of 5 M NaOH. The resin suspension was stirred for 5 minutes after each addition of base, allowed to settle, and the pH of the supernatant measured with a glass electrode. After the pH of the resin had been adjusted, it was repeatedly washed by decantation with about 10 liters of distilled water. Excess water was removed by suction and the moist resin stored in a covered container.

Double distilled water was employed throughout. All glassware was cleaned with sodium dichromate–sulfuric acid solution and washed in distilled water. International PR-1 refrigerated centrifuges were used for centrifugation. Constant temperature water baths, accurate to ±0.5°, were employed for incubation. Hydrogen ion concentration was determined with the Cambridge research model pH meter.

Methods

Titration of C′ and Its Components.—The methods employed have been described in detail elsewhere (2, 4, 5, 8). 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 sheep cells in a final volume of 1.5 ml. after 30 minutes at 37°.

The titration of individual components of complement was carried out according to the

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[1] Kindly supplied by F. Ablondi, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

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

general methods described by Bier *et al.* (4) and Kabat and Mayer (8). The 50 per cent hemolytic end-point was used in all component titrations.

The qualitative effect of various agents on C′ activity is expressed as the per cent hemolysis produced by a volume of treated serum which contained 1 hemolytic unit prior to treatment and incubation. The effect on complement components is expressed as per cent inactivation, based on the titer of each component in the corresponding untreated and unincubated serum.

Cation Exchange.—Human serum was gently swirled with an equal volume of amberlite IRC-50 (sodium cycle) for 10 minutes and decanted. Residual serum was removed with a capillary pipet. The pH of the serum remained constant within limits of ±0.3 pH. Spectrographic analyses[3] indicated that under these conditions the resin removed 99.8 and 99.9 per cent respectively of the Ca^{++} and Mg^{++} present in normal serum. Resin-treated serum therefore contained about 5×10^{-6} M Ca^{++} and 1×10^{-6} M Mg^{++}. Average normal serum values were assumed to be 4.8 m.eq. of Ca^{++} per liter and 1.7 m.eq. of Mg^{++} per liter (13).

The C′ titer of resin-treated serum was usually 5 to 10 per cent lower than in the corresponding normal serum. The drop appeared to be the result of dilution by the moist resin. The titers of C′2, C′3, and C′4 in resin-treated serum were usually the same as in the corresponding normal serum. Changes of 5 to 10 per cent, however, would not be detectable by the double dilution procedure employed in component titrations. The titer of C′1 in resin-treated serum was always lower than in normal serum. This observation is presented below in more detail.

Electrophoretic and ultracentrifugal analyses[4] of normal and resin-treated serums revealed no significant alteration of the distribution, electrophoretic mobility, or sedimentation behavior of the serum proteins as a result of treatment with the exchange resin. Electrophoretic analyses were performed in barbital buffer of pH 8.6, ionic strength 0.1; the ultracentrifugal analyses in phosphate buffer of pH 7.6, ionic strength 0.2.

Resin-treated SK solutions showed the same activity toward normal and resin-treated serums as untreated SK. In most experiments, therefore, untreated SK solutions were employed. Solutions of bovine plasmin were resin-treated as described above for human serum.

General Experimental Procedure.—Normal or resin-treated serum with or without added cations in the presence or absence of SK or bovine plasmin was incubated for 1 hour at 37°. The samples were then tested for their C′ or C′ component activities, or both. It is emphasized that all C′ and C′ component assays were performed in the presence of 1.5×10^{-3} M Ca^{++} and 5×10^{-4} M Mg^{++} in order to fulfill requirements for the hemolytic system (8, 10). Therefore, in all experiments involving the addition of SK or bovine plasmin to resin-treated serum and in which Ca^{++} and Mg^{++} were below effective concentrations for hemolysis, two opposing reactions were possible in the hemolytic test system: (*a*) hemolysis of sensitized sheep cells by the components of C′; and (*b*) inactivation of components of C′ in the presence of the Ca^{++} and Mg^{++} contributed by the hemolytic system. The first reaction would be by far the faster in the presence of minimum effective concentrations of SK or bovine plasmin. The second reaction would assume greater importance with increasing concentrations of these agents. It is clear, however, that the presence of Ca^{++} and Mg^{++} in the test system minimized the possible inhibitory effect of the absence of these ions on the inactivation of C′ by plasmin.

RESULTS

1. Comparison of the Effect of SK-Activated Plasmin on the C′ Activity of Normal and Resin-Treated Human Serum.—Aliquots of normal serum and serum treated with amberlite IRC-50 (Na cycle) were incubated for 1 hour at

[3] National Spectrographic Laboratories, Inc., Cleveland.

[4] Kindly performed by M. O. Schilling and E. Todd.

37° with one-half volumes of solutions of SK of various concentrations. The samples were tested for their C′ activity. It will be seen in Fig. 1 that concentrations of SK sufficient to inactivate C′ in normal serum had only a slight effect on C′ in resin-treated serum. Complete inactivation of C′ in normal serum could usually be accomplished by the addition of 100 units SK per ml. of serum, while 3200 units of SK per ml. was usually necessary to inactivate resin-treated serum.

2. The Effect of Various Cations on the Inactivation of C′ by SK-Activated Plasmin.—It was apparent from the above experiments that the resin removed

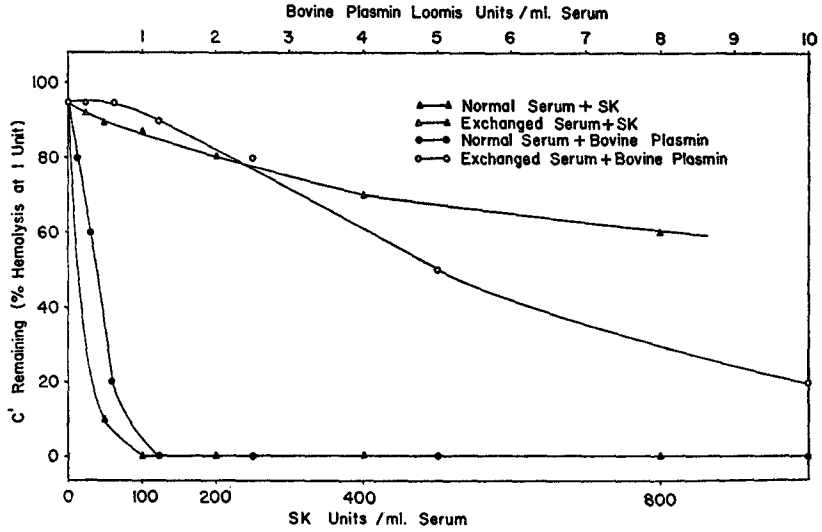

FIG. 1. The effect of SK and bovine plasmin on the C′ activity of normal and exchanged (resin-treated) serum at pH 7.7 ± 0.3 at a final dilution of serum of 1:1.5. The ionic strength was 0.10 in the experiments with SK and 0.13 with bovine plasmin.

or inactivated a substance necessary for the inactivation of C′ by SK-activated plasmin. It appeared most likely that the removal of an inorganic cation was responsible for this effect. Accordingly, Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Li^{+}, and K^{+} were tested for their ability to increase the inactivation by SK of C′ in resin-treated serum. The amount of SK used was just adequate to inactivate C′ in untreated serum. The data in Table I clearly indicated that Ca^{++} was involved in the inactivation of C′ by SK. The addition of 2.5×10^{-3} M Ca^{++} (the concentration of Ca in serum) or more to resin-treated serum resulted in the complete inactivation of C′ by the same amount of SK required for complete inactivation of C′ in normal serum. None of the other cations tested were effective to any appreciable extent.

3. The Effect of SK Concentration on the Concentration of Ca^{++} Required for Inactivation of C′.—The concentration of Ca^{++} required for the inactivation of

C′ in resin-treated serum was found to be a function of SK concentration. The results summarized in Fig. 2 demonstrated that higher concentrations of SK required lower amounts of Ca^{++} for the inactivation of C′. Thus, the optimum Ca^{++} concentration for the inactivation of C′ by the addition of 25 units SK per ml. of resin-treated serum was 10^{-2} M or greater and decreased progressively to 1.25×10^{-3} M in the presence of 3200 units SK per ml. of resin-treated serum.

4. The Effect of Ca^{++} on the Inactivation of C′ by SK-Activated Plasmin as a Function of Total Ionic Strength.—It has been shown previously (1) that the

TABLE I

*The Effect of Various Cations on the Inactivation of C′ in Resin-Treated Human Serum by SK-Activated Plasmin**

Added cation	Hemolysis at 1 unit in presence of					
	Mg^{++}	Ca^{++}	Sr^{++}	Ba^{++}	Li^{+}	K^{+}
final molarity	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	95	95	95	95	95	95
2.0×10^{-5}	95	95	95	95	95	95
2.0×10^{-4}	95	65	95	95	95	95
6.25×10^{-4}	—	20	—	—	—	—
1.25×10^{-3}	—	10	—	—	—	—
2.0×10^{-3}	85	—	85	95	95	95
2.5×10^{-3}	—	0	—	—	—	—
5.0×10^{-3}	—	0	—	—	—	—
1.0×10^{-2}	—	0	—	—	—	—
2.0×10^{-2}	75	0	95	95	95	95

* 0.5 ml. aliquots of resin-treated serum, 0.1 ml. of various dilutions of the chlorides of respective cations, and 0.15 ml. aliquots of SK containing 400 units per ml. were incubated for 1 hour at 37°. The final ionic strength of the system varied between 0.10 and 0.16. The pH was 7.8 ± 0.2. The SK concentration was constant at 120 units per ml. of serum, the amount required for complete inactivation of C′ in the normal serum used in this experiment.

inactivation of C′ by SK-activated plasmin was progressively inhibited at ionic strengths greater than 0.15 and that the inhibition could be partially overcome by increased SK concentrations. The possibility arose that part of this inhibition could be due to progressively decreasing activity coefficients of Ca^{++} in solutions of increasing ionic strength. This seemed tenable because of the observation that the Ca^{++} level of normal serum was near the minimum concentration required for the complete inactivation of C′ by minimum concentrations of SK. Accordingly, experiments were conducted with normal serum at final ionic strengths of 0.17, 0.22, 0.27, and 0.47 respectively, each containing varying concentrations of SK (25 units to 3200 units per ml. serum). In one series, the ionic strength was increased by NaCl alone while in other series, the same final ionic strengths were reached by appropriate combinations of NaCl

and $CaCl_2$. The final ionic strength of Ca^{++} varied between 3×10^{-4} and 3×10^{-2} in the latter experiments. It was found that inhibition of C′ inactivation occurred with increased ionic strengths independent of the concentration of added Ca^{++} in the system. This indicated that the effect of ionic strength was distinct from the requirement for Ca^{++} in the inactivation of C′ by plasmin.

5. The Effect of Ca^{++} on the Inactivation of C′ by SK-Activated Plasmin as a Function of pH.—It has been shown previously (1) that the inactivation of C′ and its components by SK-activated plasmin is inhibited below pH 7, whereas the reaction is promoted between pH 7 and pH 9. It appeared of interest to determine the effect of pH on the requirement for Ca^{++} in the C′-

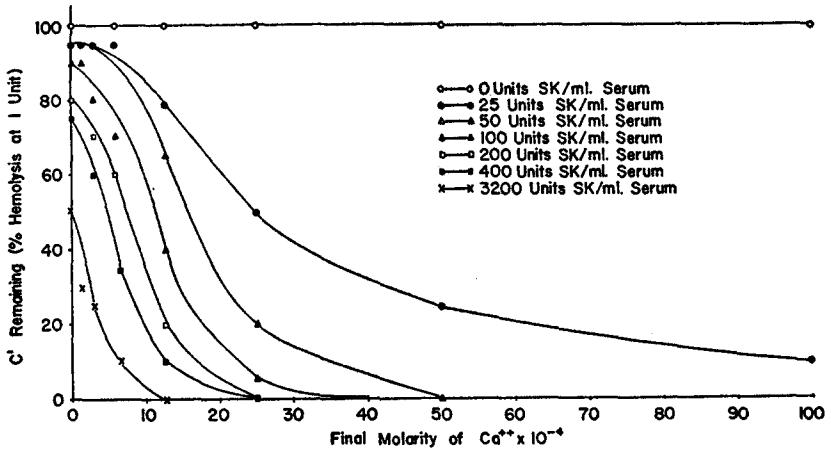

FIG. 2. Ca^{++} required for inactivation of C′ in resin-treated serum as a function of SK concentration, at pH 7.7 ± 0.3 at a final dilution of serum of 1:1.5. The ionic strength was 0.10 to 0.13.

plasmin systems. Accordingly, an experiment was performed in which resin-treated serum was incubated with SK at pH 7.8. Aliquots of this mixture, which retained most of its C′ activity, were then adjusted to varying hydrogen ion concentrations and reincubated in the presence of 5×10^{-3} M Ca^{++}. The results are given in Table II. It will be seen that more C′ was inactivated as the pH was raised from 5.9 to 8.5. The effect of pH was qualitatively the same as noted earlier (1) with normal serum, in which case the pH was varied in the initial presence of both Ca^{++} and SK.

6. Comparison of the Effect on the Components of Complement of the Addition of SK to Normal and Resin-Treated Serum.—The effect of several concentrations of SK on the titers of the individual components of C′ in normal and in resin-treated serums was determined. The data shown in Table III for normal serum confirmed previous results (1). C′2 and C′4 were most susceptible to inactivation by SK-activated plasmin, C′1 was next most susceptible, while

TABLE II

The Effect of Ca^{++} on the Inactivation of C′ by SK-Activated Plasmin as a Function of pH

Sample	Added Ca^{++}	SK units/ml. serum	Final pH	C′ activity
	final molarity			*per cent hemolysis at 1 unit*
1*	0	0	7.8	100
2*	0	150	7.8	80
1-A‡	5.0×10^{-2}	Reincubated 1 hr., 37°	5.9	95
1-B‡	"	" " " "	7.0	95
1-C‡	"	" " " "	7.7	95
1-D‡	"	" " " "	8.5	85
2-A§	"	" " " "	5.9	60
2-B§	"	" " " "	7.0	50
2-C§	"	" " " "	7.7	30
2-D§	"	" " " "	8.5	10

* 1 volume of resin-treated serum and 0.3 volume H_2O or SK containing 500 units per ml., incubated 1 hour at 37°.

‡ 1.3 ml. aliquots of Sample 1, 0.5 ml. of HCl or NaOH, and 0.2 ml. 0.05 M $CaCl_2$, reincubated 1 hour at 37°.

§ 1.3 ml. aliquots Sample 2, 0.5 ml. of HCl or NaOH, and 0.2 ml. 0.05 M $CaCl_2$, reincubated 1 hour at 37°.

TABLE III

*Comparison of the Effect of SK-Activated Plasmin on the Components of Complement in Normal and Resin-Treated Serums**

Serum	SK units/ml. serum	Inactivation of complement component			
		C′1	C′2	C′3	C′4
		per cent	*per cent*	*per cent*	*per cent*
Normal	0	0	0	0	25
	25	35	75	0	75
	50	50	95	0	100
	200	90	100	0	100
	1600	99	100	15	100
Resin-treated	0	50	0	0	0
	25	60	0	0	25
	200	75	15	0	40
	1600	85	35	0	50

* 1 ml. aliquots of normal or resin-treated serum and 0.5 ml. of various dilutions of SK were incubated for 1 hour at 37°. The final ionic strength of the system was 0.10 and the pH 7.8 ± 0.2. The per cent inactivation of each component is based upon the titers of individual components in normal and resin-treated serum prior to incubation at 37° for 1 hour.

C′3 was minimally, or not at all, inactivated. The results with resin-treated serum, however, were markedly different. As shown in Table III, C′2 and C′4

in resin-treated serum were only partially inactivated by the addition of 16 times the amount of SK effective for complete inactivation of these components in normal serum. The loss of hemolytic complement in resin-treated serum at high concentrations of SK was due primarily to the inactivation of C'1, while in normal serum this effect was due primarily to the disappearance of C'2 and C'4.

TABLE IV

*The Effect of Ca^{++} on the Components of Complement in Resin-Treated Serum in the Presence and Absence of SK**

Added Ca^{++}	SK units/ml. serum	Inactivation of complement component			
		C'1	C'2	C'3	C'4
final molarity		*per cent*	*per cent*	*per cent*	*per cent*
0	100	90	25	0	25
1.56×10^{-4}	100	90	50	0	50
6.25×10^{-4}	100	85	75	0	65
1.25×10^{-3}	100	80	90	0	90
2.5×10^{-3}	100	85	100	0	100
5.0×10^{-3}	100	85	100	0	100
2.0×10^{-2}	100	80	100	0	100
0	0	80	0	0	0
1.56×10^{-4}	0	65	0	0	0
6.25×10^{-4}	0	65	0	0	0
1.25×10^{-3}	0	50	0	0	0
2.5×10^{-3}	0	25	0	0	0
5.0×10^{-3}	0	0	0	0	0
2.0×10^{-2}	0	0	0	0	0

* 1 ml. aliquots of exchanged serum, 0.2 ml. of various dilutions of $CaCl_2$ and 0.30 ml. of H_2O or aliquots of a dilution of SK containing 333 units per ml. were incubated for 1 hour at 37°. The final ionic strength of the system varied between 0.10 and 0.16. The pH was 7.8 ± 0.2. The per cent inactivation of each component is based upon the titers of individual components in resin-treated serum prior to incubation at 37° for 1 hour.

7. The Effect of Ca^{++} on the Components of Complement in Resin-Treated Serum in the Presence and Absence of SK.—It was found, as shown in Table IV, that the addition of an optimal amount of Ca^{++} (2.5×10^{-3} M or greater) to resin-treated serum made this serum indistinguishable from normal serum with respect to the inactivation of the components of C' by SK-activated plasmin. C'2 and C'4 were completely inactivated by the addition of 100 units SK per ml. of resin-treated serum in the presence of a concentration of Ca^{++} equivalent to that found in normal serum. C'1 was also inactivated to a large extent and C'3 remained resistant to inactivation. This was found to be true when the Ca^{++} was added to resin-treated serum simultaneously with SK, as

in Table IV, and also when the Ca^{++} was added after preliminary incubation of resin-treated serum with SK, as shown in Table V.

It was further found that Ca^{++} had a protective action on C'1 in resin-treated serum in the absence of SK. A fall in C'1 of 25 to 50 per cent was observed after serum was treated with resin. The C'1 titer of the resin-treated serum decreased to 50 to 90 per cent of the latter value when incubated for 1 hour at 37° (Tables III to V). It was then observed (Table IV) that the addition of Ca^{++} to resin-treated serum protected C'1 from spontaneous inactiva-

TABLE V

The Effect of the Addition of Ca^{++} after Previous Incubation of Resin-Treated Serum with SK

Sample	Added Ca^{++}	SK units/ml. serum	C' activity	Inactivation of complement component*			
				C'1	C'2	C'3	C'4
	final molarity		*per cent hemolysis at 1 unit*	*per cent*	*per cent*	*per cent*	*per cent*
1‡	0	0	95	85	15	0	25
2§	0	100	90	90	25	0	60
2-A‖	0	Reincubated 1 hr., 37°	85	90	55	0	60
2-B‖	10^{-4}	" "	70	90	70	0	80
2-C‖	10^{-3}	" "	40	90	80	0	90
2-D‖	10^{-2}	" "	0	90	100	0	100

* The per cent inactivation of each component is based upon the titers of individual components in resin-treated serum prior to incubation at 37° for 1 hour.

‡ 1 ml. resin-treated serum and 0.5 ml. H_2O, incubated 1 hour at 37°.

§ 6.0 ml. resin-treated serum and 3.0 ml. SK containing 600 units, incubated 1 hour at 37°.

‖ 1.5 ml. aliquots of Sample 2 and 0.5 ml. H_2O or various dilutions of $CaCl_2$, reincubated 1 hour at 37°.

tion at 37°. C'1 in normal serum usually remained constant during incubation for 1 hour at 37° (Table III).

It was pointed out above that the inactivation of C' in resin-treated serum by SK-activated plasmin was primarily a result of the disappearance of C'1. It was apparent, however, from inspection of Tables III and IV that SK was less effective in causing the inactivation of C'1 in resin-treated serum than in normal serum. The precipitous drop in C'1 titer in resin-treated serum was due in large part to *spontaneous* inactivation and only partially to the action of SK-activated plasmin.

8. Comparison of the Effect of Bovine Plasmin on C' and Its Components in Normal and Resin-Treated Serum with and without Added Ca^{++}.—It has been reported (1) that large amounts of chloroform-activated bovine plasmin (16 Loomis units per ml. serum) inactivated C' in normal serum. The effect of

bovine plasmin on normal serum was reinvestigated in the course of this study. It was found, using a different preparation, that much smaller amounts of bovine plasmin (0.6 Loomis unit per ml. serum) could inactivate C′ (Fig. 1). It was further found (Table VI) that the components of C′ in normal

TABLE VI

*Comparison of the Effect of Bovine Plasmin on C′ and Its Components in Normal Serum and in Resin-Treated Serum with and without Added Ca^{++}**

Serum	Added Ca^{++}	Bovine plasmin	C′ activity	Inactivation of complement component			
				C′1	C′2	C′3	C′4
	final molarity	*Loomis units/ml. serum*	*per cent hemolysis at 1 unit*	*per cent*	*per cent*	*per cent*	*per cent*
Normal	0	0	100	0	0	0	25
	0	0.08	100	0	10	0	25
	0	0.4	30	15	90	0	90
	0	2	0	65	100	0	100
	0	10	0	90	100	50	100
Resin-treated	0	0	100	85	0	0	0
	0	0.08	100	75	0	0	0
	0	0.4	95+	85	0	0	0
	0	2	90	90	0	0	0
	0	10	10	99	50	40	0
Resin-treated	2.5×10^{-3}	0	100	0	0	0	0
	2.5×10^{-3}	0.08	95	0	50	0	25
	2.5×10^{-3}	0.4	15	65	95	0	85
	2.5×10^{-3}	2	0	85	99	0	99
	2.5×10^{-3}	10	15	95	95	40	75

* 1 ml. aliquots of normal or resin-treated serum, 0.2 ml. of H_2O or 0.019 M $CaCl_2$, and 0.3 ml. of various dilutions of plasmin in barbital buffer were incubated for 1 hour at 37°. The final ionic strength was 0.13 and the pH 7.6 ± 0.2. The per cent inactivation of each component is based upon the titers of individual components in normal and resin-treated serum prior to incubation at 37° for 1 hour.

serum were inactivated by bovine plasmin in almost exactly the same manner as by the addition of SK (Table III).

Greatly increased concentrations of bovine plasmin were required to inactivate C′ in resin-treated serum (Fig. 1 and Table VI). The inactivation was due again to the gradual disappearance of C′1.

The addition of 2.5×10^{-3} M Ca^{++} to resin-treated serum caused it to behave like normal serum with respect to the inactivation of C′ by bovine plasmin. Concentrations of bovine plasmin which inactivated C′ in normal serum also inactivated C′ in resin-treated serum to which Ca^{++} had been added.

The disappearance of C'2 and C'4 was once again the major cause of C' inactivation (Table VI). A slight inhibition was noted at 10 Loomis units per ml. of resin-treated serum in the presence of 2.5×10^{-3} M Ca^{++}.

It was apparent from these experiments that the effect of bovine plasmin on C' and its components was strikingly similar to the effect of SK, both in the presence and absence of Ca^{++}.

DISCUSSION

Evidence has been presented that Ca^{++} potentiates the destruction of human complement by plasmin, whether this enzyme has been activated by streptokinase or chloroform. Replacement of the cations of serum with Na^{+} by treatment with a cation exchange resin greatly increased the concentration of SK or plasmin required for the inactivation of C'. The addition of Ca^{++} to such resin-treated serum resulted in the inactivation of C' by SK or plasmin in amounts similar to those required for the inactivation of C' in normal serum. Earlier (1) it was demonstrated that the inactivation of C' by plasmin was primarily the result of the inactivation of two of its components, C'2 and C'4. In the present study it was observed that the inactivation of these two components by plasmin was less efficient in resin-treated serum than in normal serum. Again, the readdition of Ca^{++} to resin-treated serum restored the susceptibility of these components to inactivation by plasmin.

The stage at which Ca^{++} was utilized in the inactivation of C' by plasmin remained unclear. Previously it had been shown that Ca^{++} did not accelerate the lysis of fibrin clots by chloroform- or SK-activated plasmin (14, 15). This observation has been confirmed, using preparations which had first been depleted of Ca^{++} by the use of the cation exchange resin (16). Furthermore, the digestion of fibrinogen, like that of fibrin, was not accelerated by the addition of Ca^{++} in concentrations as high as 10^{-2} M, the highest concentration tested (16). It seems likely, therefore, that Ca^{++} does not potentiate the action of plasmin in these systems.

It had also been shown previously that Ca^{++} did not accelerate the activation of plasminogen by SK (15). These experiments have been repeated with resin-treated serum with the same results (16). However, Geiger (17) has expressed the opinion that Ca^{++} functions in the activation of plasminogen by SK.

It would appear that the effect of Ca^{++} upon the reaction between plasmin and C' must be indirect. This view is supported by the sparing effect of Ca^{++} on the requirement for SK in the inactivation of C' (Fig. 2). It is further supported by some preliminary experiments on the effect of plasmin on the components of C' in different fractions of serum (18). Ca^{++} potentiates the inactivation of the components of C' in R1, R3, and R4. However, Ca^{++} does not appear to alter the inactivation of the components of C' in the R2 fraction.

In contradistinction to R2, fractions R1, R3, and R4 have in common the presence of the non-dialyzable portion of serum which is soluble at pH 5.4 and ionic strength 0.02. Perhaps, then, the action of Ca^{++} on the inactivation of C′ by plasmin is mediated through some substance in this fraction of serum. This fraction is rich in plasmin inhibitory activity, but it is improbable that this is the full explanation for the effect of Ca^{++}, since Ca^{++} does not accelerate fibrinolysis by SK-activated plasmin in whole serum (16). Although further studies are in progress, the solution to the problem of the site of action of Ca^{++} may require a purified system.

It was also observed that treatment of serum with cation exchange resin caused spontaneous inactivation of C′1. This effect could be prevented by the addition of Ca^{++} to resin-treated serum. Thus, Ca^{++} appeared to protect C′1 from spontaneous inactivation. However, Ca^{++} did not protect C′1 from inactivation by plasmin. Presumably, in this experiment, plasmin destroyed C′1, but the possibility also remains that plasmin exerted its destructive effect on C′1 indirectly, by interfering with the protective action of Ca^{++} against the spontaneous inactivation of C′1.

The role of Ca^{++} in the immune reactions of C′ has received considerable attention (10, 19–23). However, not until recently (24) has there been a clear indication of a requirement for Ca^{++} in C′ "fixation." It is noteworthy that Ca^{++} potentiates the inactivation of C′1, C′2, and C′4 by plasmin and that these same components are involved in the "fixation" of C′ in immune hemolysis (2). These observations further emphasize the similarities mentioned earlier (1) between the action of antigen-antibody aggregates and of plasmin on the components of C′. It should be pointed out, however, that Ca^{++} may not be directly involved in the "fixation" of C′1 by specific aggregates but may serve, in some manner, to protect C′1 from spontaneous inactivation during this reaction.

SUMMARY

Ca^{++} potentiates the inactivation of human complement by streptokinase-activated plasmin and chloroform-activated bovine plasmin. The optimum concentration of Ca^{++} varies between 10^{-2} M and 10^{-3} M, decreasing with increasing concentrations of streptokinase.

The susceptibility of the components of complement to inactivation by plasmin is different in the presence and absence of Ca^{++}. C′2 and C′4 are most readily inactivated by plasmin in the presence of Ca^{++}, while C′1 disappears first in the absence of Ca^{++}. A large part of this C′1 disappearance is due to spontaneous inactivation.

Similarities are pointed out between the influence of Ca^{++} on the inactivation of the components of complement by antigen-antibody systems and by plasmin.

BIBLIOGRAPHY

1. Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H., *J. Exp. Med.*, 1953, **97,** 573.
2. Pillemer, L., *Chem. Rev.*, 1943, **33,** 1.
3. Pillemer, L., and Ecker, E. E., *Science*, 1941, **94,** 437.
4. Bier, O. G., Leyton, G., Mayer, M. M., and Heidelberger, M., *J. Exp. Med.*, 1945, **81,** 449.
5. Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, **47,** 181.
6. Pillemer, L., and Ecker, E. E., *J. Biol. Chem.*, 1941, **137,** 139.
7. Pillemer, L., Seifter, J., and Ecker, E. E., *J. Immunol.*, 1941, **40,** 89.
8. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.
9. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28,** 559.
10. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.* 1946, **84,** 535.
11. Bukantz, S. C., Rein, C. R., and Kent, J. F., *J. Lab. and Clin. Med.*, 1946, **31,** 394.
12. Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12,** 1.
13. Albritton, E. C., Standard Values in Blood, Philadelphia, W. B. Saunders Company, 1952.
14. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21,** 525.
15. Ratnoff, O. D., *J. Exp. Med.*, 1952, **96,** 319.
16. Ratnoff, O. D., unpublished data.
17. Geiger, W. B., *J. Immunol.*, 1952, **69,** 597.
18. Lepow, I. H., and Pillemer, L., unpublished data.
19. Gordon, J., Whitehead, H. R., and Wormall, A., *Biochem. J.*, 1926, **20,** 1036.
20. Gordon, J., and Atkin, W. R., *Brit. J. Exp. Path.*, 1941, **22,** 226.
21. Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1936, **30,** 417.
22. Pillemer, L., and Ecker, E. E., *J. Immunol.*, 1941, **40,** 101.
23. Gengou, O., *Ann. Inst. Pasteur*, 1952, **83,** 561.
24. Levine, L., Cowan, K. M., Osler, A. G., and Mayer, M. M., *Fed. Proc.*, 1953, **12,** 451.