A SPECIFIC INACTIVATOR OF MAMMALIAN C′4 ISOLATED FROM NURSE SHARK (*Ginglymostoma cirratum*) SERUM*

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Studies concerned with the natural antibodies and the complement system in the serum of nurse sharks included the separation of the first component ($C'1_N$) from other complement components and from antibody (1). Hemolytic assays for $C'1_N$ activity of crude preparations often exhibited typical inhibition patterns at high $C'1_N$ concentrations. Since guinea pig C′4, C′2 and C′3–C′9 were used for the hemolytic assays of $C'1_N$, the inhibition was initially believed to be due to the destructive action of $C'1_N$ for the fourth and second component of the guinea pig complement system. It was soon found, however, that the magnitude of the inhibition was independent of the $C'1_N$ titer and that, dependent on the isolation procedure used, $C'1_N$ preparations could greatly vary in this respect. These observations led to a successful attempt to separate a material from shark $C'1_N$ which specifically inactivated mammalian C′4 in vitro and in vivo. This paper deals with the generation, isolation, purification, and characterization of this C′4 inactivator and it furthermore includes some initial in vivo experiments demonstrating its usefulness as a complement blocking agent.[1]

Materials and Methods

Diluents.—The buffers used were either isotonic Veronal buffers containing Ca^{++}, 0.00015 M, Mg^{++}, 0.0005 M, and 0.1% gelatin (gelatin-Veronal) or a mixture of equal parts of gelatin-Veronal containing twice the amount of Ca, Mg, and gelatin with 5% glucose in water (glucose-gelatin Veronal) (2). When chelating buffers were required, gelatin-Veronal prepared without Ca and Mg was mixed with 0.087 M EDTA[2] to give final concentrations of 0.04 or 0.01 M EDTA. Phosphate buffered sodium chloride solutions of various ionic strengths contained 0.005 M phosphate buffer, pH 7.5 (PBS).

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[1] Reported in part at the Third International Complement Workshop, Harvard Medical School, Boston, Massachusetts, June 3–5, 1968.

[2] Reagent grade disodium ethylenediaminetetraacetate (Na_2H_2 EDTA) was titrated to pH 7.4 with NaOH to give a 0.087 M stock solution.

Sheep Erythrocytes (E).—Blood from individual sheep was collected in modified Alsever's solution (3) and stored at 4°C. For each batch, cell counts were correlated with spectrophotometrically measured hemoglobin concentrations.

Rabbit Antibody (A)-Sensitized Sheep Erythrocytes (EA).—Immunoelectrophoretically-pure IgG fractions from the serum of rabbits immunized with thoroughly washed, intact sheep E were used for optimal sensitization (3).

Guinea Pig Complement (C'), C'-Components and C'-Fractions.—Pooled guinea pig serum was used as a source of C'. The serum was either obtained from our own Hartley strain guinea pigs or was purchased from Hyland Laboratories, Los Angeles, Calif. A serum fraction rich in all components except C'1 (supernate I) and purified C'-components were prepared as outlined in reference 2.

Intermediate Cell Complexes.—The intermediate complexes $EAC'1_a$, $EAC'1_a4$, and EAC'4 were prepared as outlined in (2); the complex $EAC'1_a$-7 according to reference 4.

Nurse shark sera.—The sharks were kept in open pens at the Lerner Marine Laboratories (Bimini, Bahama Islands). For bleeding, the animals were anesthetized with ethyl-*m*-aminobenzoate methane sulfonic acid (1 g per 4000 ml of sea water). Blood was drawn from the hemal arch and was collected individually. The blood was allowed to clot at room temperature, was then chilled to 4°C and centrifuged in the cold. Sera to be used as a source of natural antibody were frozen at −75°C. To preserve their complement activity sera were either stored at 0°C or after removal of the urea at −75°C (1, 5).

Nurse Shark Antibody (A_N)-Sensitized Sheep E (EA_N).—Whole serum from one normal nurse shark was used as a source of natural antibody for the sensitization of sheep erythrocytes. EA_N were prepared by two methods: (*a*) washed sheep E in gelatin-Veronal at a concentration of 1×10^9/ml were mixed with equal volumes of heat-inactivated (30 min at 56°C) nurse shark serum diluted 1:10 in gelatin-Veronal. The mixture was first incubated in a shaking water bath for 30 min at 37°C and then at 0°C for another 30 min. (*b*) The cells were suspended and mixed with the unheated serum diluted in gelatin-Veronal, containing 0.04 M EDTA, and the mixture was incubated for 1 hr at 30°C. In both procedures the sensitized cells were then washed and stored as described for EA. Cells prepared according to these two methods were optimally reactive with nurse shark complement but were not lysed by guinea pig C' unless $C'1_N$ was supplied (1, 5).

Measurement of $C'1_N$ Hemolytic Reactivity.[3]—1 volume of EA_N 1×10^8/ml in gelatin-Veronal was incubated with 1 volume of dilutions of $C'1_N$ for 20 min at 30°C, then 2 volumes of gelatin-Veronal were added, followed by 1 volume of guinea pig supernate I diluted 1:8 in gelatin-Veronal. The reaction mixture was then incubated at 37°C for 60 min. These assays, as all other hemolytic assays mentioned in this paper, were done either in microtiter plates using a total of 5 volumes of 0.025 ml each, or in tubes with 5 volumes of 0.5 ml. In the latter case, 5.0 ml of cold saline were added to each tube at the end of the incubation period, the tubes were centrifuged in the cold and the release of oxyhemoglobin was measured spectrophotometrically in the supernatant fluid at 415 mμ. Spontaneous lysis controls, reagent controls, and 100% lysis controls were included in each assay.

Measurement of C'4 Activity in Mammalian Materials.—1 volume of dilutions of whole serum, C'4 containing serum fractions, or purified C'4 in glucose-gelatin-Veronal was incubated with 1 volume of $EAC'1_a$ 1×10^8/ml for 20 min at 30°C. 1 volume of purified C'2, enough to supply 75–100 C'H50 units per cell, was then added and incubation was continued for 10 more min. Finally 2 volumes of whole complement diluted 1:25 in 0.04 M EDTA-gelatin-Veronal (C'-EDTA) were added to the reaction mixture which was then incubated for 60 min at 37°C.

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[3] A detailed description of methods concerning the preparation and measurement of $C'1_N$ will be given elsewhere.

Measurement of C′4 Inactivator Activity.—Depending on the degree of accuracy and reproducibility required, three different hemolytic assay methods were used. Once it was established that C′4 was the only mammalian complement component affected by the inactivator, a simple and rapid method was designed. Dilutions of the inactivator were incubated for 15 min at 37°C with a dilution of whole guinea pig complement containing 5 C′H50 units per ml. Then 2 volumes of gelatin-Veronal were added and 1 volume of EA (1×10^8/ml) and incubation was continued for 60 min at 37°C. The titer of the inactivator was then expressed as the highest dilution that inactivated four out of five C′H50 units of whole complement. The two other methods, which were based on measurements of C′4 rather than whole C′ activity, are described under Results.

Sucrose Density Gradient Ultracentrifugation Analysis.[4]—The 40%-10% sucrose gradients were prepared in 0.15 M NaCl containing 0.005 M Tris buffer, pH 7.5. 0.5 ml of the test material was layered over 4.0 ml of the gradient in cellulose nitrate tubes (Beckman No. 305050). The tubes were centrifuged in a Spinco Model L ultracentrifuge for 17 hr at 35,000 rpm using the type SW 25.1 swinging bucket rotor. At the end of the run, the tubes were punctured and fractions were collected from the bottom of the gradient. The number of fractions was usually 108–115, each representing 3 drops. The fractions were collected into the wells of microtiter plates. The peak reactivities were determined by microtitrations and the corresponding S-values were calculated from the position of the reference material (6.6S rabbit anti-sheep E IgG) in comparison with an original calibration run (111 fractions).

Rabbit Antisera against Whole Nurse Shark Serum and Crude C′4 Inactivator Preparations.—New Zealand white rabbits were immunized over a time period of 3 to 4 months by subcutaneous injections of whole normal nurse shark serum or crude C′4 inactivator preparations. The latter were obtained by precipitation at 22°C as described under Results and were similar to those used as starting materials for further purification by gel filtration. Both antigens were homogenized in equal amounts of complete Freund's adjuvant and were administered subcutaneously at five sites, each receiving 0.2 ml, representing a total dose of 0.5 ml of undiluted antigen. After three such multiple injections spaced at least 1 month apart, the animals were bled. If desired, the rabbits were later boosted by intracutaneous injections of antigen without adjuvant.

Immunoelectrophoresis.—Microscope slides coated with 2.0 ml of 1.5% Noble agar in Veronal buffer (ionic strength 0.1, pH 8.6) were charged with the antigen. Electrophoretic separation was achieved in 110 min under conditions of constant current (35 to 45 ma, depending on the number of slides). The slides were read or photographed 24–48 hr after the troughs were charged with antibody. During this time they were kept in moist chambers at room temperature.

Chemicals.—Diisopropyl fluoro-phosphate (DFP) was obtained from K & K Laboratories, Plainview, New York; Trypsin (2 × crystallized) and Trypsin inhibitor (3 × crystallized, from soybean) from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Generation of the C′4 Inactivator.—

The C′4 inactivator was first detected in preparations of $C'1_N$ obtained by low ionic strength precipitation of normal nurse shark serum under conditions similar to those described for the isolation of guinea pig C′1 (2). Neither nor-

[4] The adaptation of this method to microtiter assays for materials that can be measured by immune-hemolytic titrations was developed by Dr. Ruth Fugmann in 1965. Unpublished data.

mal whole serum, nor serum from which the urea was previously removed, had any detectable inactivator activity. If the ionic strength of the serum, which was normally that of a 0.25 M NaCl solution, was lowered to 0.035–0.04 M NaCl by dilution with water at the original pH of 7.5, a precipitate formed which contained most of the $C'1_N$ activity (95–99%). The precipitate could be washed extensively in the cold with 0.04 M PBS without loss of inactivator or $C'1_N$. If the washed precipitate was dissolved in 0.25 M PBS and centrifuged to remove insoluble material, the supernatant fluid contained a mixture of $C'1_N$ and the C'4 inactivator. It soon became apparent that the content of inactivator in such preparations depended greatly on (*a*) the temperature at which the initial precipitation was carried out and (*b*) how the precipitate was treated before it was dissolved.

A. *Precipitation at 22°C.* One volume (50 ml) of freshly obtained shark serum was mixed with six volumes of distilled water and stirred for 30 min at 22°C. The ionic strength of the mixture was that of a 0.036 M NaCl solution. The precipitate which formed was centrifuged and washed twice in 40 ml of 0.036 M PBS at the same temperature. The final precipitate was then chilled to 0°C and dissolved in 10 ml of ice cold PBS 0.25 M. 1.0 ml portions of the dissolved precipitate were then incubated at 30°C for various periods of time, mixed with 4.0 ml 0.25 M PBS (0°C) and centrifuged in the cold. The clear supernatant fluid was tested for $C'1_N$ and inactivator activity. One portion of the dissolved precipitate was diluted 1:5 in cold 0.25 M PBS and kept at 0°C at all times.

B. Precipitation at 0°C. Exactly the same procedure was followed except that the temperature was kept at 0°C at all times and the final precipitate was dissolved directly at 0°C (to the original serum volume).

C. Precipitation at 37°C. Precipitation at 37°C was exactly the same as in B, except for the difference in temperature during precipitation. The precipitate was washed at room temperature, then chilled, and dissolved at 0°C.

D. Precipitation at 0°C followed by incubation of the suspended precipitate at different temperatures. Precipitation was done as in section *B* above. The final precipitate, however, was not dissolved but suspended in the original volume (serum volume) in 0.036 M PBS at 0°C. Equal volumes of this suspended precipitate were then incubated at 0°, 30°, and 37°C for 30 min, then chilled to 0°C, and centrifuged cold. The supernatant fluids were tested, the sediments were dissolved in ice cold 0.25 M PBS, centrifuged, and also tested for inactivator and $C'1_N$ activity.

Table I contains representative data which were gathered over a period of several months. It summarizes inactivator and $C'1_N$ activities obtained by various precipitation procedures; also listed is the OD_{280} as an indication of purity. It is not clear at present by what mechanism the inactivator is formed. The following observations taken from the table seem to indicate that the

generation process is complex and probably involves enzymatic activation or degradation of a precursor. (*a*) If the low ionic strength precipitation, the washing of the precipitate, and its solution were performed strictly at 0°C, no inactivator was formed. The low grade inactivation of C′4 that disappeared upon heating to 56°C for 2 min (see below) was presumably due to

TABLE I

Generation of C′4 Inactivator under Various Conditions

Conditions of formation (see text)	Inactivator titer		$C'1_N$ titer		OD_{280} of inactivator preparations (initial serum volume)
	Unheated	56° 2 min	Unheated	56° 2 min	
Precipitation at 22°C					
ppt. dissolved at 0°C	500	500	6,400	0	1.65
then incubated at 30°C					
for 5 min.	500	500	6,400	0	1.75
for 15 min.	500	500	6,400	0	1.69
for 60 min.	500	500	6,400	0	1.75
Precipitation at 0°C					
ppt. dissolved at 0°C	256	0	6,400	0	3.29
Precipitation at 37°C					
ppt. dissolved at 0°C	500	500	6,400	0	1.65
Precipitation at 0°C					
ppt-suspension 30 min. at:					
0°C sup.	0	0	20	0	0.29
ppt.	256	0	5,000	0	2.90
30°C sup.	0	0	0	0	0.58
ppt.	500	500	10,000	0	2.18
37°C sup.	6,000	6,000	0	0	0.75
ppt.	256	256	40	0	2.50

the $C'1_N$ content of the material. (*b*) Incubation of the dissolved precipitate, no matter at what temperature it was obtained, did not increase the inactivator activity. (*c*) The inactivator was formed if the serum was precipitated at room temperature or at 37°C. In both cases it was found in the precipitate, insoluble even in large volumes of low ionic strength PBS. When the precipitate was dissolved in 0.25 M PBS it went into solution together with $C'1_N$, from which it could be distinguished by its greater heat stability at 56°C. (*d*) Inactivator preparations with the highest specific activity were obtained if the 0°C precipitate was suspended in low ionic strength buffer and incubated at 37°C. Only in this case the activity was found in the low ionic strength buffer even after the incubated suspension was chilled and centrifuged.

Since the last mentioned procedure was developed recently, experiments reported in this paper, unless otherwise stated, were performed with inactivator preparations that were obtained by precipitation at 0.035–0.04 M, pH 7.5, at room temperature (22°C). The washed precipitate was dissolved in 0.25 M PBS, stirred at 30°C for 1 hr, centrifuged, and stored at 0°C overnight.

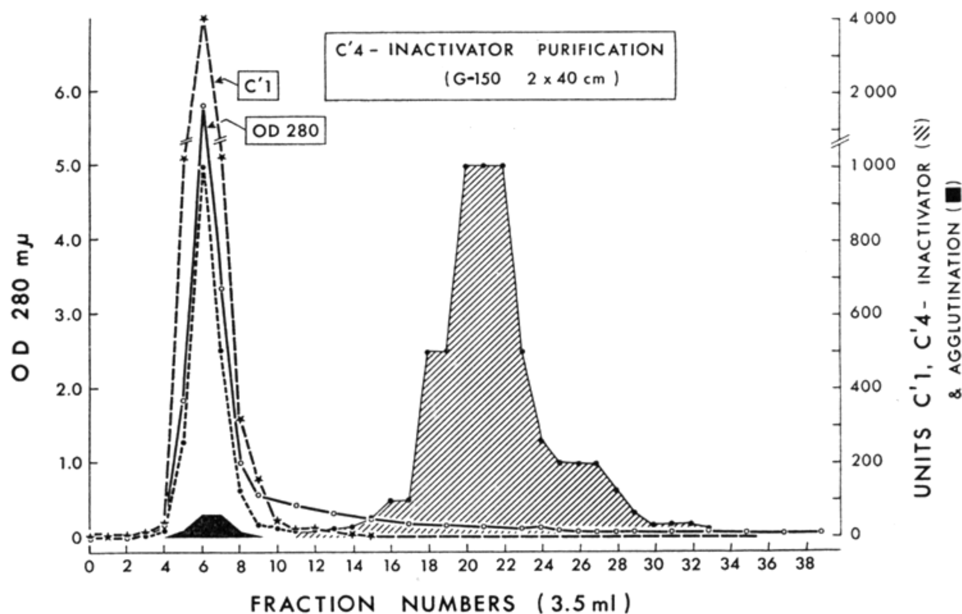

FIG. 1. (Inactivator column I-1) Pooled normal nurse shark serum was precipitated at room temperature (as described in the text) and the dissolved precipitate was concentrated by pressure filtration through Diaflo membrane No. 1.* The concentrate, cleared by centrifugation, served as starting material for a 2 × 40 cm Sephadex G-150 column, equilibrated with 0.15 M PBS. The starting material had an OD_{280} of 15.0, a $C'1_N$ titer of 1:12,000, an inactivator titer of 1:10,000 and a sheep E agglutination titer of 1:640. 3 ml of this material were applied to the column, which was run at room temperature at a flow rate of 25 ml/hr. 25 ml of the effluent were discarded before the collection of 3.5 ml fractions was begun.

During the cold storage, bulky precipitates formed, which were soluble at 30°C, contained up to 25% of the total protein, and could be removed without loss of inactivator or $C'1_N$ activity. Such preparations could be kept at −75°C or 0°C for several months without detectable loss of activity; they served as starting materials for further purification procedures.

Purification of the C'4 Inactivator.—These crude preparations contained very little whole complement reactivity but were contaminated with 10% to 30% of the natural anti-sheep E antibody and, as mentioned earlier, with more

* Amicon Corp., Lexington, Mass.

than 90% of the total $C'1_N$. Practically all remaining shark complement components (as measured by the reactivity with $EA_NC'1_N$) and most of the natural antibody remained in the supernatant fluid. Purification of the inactivator was therefore primarily concerned with its separation from the known hemolytically active materials, $C'1_N$ and antibody.

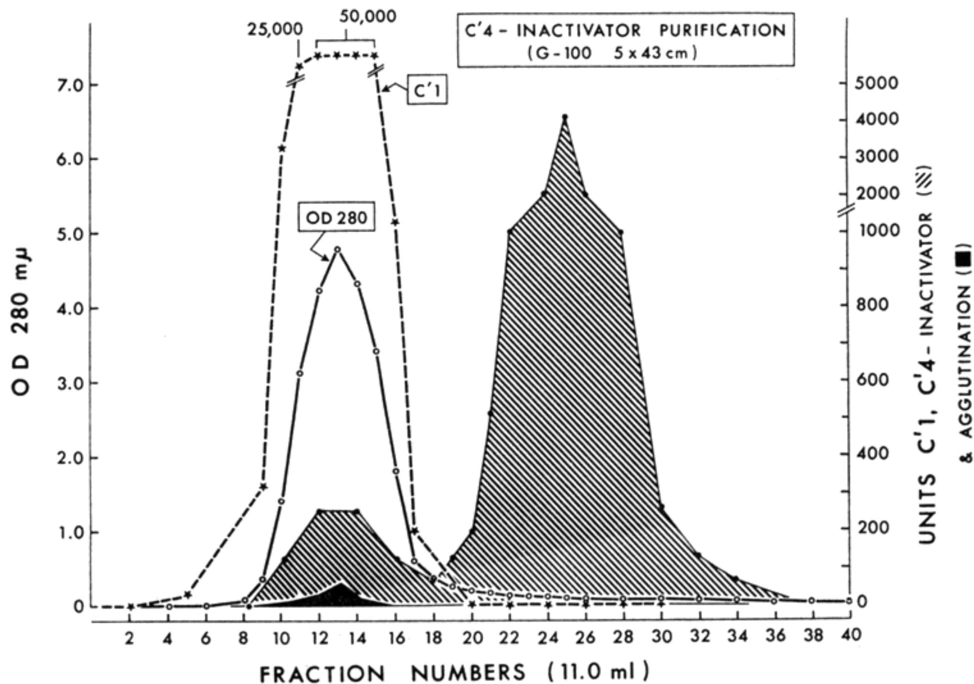

FIG. 2. (Inactivator column VI-1) Pooled normal nurse shark serum was precipitated as before. The precipitate was dissolved in 1/6 the original serum volume in 0.25 M PBS, chilled to 0°C, centrifuged in the cold, and used as starting material for a 5 × 45 cm Sephadex G-100 column, equilibrated with 0.25 M PBS. The starting material had an OD_{280} of 12.0, a $C'1_N$ titer of 1:100,000, an inactivator titer of 1:10,000, and a sheep E agglutination titer of 1:200. 25 ml of this material were applied to the column which was run at 4°C with a flow rate of 90 ml/hr. 100 ml of the effluent were discarded before the collection of 11.0 ml fractions was begun.

Separation by Gel Filtration.—Figs. 1 and 2 show the behavior of the C'4 inactivator in its relation to antibody and $C'1_N$ on gel filtration using Sephadex G-150 and G-100 respectively. Both the column profiles show good separation of the inactivator from antibody and from $C'1_N$. The yield was usually between 50% and 80%. The specific activity of the pooled inactivator fractions was approximately 25 times that of the starting material of the columns. All of the applied $C'1_N$ and antibody was associated with the first protein

peak. Some C'4 inactivating activity was almost always found in the high $C'1_N$ fractions. About 30% of this activity disappeared on heating and could therefore be contributed to $C'1_N$. The remainder however, showed the same heat stability as the later eluting C'4 inactivator. It is not clear at present whether this large molecular material is an aggregation product of the inactivator or whether it is an unrelated substance with similar properties.

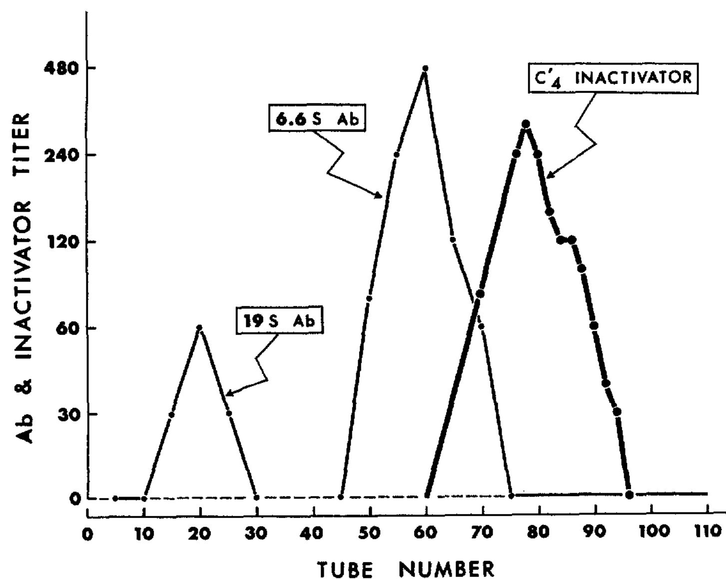

FIG. 3. Sucrose density gradient ultracentrifugation: 10%–40% sucrose gradient; 17 hr at 35,000 rpm Spinco Model L ultracentrifuge. Reference materials: Rabbit anti-sheep E IgG and IgM. Inactivator: preparation II-1: pool from Sephadex G-150 column, concentrated 4.5 times, OD_{280} 1.8, titer 1:3200. Tube i) IgG + inactivator + buffer; tube ii) IgM + inactivator + buffer; tube iii) IgG + IgM + inactivator. After compensating for differences in fraction numbers 109, 113, 108 for i, ii, iii, respectively, the various peak activities were found to reside in the same fractions in all three tubes.

Further purification of the active fractions was possible by ion exchange chromatography on cellulose columns, but the yield was poor. It was therefore decided to use the functionally pure, but only chemically partially purified preparations obtained from Sephadex columns for further characterization of the properties of the inactivator.

Physico-Chemical Characteristics.—

A. Estimation of molecular weight: From the known parameters of the Sephadex column used in Fig. 1, it was apparent that the inactivator was retarded more than guinea pig serum albumin and therefore had probably a

smaller molecular weight. Sucrose gradient ultracentrifugation supported this assumption.

Fig. 3 is a composite graph of the sedimentation characteristics of the C′4 inactivator and rabbit anti-sheep E IgM, and IgG. The calculated S-value for the inactivator was 3.3S based on comparison with 6.6S Rabbit IgG. $C'1_N$ (not shown) sedimented with the rabbit IgM.

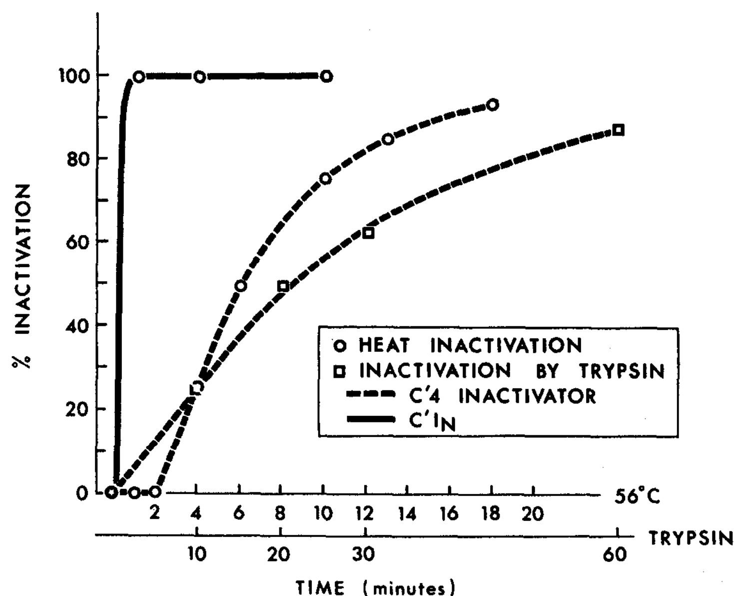

FIG. 4. Effect of heat treatment on the C′4 inactivator and on $C'1_N$; effect of trypsin treatment on inactivator; a series of tubes containing 0.5 ml of C′4 inactivator (II-1, titer 1:6400) of whole shark serum ($C'1_N$ titer 1:12,000) or purified $C'1_N$ (titer 1:6.400) were prewarmed to 37°C and placed in a 56°C waterbath at zero time. At the indicated times, duplicate tubes were removed from the bath, chilled, and the residual activity assayed.

The same inactivator preparation was diluted 1:25 in 0.15 M PBS pH 7.5. One ml samples were mixed with 0.5 ml of the same buffer containing 0.5 mg trypsin per ml and incubated at 30°C for the indicated periods of time, then 0.5 ml of an adequate concentration of soy bean trypsin inhibitor* was added to each tube and the residual activity was assayed and compared with a control tube that received PBS instead of trypsin.

B. Inactivation by heat (56°C) and trypsin: Fig. 4 shows the differences in heat sensitivity of the inactivator and of $C'1_N$, and the effect of trypsin on the inactivator.

C. Influence of pH, dilution, and freezing on stability of the inactivator: The inactivator was stable at 0°C, room temperature, and at 30°C in the pH range

* Soy bean trypsin inhibitor has no effect on the C′4 inactivator.

of pH 5.0–10.0, whether it was submitted to these conditions in concentrated form or at high dilutions without the addition of protective proteins. Incubation for 30 min at 30°C at pH 2.0 or pH 3.0 resulted in 75% loss of activity, at pH 11.0 in more than 90%. Repeated freezing and thawing at −20°C or −75°C did not affect neutral dilute or concentrated inactivator preparations.

The Anticomplementary Activity of the C'4 Inactivator and its Specificity.—

A. In vitro: As mentioned above, the anticomplementary activity of low ionic strength precipitates obtained from nurse shark sera was first observed when these materials were tested for $C'1_N$ hemolytic reactivity. In these tests EA_N were incubated with dilutions of the dissolved precipitate for 30 min at 30° to form EA_N $C'1_N$ which were then brought to lysis with guinea pig supernate I or whole guinea pig C' in a dilution of 1:8 or 1:25 respectively. The degree of lysis was proportional to the concentration of the crude $C'1_N$ preparations except in some instances, where high concentrations showed strong inhibition of the lytic reaction. This inhibition could be dramatically increased if the guinea pig C' or supernate I was preincubated with the precipitate dilutions before the cells were added. This indicated that the anticomplementary action was directed against the guinea pig complement components rather than against the cells or $C'1_N$. Since it was not clear at this time whether the anticomplementary activity was due to the shark $C'1_N$ or to a different material contained in the precipitate, fractionation of the precipitate was attempted. The partially purified inactivator that resulted from these attempts (see above) was completely free of $C'1_N$, shark natural antibody, and to our present knowledge, other shark complement components.

In order to determine which guinea pig component or components were affected by such functionally pure preparations, the lowest inactivator concentration was chosen, that, if diluted 1:10 in whole guinea pig serum, completely inhibited its hemolytic activity after incubation at 37°C for 20 min. Intermediate cell complexes were then treated with that inactivator concentration and, after washing, tested for their reactivity in comparison with control cells which were incubated without the inactivator. Neither optimally sensitized sheep erythrocytes, nor $EAC'1_a$, $EAC'1_a4$, EAC'4, or EAC'1–7 were detectably affected in their sensitivity by this treatment. When the treated guinea pig serum was analyzed in terms of residual component activity, it was found that no C'4 hemolytic reactivity was detectable. C'1, C'2, C'5, C'8, and C'9 which were measured hemolytically and C'3 as measured by immune adherence were unaffected. Since C'6 and C'7 could not be tested individually, C'-EDTA was made with the inactivator treated and the untreated control serum and both were titrated with $EAC'1_a4$ and constant amounts of C'2 and with $EAC'1_a42_a$. Both C'-EDTA titers were similar. These results allowed the conclusion that the inactivator was specifically directed against the fourth

component of the guinea pig complement system. Since highly purified guinea pig C′4 was at least as susceptible to the inactivator as C′4 in whole serum, it was apparent that no cofactor was required for the inactivation.

In a similar manner, using the same intermediate cell complexes and guinea pig complement components, the inactivator's anticomplementary action for human and dog serum were also found to be due to its specific inactivation of C′4.

B. In vivo: If inactivator concentrations equivalent to those used in vitro were injected intravenously into dogs or guinea pigs, no apparent reaction was observed in the animals. Within 5 to 15 min after the injection, no whole serum complement activity was detectable. Again the anticomplementary effect was solely due to the inactivation of the fourth complement component.

Measurement of C′4 Inactivator Reactivity.—

A. By residual C′4 titration: When it was established that the inactivator was specifically directed against the fourth complement component and that no other serum constituents were required for its action, an assay system was developed which was based on measurements of residual C′4 activity rather than of residual whole complement activity of inactivator treated material.

Initial experiments in which purified C′4 was incubated with dilutions of the inactivator and then titrated using $EAC'1_a$, purified C′2, and C′-EDTA showed that reproducible titers could be obtained. However, inactivation of C′4 proceeded very rapidly and the inactivator titers were extremely high if limited amounts of C′4 were used in the assay (see below). Rather than using large amounts of purified components, a standard assay was developed using guinea pig supernate I as a source of C′4 and C′2. One volume inactivator dilutions in glucose-gelatin-Veronal was mixed with one volume of a 1:25 dilution of supernate I in the same buffer. The mixture was incubated for 20 min at 37°C; then one volume of $EAC'1_a$ (1×10^8 cells per ml in glucose-gelatin-Veronal) was added and incubation continued for 20 min at 30°C. Finally, two volumes of C′-EDTA 1:25 were added, followed by incubation for 60 min at 37°C. The assays were either performed in microtiter plates or in tubes. The concentration of supernate I contained approximately 200 C′H50 units of C′4 per ml and 125 units of C′2. Since addition of purified C′2 did not alter the results, this step was omitted. The titer of the inactivator was expressed as that dilution which inactivated all but one of the available C′H50 units of C′4. The time allowed for the inactivation at 37°C was derived from several kinetic experiments, of which Fig. 5 is an example. It should be kept in mind, however, that the actual time during which the inactivation of C′4 occurred in this kind of assay was the preincubation time, plus an undefined time interval during which inactivation still proceeded while $SAC'1_a4$ were formed.

The data illustrated in Fig. 6 were obtained using a modification of the above assay method. In order to decrease the number of variables involving temperature changes and changes of reaction mixture volumes, the following two step procedure was used. To 0.5 ml of prewarmed (37°C) inactivator dilutions (inactivator VI-1 pool 5 × concentrated) 0.5 ml of also prewarmed supernate I (1:25) were added at timed intervals. Without interrupting the incubation in the waterbath shaker 1.5 ml of 0.333 × 10^8/ml EAC'1_a were

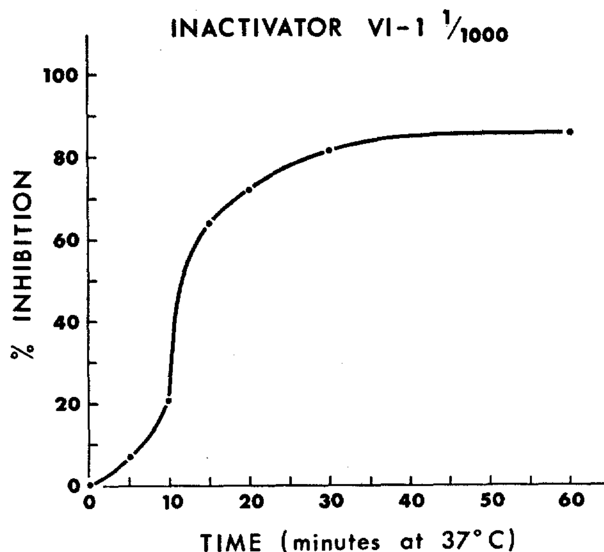

FIG. 5. Inactivation of C'4 hemolytic reactivity of guinea pig supernate I. Constant C'4 inactivator concentration, variable time of inactivation at 37°C. See text for assay procedure.

pipetted into each tube and incubation was continued for 60 min at 37°C. Finally 5.0 ml of ice cold EDTA-NaCl were added, the tubes were immediately centrifuged, and the optical density of the supernatant fluid was recorded at 415 mμ. Tubes containing no inactivator showed 100% lysis, those containing inactivator, but no supernate I showed the same low degree of lysis as the spontaneous lysis controls.

Fig. 5 shows that, after an apparent initial lag period, the inactivation of C'4 proceeded rapidly, then slowing down and approaching a plateau at about 20 min.

The kinetic data plotted in Fig. 6 demonstrate that the lag periods decreased with increasing inactivator concentrations and that the time required for the inactivation of all but one C'H50 unit of C'4 (50% lysis) was roughly proportional to the inactivator concentrations. This relationship was also expressed by the velocity of C'4 inactivation between 80% and 60% lysis.

Since we are at present unable to stop the inactivator action at a given moment, it was not possible, using these assay methods, to really titrate residual C′4. What was actually measured was the activity of the last few C′H50 units of C′4 that remained hemolytically active after at least 190 units were inactivated. This accounts for the longer lag periods at low inactivator concentrations. The early plateauing that can be seen in Fig. 5 and 6 and the slow approach to 100% inhibition or 0% lysis respectively, could have various reasons—among them, the possible presence of inhibitory substances in the

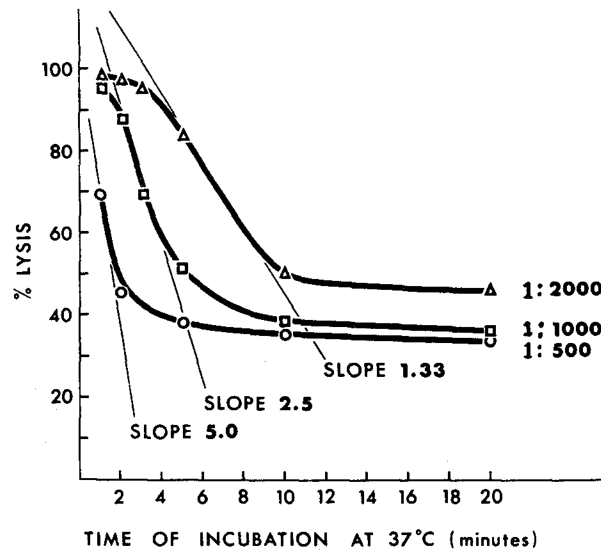

FIG. 6. Inactivation of C′4 hemolytic reactivity of guinea pig supernate I. Two-fold C′4 inactivator dilutions; variable time of inactivation at 37°C. See text for assay procedure.

guinea pig supernate I, and the highly efficient lytic system in the absence of EDTA (Fig. 6). In order to avoid both these difficulties, similar kinetic experiments with limited amounts of purified C′4 were performed (Fig. 7). Four concentrations of a purified C′4-inactivator preparation were incubated at 37°C with 10 and 15 C′H50 units of purified C′4. After the given time intervals EAC′1_a were added to each tube, followed by C′2 and C′-EDTA as in a standard C′4 assay. For each preincubation time, the degree of inhibition, when plotted against the dilutions of the inactivator, described a curve which was concave to the dose scale. In Fig. 7 this relationship was converted to one of direct proportionality by plotting arithmetically the negative natural logarithm of the lysed cells, i.e., $-\ln(1-\%$ inhibition) against the dilutions of the inactivator.

It is apparent from Fig. 7 that this type of assay is strongly influenced by

the time of inactivation and also by small differences in C'4 concentrations. On the other hand, if these two variables were kept constant, straight line plots over a wide range of inactivator concentrations were obtained, provided that no large excess of C'4 was used. With still smaller amounts of C'4 (less than 10 units), inactivation was so rapid that impractically high dilutions of

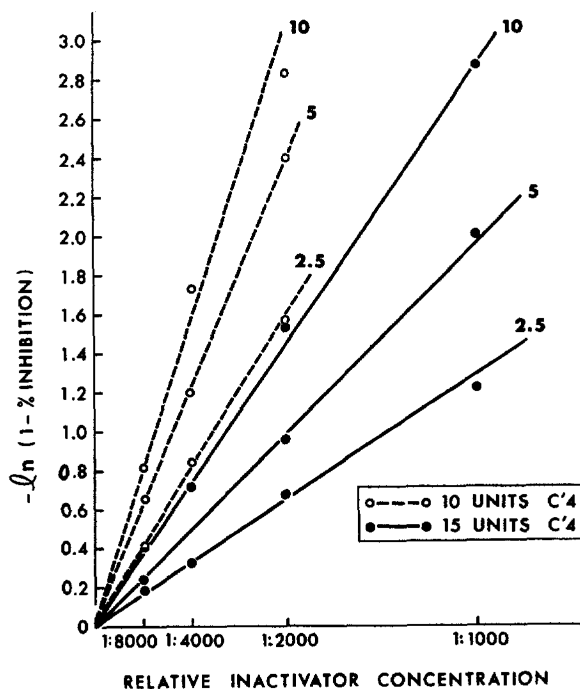

FIG. 7. Inactivation of purified guinea pig C'4 by two-fold C'4 inactivator dilutions. 10 and 15 C'H50 units of C'4 were incubated with the inactivator for 2.5, 5, and 10 min. See text for assay procedure.

the inactivator had to be used in order to obtain a measurable degree of lysis.

B. Hydrolysis of TAME[5] *by the C'4 inactivator:* Preliminary experiments indicated that addition of purified C'4 inactivator to a weakly buffered TAME solution resulted in a linear decrease of pH values with time. To 1.0 ml of a 0.05 M solution of TAME in 0.15 M PBS were added 0.15 ml of various inactivator concentrations at zero time and 22°C. The pH was recorded in the constantly stirred mixture. Initial velocities could be easily and quite reliably obtained by this simple method. For example, a preparation of inactivator

[5] TAME, *p*-toluene sulfonyl-L-arginine methyl ester hydrochloride. A grade Calbiochem, Los Angeles, Calif.

VI-1 (5 × concentrated) lowered the pH in a linear fashion from pH 7.57 to pH 7.4 in 10 min under these conditions. Initial velocities were found to be proportional to the concentrations of the inactivator. Samples from two purified inactivator pools (VII-1) were kindly tested by Dr. Robert Stroud for

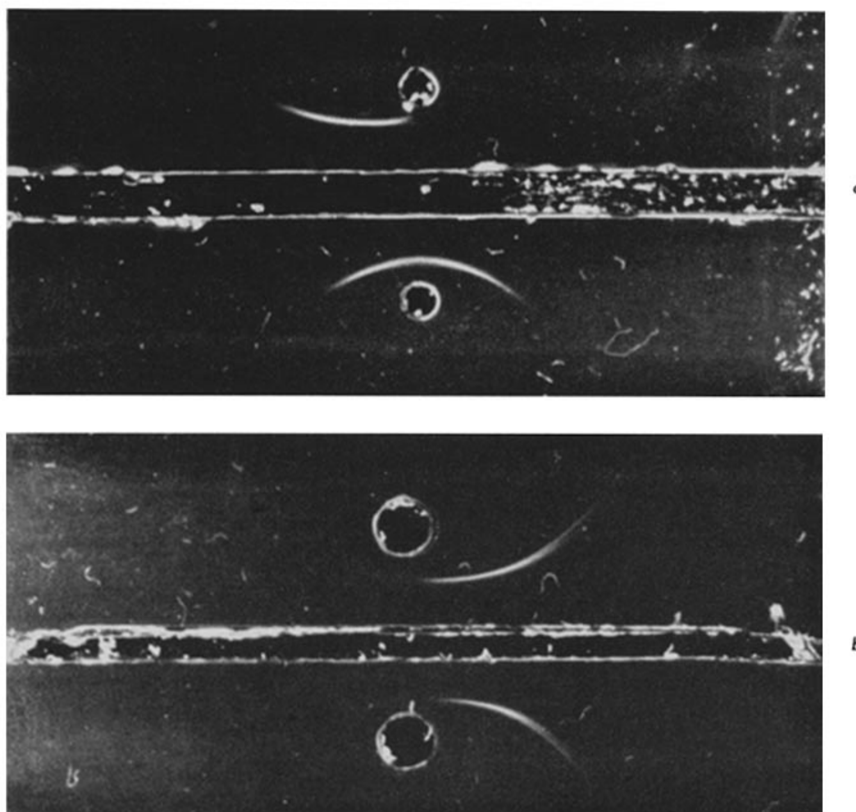

FIG. 8. Immunoelectrophoresis of purified human C′4 (*a*), and human serum (*b*). Upper wells: treated with C′4 inactivator; lower wells: untreated controls. The troughs contained rabbit anti-human C′4. The anode is to the left.

their TAME-esterase activity by a newly developed spectrophotometric method (6). The samples which had an OD_{280} of 0.04 were comparable in their activity with a purified human C′1-esterase preparation of an OD_{280} of 0.1. Their different initial velocities reflected their different titers in terms of C′4 inactivation.

The Mechanism of C′4 Inactivation.—Several observations seem to indicate that the inactivation of C′4 by the C′4 inactivator is enzymatic. This view is

supported by the velocity studies with C′4 as substrate and by the preliminary experiments with TAME. In addition, the inactivation of C′4 was found to be strongly temperature dependent. For example, a given inactivator concentration preincubated with C′4 for 20 min at 0°C caused 35% inhibition (in a standard titration), as compared with 90% if the temperature was 37°C. For a 10 × lower concentration of the inactivator under otherwise identical conditions the values were: less than 1% and 60% respectively. Furthermore, it was found that the inactivator could be inhibited by DFP (see under discussion).

In an attempt to demonstrate directly an alteration of the C′4 molecule as a result of inactivator treatment, an immunoelectrophoretic analysis of highly purified human C′4[6] before and after inactivation was undertaken. Fig. 8a shows the results. The treated C′4 formed a foreshortened precipitation arc with rabbit anti-C′4[6] and an anodic shift indicating faster anodic movement. Surprisingly, no such change could be detected if normal whole human serum was treated and analyzed in a similar manner Fig. 8b. In both cases no residual hemolytic C′4 activity was detectable after treatment with the inactivator.

Though most of the experimental data seem to favor an enzymatic destruction of C′4 as cause for its inactivation an alternate explanation, namely the formation of an inactivator-C′4 complex, can not be completely ruled out at the present time.

Immunoelectrophoresis of the C′4 Inactivator (Fig. 9).—Peak inactivator fractions from a Sephadex G-100 column (VII-1) were pooled, concentrated, and analyzed by immunoelectrophoresis in comparison with the starting materials of the column and with whole serum. The antibody preparations that were used were sera from rabbits immunized (*a*) with crude inactivator, containing as known contaminants, $C'1_N$ and natural antibody and (*b*) with whole nurse shark serum (see Materials and Methods). At present, we interpret these findings (Fig. 9) to mean (*i*) that whole serum and also the crude inactivator preparations contain a precursor of the C′4 inactivator, (*ii*) that the partially purified inactivator is contaminated with the precursor which is precipitated by both antisera (3*a*,*b*); (*iii*) that during inactivator generation, the precursor is partially cleaved in two antigenically new fragments, of which one is slower, the other faster moving than the precursor, and (*iv*) that the slower moving material (2*b*, 3*a*) is the inactivator. At the moment, this is a working hypothesis which requires for its verification the isolation of the precursor and of the enzyme by which it is cleaved.

The C′4 Inactivator as Anticomplementary Agent In Vivo.—The inactivator's unique specificity against only one complement component and its apparent

[6] The human C′4 preparation and the corresponding rabbit antibody was kindly provided by Dr. Hans Müller-Eberhard.

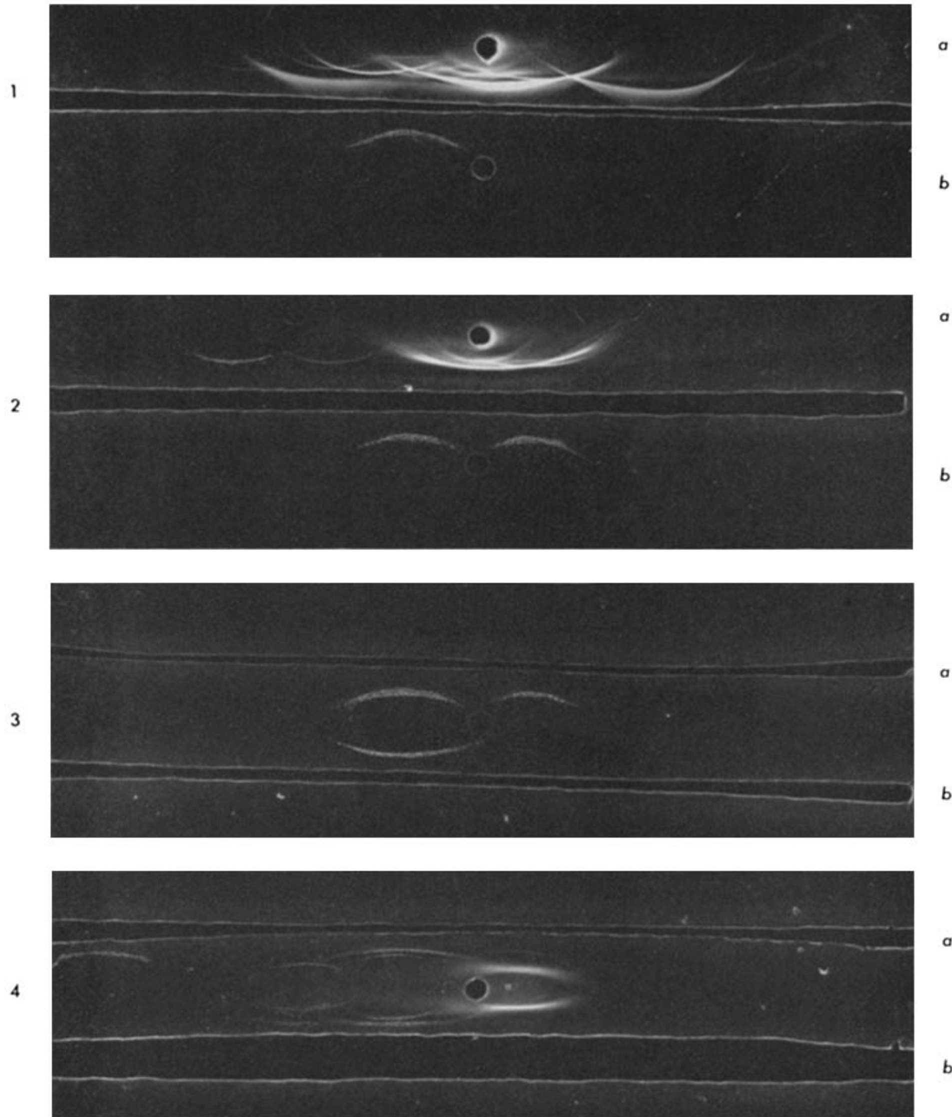

FIG. 9. Immunoelectrophoresis of partially purified C′4 inactivator and whole nurse shark serum. In Fig. 9 (1) and (2) the upper antigens (*a*) were whole shark serum; the lower (*b*) partially purified inactivator (peak fractions from G-100, 10 × concentrated). The antiserum for (1) was anti whole shark serum for (2) anti crude inactivator. Two distinct precipitation arcs can be seen in Fig. 9 (2*b*), but only one in (1*b*). The latter corresponds with the more anodic one of the two former ones.

This relationship is more obvious in Fig. 9 (3), where the antigen was the inactivator and the two antisera were compared. In Fig. 9 (4) the antigen was the crude inactivator, in the upper trough was the homologous antiserum (*a*), in the lower, anti whole shark serum (*b*). Fig. 9 (4*a*) shows two arcs that correspond to those formed by the purified inactivator with the same antiserum, in addition several others that also appear in (4*b*). A comparison of (4*a*) and (4*b*) reveals however, that there are two arcs that are formed with the anti inactivator and not with the anti whole shark serum. One is the slower moving of (2*b*) or (3*a*), the other is a very fast moving new arc. The anode is to the left.

lack of toxicity for biological systems at high, fully effective concentrations prompted various investigations concerned mainly with the participation of the complement system in immune and immune-pathological situations. Though most of the results are preliminary and were in part obtained in collaboration with other laboratories, it was decided to briefly discuss them here in order to demonstrate the usefulness of an anticomplementary agent with the characteristics of the C'4 inactivator. Detailed reports will follow at a later date.

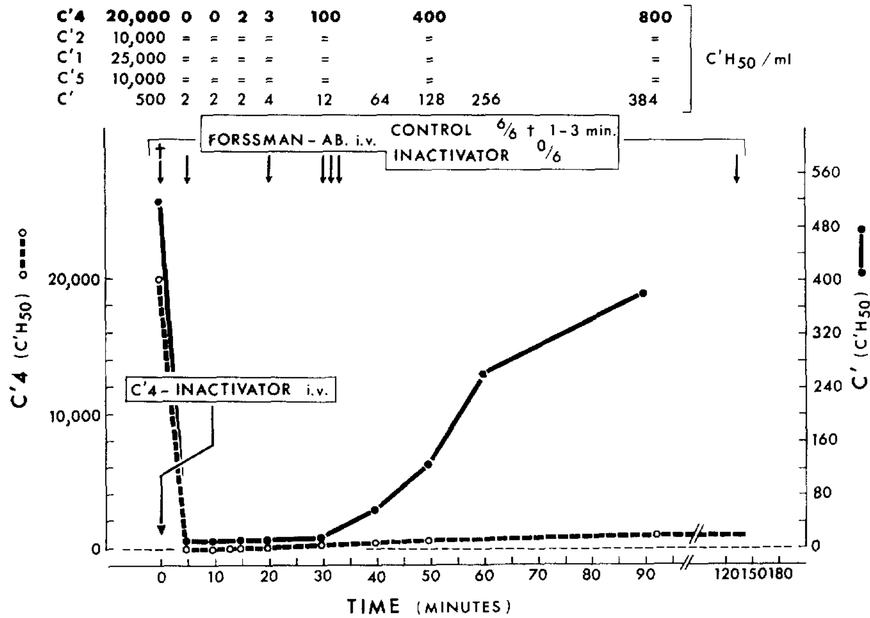

FIG. 10. Protection of guinea pigs against Forssman shock by a single intravenous injection of the C'4 inactivator. Effect of the inactivator on C' and C' components in vivo. See text for details.

A. Prevention of Forssman shock by treatment with the C'4 inactivator: Guinea pigs weighing 500–600 g were injected intracardially with the inactivator at a concentration that was estimated to reduce the C'4 activity to a titer of less than 1:10. At various time intervals, the animals were challenged with intravenous injections of rabbit anti-sheep E serum in amounts that caused lethal shock within 3 min in control animals. Several inactivator treated animals were not shocked but bled at various time intervals and the serum titers of whole C', C'1, C'4, C'2, and C'5 were measured. Fig. 10 is a summary of these experiments. It can be seen that one injection of inactivator protected the animals from 5 min until 135 min after the injection (longer time intervals

were not tested). Challenge simultaneously with the treatment resulted in death. During the time of observation, the serum levels of C′1, C′2, and C′5 did not change; the whole complement titer however dropped to 1:2 from its pretreatment level of 1:500, stayed low until 30 min postinjection, and climbed then rapidly to almost normal titers, while the animals were still fully protected. The rapid normalization of the whole complement reactivity was quite puzzling at first and raised the question of a possible in vivo reactivation of the complement system, which we had never observed in vitro. All attempts to reactivate serum treated in vitro or in vivo by incubation with various minced guinea pig tissues failed. However, measurements of C′4 activity in the serum of inactivator-treated animals explained our findings. Hemolytic C′4 was dramatically lowered by the treatment and recovered very slowly, reaching 2% of its initial value at 50 min and only 4% at 90 min. But since these 4% represented 800 C′H50 units of C′4 and since no other components of the complement system were affected by the inactivator, it was no longer surprising that the whole complement activity was almost normal at this time. This situation illustrates rather convincingly how misleading estimations of the reactivity of the complement system can be if one relies on hemolytic measurements of whole complement only. Highly significant depletion or destruction of a single complement component (more than 95% in our case) may have an insignificant effect on the whole complement titer.

Arthus reaction: Initial experiments indicated, as expected, that direct and reversed passive Arthus reactions could be suppressed if the C′4 reactivity was kept low for an extended period of time.

B. Passive cutaneous anaphylaxis (PCA): Under none of various experimental conditions was it possible to influence the PCA reaction in guinea pigs using rabbit anti-BSA and BSA. Whether the animals were treated so that C′4 levels were undetectable during the whole experimental period, whether the inactivator was in addition injected locally with the antibody or intravenously with the antigen, no suppression of the PCA reaction could be observed.

C. Prolongation of xenograft rejection times: The rejection of pig kidneys by dogs, normally occurring within 10 min after anastomosis, was dramatically delayed when the dog was treated with one intravenous injection of C′4 inactivator prior to grafting. The prolongation was of the same magnitude as that obtained by in vivo absorption of the dog's natural anti-pig antibodies (7) or by treatment with the anticomplementary cobra venom factor (8).

The C′4 Inactivator and the Formation of Biologically Reactive Materials in Guinea Pig Serum.—The inactivator itself did not increase the capillary permeability in the guinea pig skin, nor did it cause contractions of the terminal guinea pig ileum. Guinea pig serum treated in vitro with the inactivator to complete inactivation of C′4 caused a very slight increase in capillary permea-

bility at a dilution of 1:2 and none at a dilution of 1:20. The serum so treated exhibited no smooth muscle contracting activity. Treatment of the inactivated serum with cobra venom factor resulted in the formation of anaphylatoxin; there was no apparent difference between the inactivator-treated and the untreated control serum (9). However, in contrast to normal serum, anaphylatoxin could not be generated in the C′4 deficient serum if it was treated with immune precipitates. In collaboration with Dr. Snyderman and in confirmation of our former findings (10), it was found that the same relationship holds true for the generation of a chemotactic factor generated in guinea pig serum upon incubation with endotoxic lipopolysaccharide (LPS). Whereas the chemotactic factor could be generated in inactivator-treated serum with cobra venom factor, its generation with LPS was not possible unless C′4 was added to the serum. Relatively small amounts of either residual C′4 in insufficiently treated serum or of purified C′4 added to completely deficient serum was enough to allow the formation of the chemotactic factor.[7]

In Vitro Synthesis of C′4 by Guinea Pig Liver (11).—The complete depletion of C′4 in the tissues of inactivator treated guinea pigs made it possible to observe the synthesis of C′4 in minced organ cultures of such animals. All untreated cultures of spleen and liver showed decreasing titers of C′4 and C′2 in the culture fluid due to leakage from the tissues. However, the inactivator-treated cultures contained no detectable C′4 initially but showed comparable C′2 titers. Subsequently, C′4 titers increased from 20–40 hr to peak titers of 1:48 in all treated liver cultures, (not in the spleen cultures) except those containing puromycin. It was estimated that the guinea pig liver could synthesize all serum C′4 in 24–48 hr.[8]

DISCUSSION

In recent years three specific inhibitors or inactivators of single complement components have been isolated from various mammalian sera. The C′1 inactivator from human serum (12–14) and from guinea pig and rabbit serum are known to destroy $C'1_a$ in the fluid phase and the C′1 site of $EAC'1_a$ and $EAC'1_a4$. The C′3 and C′6 inactivators which have been found in normal human, rabbit, and guinea pig sera have no known effect on the corresponding components in the fluid phase but specifically destroy the hemolytic activity and in the case of the C′3 inactivator, also the immune adherence reactivity of the respective active sites (15). It has been suggested that the existence of specific inactivators of complement components in normal serum may constitute an important control mechanism for the complement system (16).

The C′4 inactivator described in this paper apparently has no such function.

[7] Snyderman, R., J. A. Jensen, and H. Gewürz. In preparation.

[8] Jensen, J. A., and R. Stolfi. In preparation.

No effect on the complement activity of the shark serum could be observed even at inactivator concentrations which rapidly and completely inactivated mammalian C′4 in whole serum and highly purified preparation of C′4. Furthermore, in contrast to the known inactivators, ours is directed exclusively against fluid phase C′4, has no effect on the EAC′1_a4 site, and is not detectable in whole serum. The conditions for its generation in low ionic strength precipitates, seem to indicate that a precursor might be enzymatically transformed to yield a material with new properties. The mechanism of its generation requires further investigation.

TABLE II

Characteristics	C′4 inactivator	C′1-esterase	
Inactivation of C′4			
at 37°C	Rapid	Rapid	
at 0°C	Slow	Rapid (>37°C)	(18)
Inactivation of C′2	No	Yes	(18)
Inactivation of C′4 in vivo	Rapid	?	
S-value	3.3	3.7	(19)
Half life at 56°C	5 min	30 min	(18)
Stability at pH 5.0	Stable	Unstable	(18)
Hydrolysis of TAME	Yes	Yes	
Inhibited by DFP	Yes*	Yes	

*Assuming a molecular weight of 40,000 to 60,000 for the C′4 inactivator and an approximate mole per mole inhibition of esterases by DFP, our purest preparation should have been completely inhibited by 10^{-6} M DFP. However, the concentration required for 50% inhibition was found to be 1000 times greater.

Recently Opferkuch, Ringelmann and Klein reported on a C′4 inactivator which was generated in guinea pig euglobulin fractions under essentially similar conditions (17). Their material which hydrolyzed TAME also specifically inactivated C′4 in the fluid phase, but not C′2. These investigators searched for a relationship between their C′4 inactivator and guinea pig C′1. Their data were not compatible with the view that C′1_a acted as a precursor and that their C′4 inactivator could therefore be C′1 esterase.[9] A complete comparison of the two inactivators has to wait for a more detailed report from the authors. The possible relationship of our inactivator to C′1 esterase as described in the human system requires some discussion. There are certain similarities but also striking dissimilarities. Table II compares our data with those published for human C′1 esterase.

Obviously, shark C′1 esterase need not be similar in every respect to human C′1 esterase, but even with this reservation in mind, the following observations

[9] Klein, P. G. Personal communication.

seem incompatible with the assumption that the C′4 inactivator could really be shark C′1 esterase: (*a*) The inactivator could be generated and then separated from $C'1_N$ without loss of $C'1_N$ activity. (*b*) The generation of the inactivator was inhibited rather than enhanced in the presence of EDTA. (*c*) In spite of the fact that $C'1_N$ like guinea pig $C'1_a$ activated guinea pig C′4 and C′2, the C′4 inactivator did not inactivate C′2. Even concentrations that completely inactivated 10^{13} effective molecules of C′4 in a few minutes had no detectable effect on the hemolytic activity of C′2. These findings do not absolutely exclude the possibility that the inactivator could be associated with the first component of the shark complement system. Further research concerning this possibility had to wait for preparations of highly purified $C'1_N$ and is being conducted at the present time.

Rather than adding it to the list of the above mentioned component inactivators it seems to be more logical to classify our C′4 inactivator together with other anticomplementary materials found in nonmammalian biological systems.

If compared with the most thoroughly investigated member of this group, the cobra venom factor (20, 21), the uniqueness of the C′4 inactivator becomes apparent. While the former is relatively nonspecific, affects C′3, C′5, and in high concentrations, C′6–C′9 (22),[10] is toxic due to the in vivo generation of anaphylatoxin, and does not inactivate purified complement components because of its cofactor requirement (22),[10] the latter is highly specific, nontoxic, and independent of other serum constituents.

So far we have not found a specific blocking agent for the C′4 inactivator which would allow precise kinetic studies under various conditions with C′4 as "substrate." At relatively high concentrations, the initial velocity of C′4 inactivation seems to be proportional to the concentration of the inactivator. Proportionality was also observed between initial velocities of TAME hydrolysis and inactivator concentrations. Based on the temperature dependence of inactivation, the protein nature of the inactivator, and its effect on the electrophoretic mobility of human C′4, we are inclined to believe that it enzymically destroys C′4. Furthermore, recent experiments indicate (*a*) that depletion of the inactivator is much slower than one would expect if C′4 were inactivated by complex formation and (*b*) that a fragment of C′4 is formed during its inactivation. Whether the hydrolysis of TAME is an expression of the same enzymatic activity however, is not beyond question.

As we learn more about the mode of action of the C′4 inactivator we are bound to learn more about C′4; and as we attempt to elucidate its generation and identify its precursor we must be aware of its possible relation to C′1. If C′1 esterase and the C′4 inactivators from shark or guinea pig serum are two independently different substances, then, considering their generation under

[10] Jensen, J. A. Unpublished data.

identical conditions in euglobulin fractions, they are most certainly mutually cross-contaminated—at least initially—during preparatory procedures. If they are both derived from the C′1 macromolecule, one must find a basis for their dissimilarities and define their functions.

Independent of these still unanswered questions, the shark C′4 inactivator has proven to be a valuable tool in vitro as well as in vivo to interrupt the complement system's sequential action by destroying an early link in the chain without affecting later ones. Its properties as a specific, efficient nontoxic, anticomplementary agent will be helpful in further elucidating the function of the complement system and its participation in immune pathological syndromes.

SUMMARY

A material which specifically inactivates mammalian C′4 was isolated from low ionic strength precipitates of nurse shark serum. The C′4 inactivator was not detected in whole serum. The conditions of its generation and its immunoelectrophoretic behavior seem to indicate that it is an enzymatically formed cleavage product of a precursor contained in whole shark serum. The inactivator was partially purified and characterized. It had an S-value of 3.3 (sucrose gradient) which was in agreement with its retardation on gel filtration, was stable between pH 5.0 and 10.0, had a half-life of 5 min at 56°C, pH 7.5, was inactivated by trypsin and was nontoxic. Its powerful anticomplementary activity in vitro and in vivo was solely due to the rapid inactivation of C′4; no other complement components were affected. No cofactor requirement was observed for the equally rapid inactivation of highly purified human and guinea pig C′4. The kinetics of C′4 inactivation and TAME hydrolysis, the greater anodic mobility of inactivated human C′4, and the influence of temperature on the rate of inactivation suggest that the inactivator is an enzyme and C′4 its substrate. This conclusion was supported by the more recent detection of a split product of C′4. Intravenous administration of the C′4 inactivator could prevent lethal Forssman shock and suppress the Arthus reaction in guinea pigs; it prolonged significantly the rejection time of renal xenografts but had no detectable effect on passive cutaneous anaphylaxis. Anaphylatoxin could be generated in C′4 depleted guinea pig serum with the cobra venom factor, but not with immune precipitates. The possible relationship between C′1 esterase and the C′4 inactivator is discussed on the basis of similarities and dissimilarities.

I wish to thank Dr. M. Michael Sigel, who was instrumental in initiating my interest in the shark's complement system. He and his staff at Variety Children's Research Foundation provided the facilities and the materials without which this investigation would not have been possible. The methods for the isolation, purification, and measurement of the first component of shark complement was developed in collaboration with Gordon D. Ross, a graduate student of the Department of Microbiology. The technical assistance of Elena Iglesias throughout this study has been invaluable. The technical assistance of Kathleen Travino and

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