COMPONENTS OF GUINEA PIG COMPLEMENT

II. Separation of Serum Fractions Essential for Immune Hemolysis

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The results in the preceding paper (1) provide strong evidence that the complement (C′) component requirements for immune hemolysis are more complex than those for immune adherence (I-A). The first C′ component to react with erythrocytes in the state EAC′1,4,2 was termed C′3c, and the resulting unlysed complex, EAC′1,4,2,3c, was found to be reactive in I-A.

In order for cells in this intermediate state to proceed to lysis, three additional fractions of guinea pig serum have been found essential. The present experiments clarify the sequential reactivity of these fractions and provide other data concerning their properties, including information regarding their depletion by antigen-antibody complexes.

Methods and Materials

Most of the reagents, methods, and abbreviations employed were described in the previous paper (1).

Continuous Gradient Cellulose Chromatography.—One-hundred gm of DEAE cellulose was washed once with 2 liters of 0.5 M NaCl and twice with 2 liters of 0.01 M NaCl. After adjustment of the pH to 7.6 with HCl, the cellulose was packed into a glass column 8 cm in inside diameter and 60 cm long, and after washing with 2 liters of 0.01 M NaCl the column was placed in a cold room.

One-hundred ml of chilled guinea pig C′ was diluted with 300 ml of cold water and held at 0°C for 30 minutes, after which the resulting precipitate was removed by centrifugation. The supernatant fluid was then applied slowly to the column, followed by 800 ml of 0.01 M NaCl, and the eluate discarded. After this, the column was connected to a varigrad apparatus through a micropump, by means of which the flow rate was regulated to 50 ml/hour. The desired gradient change was obtained by using 8 of the 9 chambers of the varigrad apparatus, each filled with 300 ml of fluid as follows: chamber 1, water; 2, 0.15 M NaCl; 3, 0.03 M NaCl; 4, water; 5, 0.3 M NaCl; 6, 0.03 M NaCl; 7, 0.06 M NaCl; 8, 0.3 M NaCl.

The first 500 ml of eluate was discarded, and then 140 fractions of 13.5 ml each were collected automatically. After adjustment to 0.15 M with 3.0 M NaCl, each fraction was assayed for C′3c, C′3b, C′3a, and C′3d activity. C′3b and C′3c assays were carried out after heating aliquots of the fractions for 30 and 60 minutes respectively at 56°C, to destroy C′3a.

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Preparation of C'3a.—Fractions were collected from a continuous gradient DEAE cellulose column as described above. After appropriate assays had been carried out, the fractions (approximately 0.065 to 0.073 M) showing a high C'3a activity with minimal or no C'3c, C'3b, or C'3d activity were pooled. The resulting preparation was brought to pH 3.5 in the cold by addition of 0.15 N HCl, and allowed to remain 8 hours or overnight at 0°C in order to inactivate any residual C'3c or C'3b. The pH was then adjusted to 7.2 with 0.15 N NaOH and the resulting C'3a preparation was stored at 0–4°C, where activity remained for several months. Preparations made in this manner usually showed little or no C'3c, C'3b, or C'3d activity when tested undiluted, and contained small but variable amounts of C'2. C'3a activity was present at dilutions of several thousandfold.

Preparation of C'3b.—Two columns were employed, of 5 cm inside diameter and 60 cm length. For column 1, 25 gm of DEAE cellulose were washed twice with about 500 ml of 0.5 M NaCl and once with 0.02 M NaCl. The cellulose was then resuspended in a liter of 0.02 M NaCl, adjusted to pH 7.6, packed tightly into the column under pressure, and topped with glass wool and glass beads. The column was washed with about 1 liter of 0.02 M NaCl, and placed in a cold room. Column 2 was prepared in the same manner, but using 0.095 M NaCl instead of 0.02 M.

Twenty ml of guinea pig C' was diluted with 60 ml of cold water, and let stand for 30 minutes at 0°C. The resulting precipitate was removed by centrifugation, and the supernatant fluid was applied to column 1 under pressure. (This was done at room temperature, but the columns and solutions were kept cold until use.) As soon as the fluid level had reached that of the glass beads, the following solutions were passed through the column successively, under pressure, and discarded: 750 ml of 0.02 M NaCl; 1 liter of 0.07 M NaCl; 1 liter of 0.08 M NaCl; 2 liters of 0.095 M NaCl. Then 1 liter of 0.2 M NaCl was passed through the column and 1 liter of effluent collected. This was added to 1.1 liters of cold water, mixed well, and applied to column 2. The effluent was discarded, as was 500 ml of 0.095 M NaCl applied as a wash. C'3b was eluted with 250 ml of 0.5 M NaCl, the first 125 ml of effluent being discarded and the next 75 ml collected. The molarity was adjusted to 0.15 by dilution with water, and the preparation was then heated 30 minutes at 56°C to inactivate most of the C'3a present. The activity of preparations made in this manner persisted well over a storage period of several months at 0–4°C. They contained only a trace of C'3a and C'2 activity when tested undiluted, but somewhat larger amounts of C'3c and C'3d; C'3b activity was present at dilutions of several hundredfold or thousandfold, depending upon the method of assay.

C'3d.—Before its recognition as a distinct component in the C' system, C'3d was supplied in an unrecognized form in all component assays, because it was present in the "purified" C'2 preparations being used, and was thus carried over with the EAC'1,4,2, which was not washed after incubation with C'2. After this fact came to light, C'2 in EDTA was used as a temporary source of C'3d until further investigation revealed that fraction IV of bovine serum, prepared according to the method of Cohn *et al.* (2), was a potent and functionally pure source of C'3d.

Titration of C'3b.—Into test tubes were placed 1.0 ml of dilutions of the unknown in buffer with EDTA, and 1.0 ml of a high but arbitrarily chosen concentration of C'3c + a + d (*i.e.* C'3c + C'3a + C'3d). Then 0.5 ml of unwashed EAC'1,4,2 (7.5×10^7/ml) was added to each tube, followed by incubation for 90 minutes at 37°C. Following this, 2.5 ml of cold buffer with EDTA was added to each mixture, and after centrifugation the optical density was determined spectrophotometrically at 415 mμ. The approximate $C'H_{50}$ for a specified set of test conditions was determined by plotting the negative logarithm of the unlysed cell concentration, $-\ln(1-y)$, against the dilution on logarithmic paper, and reading the dilution 1 ml of which would produce 50 per cent lysis.

Since the amounts of C'3c, C'3a, and C'3d employed were arbitrarily chosen and not in excess, it must be emphasized that the C'3b "titer" of any preparation is applicable to a given

set of test conditions only, and has no meaning in absolute terms or in relation to similar "titers" of C'3c, C'3a, or C'3d. These important limitations are equally true for the hemolytic assay of the other three components. Whenever it was desired to compare two or more preparations, they were always assayed simultaneously under identical conditions.

It should be mentioned that in some of the earlier work only half as high a concentration of EAC'1,4,2 was employed, and that before the discovery of C'3d this component was supplied unrecognized along with the unwashed EAC'1,4,2. When C'3b assays were carried out on fractions from continuous gradient chromatography, aliquots were heated 30 minutes at 56°C to destroy C'3a, before dilution and testing.

Titration of C'3c and C'3a.—These components were usually titrated in a manner similar to that just given for C'3b, except that to dilutions of the unknown in buffer with EDTA were added aliquots of an arbitrary but relatively high concentration of C'3b + a + d for assay of C'3c, or of C'3c + b + d for assay of C'3a. Titration of C'3c by means of I-A is described in reference 1, and an alternative method for measuring C'3a will be presented below.

Physical and Chemical Treatments.—

(*a*) *Dialysis:* Aliquots of separated C' components were dialyzed for 24 to 48 hours in the cold against several 4-liter volumes of veronal-buffered saline. In addition, 2 ml of whole C' was placed in a dialysis tube along with a small empty test tube to keep the serum in a thin layer next to the membrane. Dialysis was carried out for 48 hours in the cold against 10 ml of veronal buffer, and the buffer was then tested for C' component activity.

(*b*) *Sephadex chromatography:* A 2 × 4 cm column of sephadex G-75 in saline was set up in a chromatography tube. Three ml of a mixture of C'3c, C'3b, C'3a, and C'3d was passed through the column, and fractions were assayed for each of these components.

(*c*) *Heat:* One-half ml aliquots of various separated components were placed in small test tubes, stoppered, and heated for varying times at 56°C. They were then cooled in an ice bath or by adding several volumes of cold diluent, and tested.

(*d*) *Low pH:* Preparations were chilled, then brought to the desired pH with 0.15 N HCl. After incubation at 0°C for the desired time, they were neutralized with 0.15 N NaOH, and assayed.

(*e*) *Freezing:* Preparations of C'3c, C'3b, and C'3a were diluted 1:10 with veronal buffer or with veronal buffer containing 1 per cent gelatin. Aliquots were then sealed in glass ampoules and stored at 0–4°, −35° or −70°C. After 6 weeks all were tested for activity, and compared with the undiluted stock preparations which had been kept at 0–4°C.

(*f*) *Trypsin, hydrazine, and ammonium hydroxide:* One ml of each separated fraction in veronal buffer (pH 7.5) was mixed with 1 mg of Difco 1:250 trypsin in 1 ml of saline and incubated 1 hour at 37°C, followed by dilution and testing. Other aliquots were treated for 1 hour at 37°C with an equal volume of 0.1 M hydrazine, then neutralized with twice as many moles of propionaldehyde, followed by dialysis for 24 or 48 hours in the cold against 2 4-liter volumes of veronal-buffered saline. Two-ml aliquots of the various fractions were treated with 0.5 ml of 0.15 N NH_4OH for 1 hour at 37°C, followed by neutralization with HCl. Controls were included for all treatments, to correct for any effect of the reagents on the assay system.

C' Component Depletion.—

(*a*) *C'3c depletion:* Three ml of washed EAC'1,4,2 (1.5×10^8/ml) was mixed with an equal volume of precooled or prewarmed C'3c, so that the final mixture contained approximately 12 $C'H_{50}$ of C'3c/ml. During incubation at 0° or 30°C, samples were withdrawn and centrifuged after 15 minutes and after 2 hours. Dilutions of the resulting supernatant fluids were assayed hemolytically for C'3c much as in a quantitative C'-fixation test, while the cells were washed and aliquots were then tested for EAC'1,4,2,3c activity by the addition of C'3b + a + d (*i.e.* C'3b + C'3a + C'3d), with or without C'2, in buffer without EDTA.

(*b*) *C'3b depletion:* This was carried out in essentially the same manner as described above under C'3c depletion, except that EAC'1,4,2,3c was substituted for EAC'1,4,2. Buffer without EDTA was used so that in some reaction mixtures purified C'2 could be used to regenerate decaying SAC'1,4,2,3c. After samples were centrifuged the supernatant fluids were assayed for C'3b, and the cells were washed and tested for EAC'1,4,2,3cb activity by the addition of C'3a + d.

(*c*) *C'3a depletion:* C'3a in an amount approximating only 1.5 $C'H_{50}$/ml was incubated with EAC'1,4,2,3cb at a final concentration of 6×10^8/ml, and samples were withdrawn and centrifuged at intervals. The supernatant fluids were tested for C'3a activity, and the washed cells were incubated with C'3d in order to reveal the presence of EAC'1,4,2,3cba.

RESULTS

Discovery of an Unrecognized Component (C'3d) in C'2 and in Bovine Serum Fraction IV.—When C'3c, C'3b, and C'3a in EDTA were added to EAC'1,4,2 which had not been washed since the addition of C'2, the cells lysed. However, it was found that when the EAC'1,4,2 was washed just prior to addition of C'3c, C'3b, and C'3a, very little lysis took place. Since previous work had indicated that C'2 preparations were almost completely free from C'3c, C'3b, and C'3a, an additional factor was suspected, and tentatively termed C'3d.

An initial method for assay of this factor was developed by preparing EAC'1,4,2 cells in the usual manner (1), then washing them twice and resuspending in cold buffer with EDTA. Aliquots of these cells were then incubated at 37°C with dilutions of the unknown and a 1:100 dilution of C'3c + b + a (*i.e.* C'3c + C'3b + C'3a). When it became possible to prepare the intermediate complex EAC'1,4,2,3cba (see below), C'3d was measured by incubating 2 ml aliquots of dilutions of the unknown in buffer with EDTA, for 90 minutes at 37°C with 0.5 ml of EAC'1,4,2,3cba (7.5×10^7/ml). Then 2.5 ml of cold buffer was added, the tubes centrifuged, and the supernatant fluids read spectrophotometrically.

By these methods evidence was obtained suggesting that C'3d differed from C'2 in being less labile at 56°C, in not being consumed during incubation at 37°C with EAC'1,4, and in not requiring the presence of divalent cations. Three different lots of C'2 all showed about the same C'3d activity.

Since C'2 in EDTA did not appear to be an advisable source of C'3d for routine use, and since bovine serum was found to have a high C'3d activity, various fractions of bovine serum prepared by Dr. R. M. Zarco according to the method of Cohn *et al.* (2), were tested for this component. Fraction IV proved to have about 1000 times as much C'3d activity as our C'2 preparations, and to be lacking in C'2, C'3b, and C'3c activity, with only traces of C'3a when tested undiluted. For this reason, bovine serum fraction IV was used as a convenient source of C'3d. It was dialyzed extensively against 0.15 M NaCl in the cold, and stored at −35°C until use.

Comparative Elution Characteristics of C'3c, C'3b, C'3a, and C'3d from DEAE Cellulose.—Evidence for the separate nature of each of the four components under study was obtained by parallel assays for each, carried out on fractions

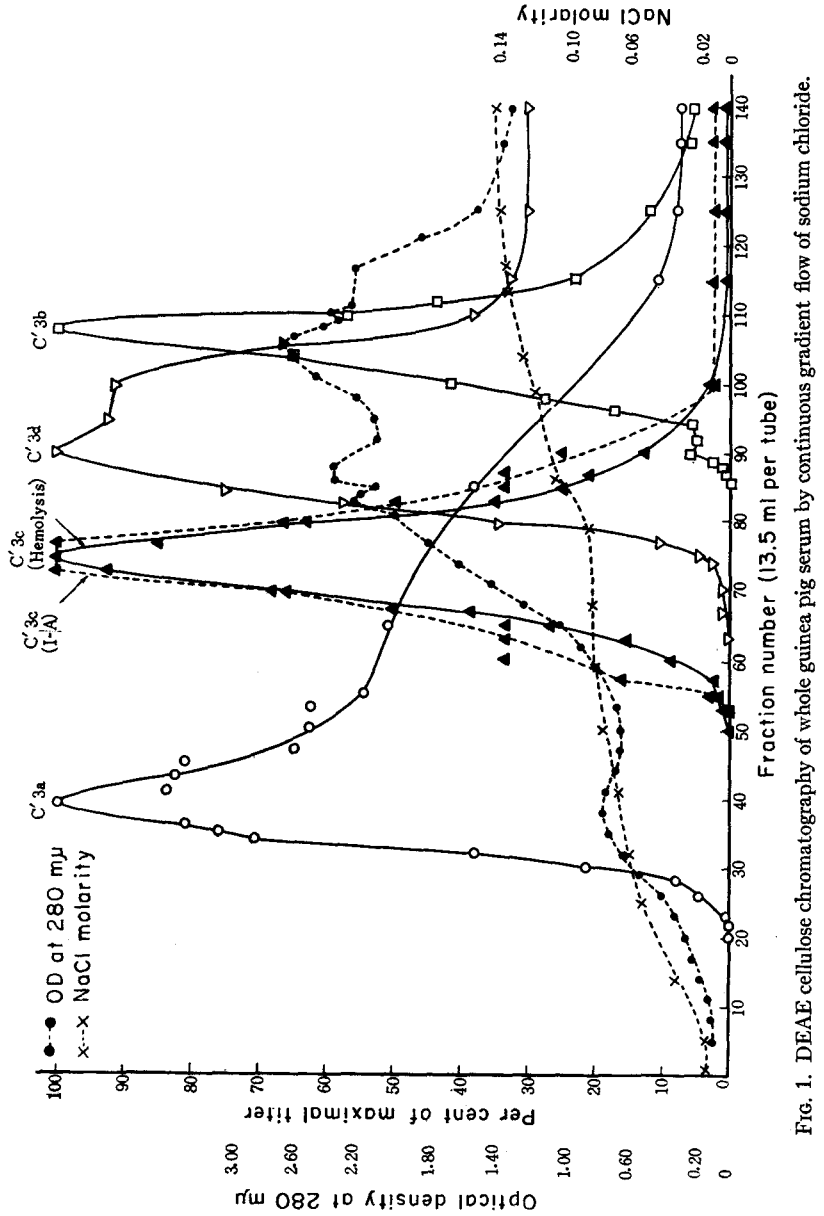


FIG. 1. DEAE cellulose chromatography of whole guinea pig serum by continuous gradient flow of sodium chloride.

eluted from a single DEAE cellulose column using a continuous gradient flow of NaCl (described in Methods and Materials). Fig. 1 shows the elution characteristics of each component. It will be noted that initial and peak elutions of each took place at a different molarity, while a comparison of the I-A and hemolytic assays for C'3c shows that both methods appear to measure the same substance.

Pooled C'3a and C'3c reagents were prepared by combining appropriate fractions from this column, followed by heat or low pH treatment as described under Methods and Materials or in reference 1. A detailed comparison between these reagents and certain others in use is given in Table I, where it can be seen

TABLE I

Relative Activities of Some Recent C' Component Preparations

Preparation	C'I-A_{50}/ml	C'H_{50}/ml					
	C'3c	C'3c	C'3b	C'3a	C'3d	C'2	C'1,4*
C'3c-15	400,000	1250	0	<1 (14.9)‡	11	0	0
C'3b-14	200	11	2050	<1 (28.8)	29	<1 (11.6)	0
C'3a-15	0	<1 (15.7)	0	1700	0	<1 (15.8)	0
C'3d-2	0	0	0	<1 (12.9)	7600	0	0
C'2-6	0	0	0	0	7	2100	0

* Fractions tested undiluted with EA + C'2 + C'3c + b + a + d (i.e. C'3c + C'3b + C'3a + C'3d).

‡ Less than 50 per cent lysis when tested undiluted (actual amount of lysis in parenthesis).

that while C'3a and C'3d preparations have a high degree of functional purity, C'2, C'3c, and C'3b contain small amounts of certain other components.

Properties of the Four Components.—C'3c, C'3b, C'3a, and C'3d were found to be non-dialyzable, and there was no evidence for their retardation by sephadex G-75 so by inference all have molecular weights in excess of about 40,000. As may be seen from Table II, C'3c is exceptionally resistant to heating at 56°C. C'3a is extremely heat-labile, while C'3d is less so and C'3b is moderately heat-resistant.

C'3c and C'3b are rapidly inactivated at low pH, which has a lesser effect on C'3d and essentially no effect on C'3a. Freezing at −35° or −70°C caused a pronounced drop in the activity of C'3c, C'3b, and C'3a (C'3d was routinely stored at −35°C), but especially affected C'3c and C'3b. Surprisingly, the loss was greater at −70°C than at −35°C in every case. One per cent gelatin provided moderate protection against freezing for C'3b and C'3a, but was of little value with C'3c. C'3c was found to be markedly more susceptible to hydrazine than the other components tested, and was also readily inactivated by ammonium hydroxide.

In all of the above inactivation procedures the loss of C'3c activity was very nearly the same whether measured by I-A or by immune hemolysis. This is considered strong evidence that the same substance was being measured by both tests.

Component Activities of Heterologous Sera.—Since a preliminary assay showed sera from several species to have high C'3d activity, a more comprehensive survey was carried out in which sera from several species were tested for C'3c, C'3b, C'3a, and C'3d. Cell controls of EAC'1,4 were incubated in buffer with EDTA

TABLE II

Inactivation of C'3c, C'3b, C'3a, and C'3d by Various Methods

Treatment	Temperature	pH	Time	Inactivation of:				
				C'3c*	C'3c‡	C'3b‡	C'3a‡	C'3d‡
	°C			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Dialysis	0–4	7.0–7.5	24–48 hrs.	0	0	0	0	
Low pH	0	4.0–4.1	30 min.	92	93	81	0	45
	0	3.5	30 min.	>99.9	99.4	>98		
Freezing	−35	7.0–7.5	6 wks.	65	75	80	65	
	−70	7.0–7.5	6 wks.	99.8	>98	95	90	
Trypsin	37	7.5	1 hr.	98.5		98	>99	
Hydrazine	37		1 hr.	>99.9	>98	41	33	0
NH_4OH	37		1 hr.	>99.9		92	37	
Time necessary for 50 per cent inactivation at 56°C, *in min*.....				>600	>600	60	2	12

* Assay by I-A.

‡ Assay by immune hemolysis.

with the lowest dilution (1:10) of each serum tested, and showed no lysis with the exception of shark serum (1.5 per cent) and snake serum (4.4 per cent). Except for the land crab hemolymph, all sera had been stored at −35° or − 70°C for less than 1 month at the time of testing, and most for less than 2 weeks. The results are listed in Table III.

Since 10-fold dilutions were employed for all except the guinea pig serum, the $C'H_{50}$ values listed are crude estimates of activity. The magnitudes of these titers have no significance either in absolute terms, or with respect to the relationship between the apparent titers for one component and those for any other component. For any one component the relative reactivities of sera from different species may be compared, but it must be kept in mind that these all represent reactions between EAC'1,4,2 prepared with guinea pig C' components, and a mixture of guinea pig and heterologous components other than C'1, C'4 and C'2. In addition, the reactions were carried out under conditions of temper-

ature, pH and osmotic strength only known to be satisfactory for the guinea pig C′ system.

Non-mammalian sera (except duck) showed little or no activity except for the surprising C′3a activity of water snake serum. Of the mammalian sera tested, horse serum alone failed to show any detectable reactivity in EDTA with EAC′1,4,2 made from guinea pig components. Guinea pig, swine, and rat sera showed the highest overall activity for the components tested, with the homologous (guinea pig) system showing the greatest activity in every case. C′3b and

TABLE III

Component Activities of Whole Sera from Various Species

Source of serum	Approximate $C'H_{50}$ per ml			
	C′3c	C′3b	C′3a	C′3d
Human	300	1500	100	>10,000
Horse	<10	<10	<10	<10
Ox	20	2000	10	>10,000
Fetal calf	<10	800	<10	>10,000
Goat	50	3000	30	4,000
Swine	600	25,000	20,000	>10,000
Rabbit	300	5000	30	>10,000
Rat (Fischer)	800	2000	3000	>10,000
Hamster	60	4000	300	>10,000
Mouse (C57Bl/6)	<10	10	50	>10,000
Duck	100	300	30,000	>10,000
Carp	<10	<10	<10	100
Shark	<10	<10	<10	10
Snake (*Natrix cyclopion*)	<10	<10	3000	<10
Land crab (*Cardisoma* sp.)	<10	<10	<10	<10
Guinea pig	4100	28,000	68,000	80,000

C′3d activities were the most widely distributed, being high in all mammalian sera except those from the horse (deficient in both) and the mouse (low in C′3b).

Formation of the Intermediate Complex EAC′1,4,2,3cb.—After development of methods for the preparation of EAC′1,4,2,3c (1), it was not difficult to show that C′3b reacts with this intermediate complex to produce another intermediate form of different properties, termed EAC′1,4,2,3cb. As discussed in reference 1, in the absence of free C′2, cells in the state, EAC′1,4,2,3c decays rapidly at 30–37°C to a form which will no longer react with C′3b. Since the decay competes with the reaction between SAC′1,4,2,3c and C′3b, the latter does not proceed very far at elevated temperatures in the absence of free C′2, unless a high concentration of C′3b is employed. Although decay would be minimal at 0°C, C′3b fails to react significantly at this temperature except in

very high concentrations. However, since most of the sites of decayed SAC'1,4,2,3c are readily "regenerated" by fresh C'2, the reaction between C'3b and EAC'1,4,2,3c proceeds quite well at 30°C in the presence of free C'2. (See Table IX for more data on this point.)

The effect of temperature on the formation of EAC'1,4,2,3cb was investigated by mixing prechilled or pre-warmed, washed EAC'1,4,2,3c (1.5×10^8/ml) with equal volumes of a 1:20 dilution of C'3b to which had been added more than 300 effective molecules of C'2/cell. These mixtures were incubated for 3 hours at 0°, 30° and 37°C, and samples were removed at

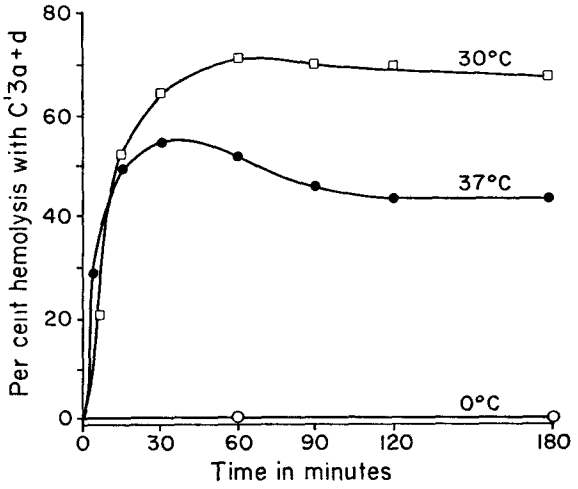


FIG. 2. Formation of EAC'1,4,2,3cb from EAC'1,4,2,3c and C'3b at 0°, 30° and 37°C.

intervals. These were quickly diluted with several volumes of ice cold buffer to stop the reaction, then centrifuged, and the cells washed twice and resuspended to a concentration of 7.5×10^7/ml. All cell preparations were then held at 0°C until sampling was completed, after which 0.5 ml aliquots were incubated for 90 minutes at 37°C with 2.0 ml of a mixture of C'3a and C'3d. Separate cell blank and water lysis controls were included as usual, for each cell preparation.

It will be noted in Fig. 2 that more reactive preparations were formed at 30°C than at 37°C. This may reflect a more favorable relationship at 30°C between the decay of EAC'1,4,2,3c cells and their reaction with C'3b, as occurs in the reaction between EAC'1,4 and C'2 (3, p. 182). Although the reaction progressed quite rapidly at first, maximum reactivity was attained only after a period of from 30 to 60 minutes. At the concentration of C'3b employed in this experiment, no detectable reaction occurred at 0°C. It has since been found that when much higher concentrations are used (*ca.* 4 to 10 times as much C'3b), detectable EAC'1,4,2,3cb formation does take place at 0°C, but it is very slow and limited in extent.

It can also be seen from Fig. 2 that cell reactivity declined after the peak had been attained, this loss being most noticeable at 37°C. This gradual loss of activity during continued exposure to the C'3b preparation was in sharp contrast to the rapid and nearly complete inactivation of EAC'1,4,2,3c in the continued presence of C'3c preparations (1).

In a further study of this spontaneous inactivation, EAC'1,4,2,3cb was first prepared at 30°C, washed, then incubated at 0° or 37°C for 2 hours, washed again, and restandardized to 7.5×10^7/ml. One-half ml aliquots of these cells were then incubated for 90 minutes at 37°C with 2.0 ml of a mixture of C'3a and C'3d in EDTA. It will be noted that the slow inactivation of EAC'1,4,2,3cb at 37°C does not depend on the simultaneous presence of free C'3b, since this inactivation was about the same whether the cells were washed (Table IV) or not (Fig. 2).

TABLE IV

Immune Lysis of Washed EAC'1,4,2,3cb after Incubation at 0° or 37°C

2 hr. incubation	Lysis with C'3a + C'3d
°C	*per cent*
0	80.3
37	73.0

Another difference between EAC'1,4,2,3c and EAC'1,4,2,3cb is that after heating for 2 hours at 37°C the former becomes unable to react with the next C' component (C'3b), unless "regenerated" by the addition of fresh C'2 (1). Table IV shows that such heating has only a slight inactivating effect on EAC'1,4,2,3cb and that C'2 is not required for heated cells to react with C'3a and C'3d.

Sequence of Reaction of C'3a and C'3d.—

EAC'1,4,2,3cb (7.5×10^7/ml) was incubated for 30 minutes at 30°C with equal volumes of 1:75 dilutions of C'3a or C'3d. After washing three times in the cold, half of each preparation was held at 0°C and the rest incubated 30 minutes at 30°C with the same concentration of C'3d or C'3a, whichever was omitted during the first incubation. Those cells treated first with C'3a and then with C'3d lysed during the second incubation. The remaining preparations were washed thoroughly, standardized to a concentration of 7.5×10^7/ml, and 0.5 ml aliquots were incubated 90 minutes at 37°C with 2 ml of diluent containing the same amount of C'3a, C'3d, or C'3a + C'3d.

The resulting lysis is presented in Table V, and shows clearly that EAC'1,4,2,3cb reacts next with C'3a to form a complex, termed EAC'1,4,2,3cba, which can be washed and which will then lyse when exposed

to C'3d. Thus, the sequence of reaction of the C' components after C'2 appears to be C'3c → C'3b → C'3a → C'3d.

TABLE V

Hemolysis of EAC'1,4,2,3cb Pretreated with Various Combinations of C'3a and C'3d

Pretreatment of the cells at 30°C	Lysis with:		
	C'3a	C'3d	C'3a + d
	per cent	*per cent*	*per cent*
EAC'1,4,2,3cb/wash	6.2	1.8	100
EAC'1,4,2,3cb + C'3d/wash	7.8	2.3	100
EAC'1,4,2,3cb + C'3d/wash/+ C'3a/wash	3.0	99.1	98.2
EAC'1,4,2,3cb + C'3a/wash	3.9	100	100

Formation of the Intermediate Complex EAC'1,4,2,3cba.—The effects of time and temperature upon the C'3a reaction were studied by bringing EAC'1,4,2,3cb to reaction temperature (0°, 30° or 37°C), then adding an equal volume of prewarmed or precooled C'3a diluted 1:25. During incubation, 2.0 ml samples were removed at intervals, diluted with cold buffer, centrifuged, washed, and resuspended. All preparations were then held at 0°C until sampling had been completed, after which 0.5 ml aliquots of each were incubated 90 minutes at 37°C with 2.0 ml of C'3d diluted 1:2000.

The data from this experiment indicated that C'3a reacts very rapidly even at 0°C. Maximum EAC'1,4,2,3cba activity was found in the first sample, removed, and washed after only 5 minutes at 0°C. Mixtures of EAC'1,4,2,3cb and C'3a incubated at 30° or 37°C did not produce EAC'1,4,2,3cba of any greater reactivity, and in fact cells removed after a prolonged incubation at these higher temperatures were somewhat reduced in their EAC'1,4,2,3cba activity, especially those treated at 37°C. This decreased activity was investigated further with washed EAC'1,4,2,3cba, which was incubated at 0°, 30° or 37°C. Samples removed after 30 minutes and 2 hours were washed and resuspended in cold buffer, and 0.5 ml aliquots were incubated 90 minutes at 37°C with 2.0 ml of a 1:5000 dilution of C'3d. As previously observed with EAC'1,4,2,3c and EAC'1,4,2,3cb, spontaneous inactivation of these cells was more rapid at 37°C but also occurred at 30°C (Table VI). The reason for this loss of activity is not known.

EAC'1,4,2,3cba is now prepared as routine by incubating washed EAC'1,4,2,3cb (1.5×10^8/ml) with an equal volume of C'3a diluted 1:50 or 1:100, for 15 minutes at 0°C in buffer with EDTA. The cells are then washed, resuspended to a concentration of 7.5×10^7/ml, and stored at 0°C. They are usually used the same day or the following day, but retain their ability to be lysed by C'3d for many days at 0°C. It is of interest that these cells have a

higher rate of spontaneous lysis than do any of the other intermediate complexes which have been prepared, showing from 8 to 15 per cent lysis after 90 minutes at 37°C. If the cells are stored overnight at 0°C, washed again, and then tested, spontaneous lysis at 37°C is somewhat lower (*ca.* 5 to 8 per cent), indicating that E* formed during preparation may contribute to the lysis observed shortly

TABLE VI

*Immune Lysis of Washed EAC'1,4,2,3cba after Incubation at 0° or 37°C (per cent Lysis by C'3d 1:5000)**

Incubation	Incubation Time	
	30 min.	2 hrs.
°C	*per cent*	*per cent*
0 (control)		59.6
30	61.6	55.4
37	56.0	43.7

* Separate cell blank and lysis controls included for each sample to allow correction for spontaneous lysis of about 12 per cent in 90 minutes at 30–37°C.

TABLE VII

Lysis of EAC'1,4,2,3c, EAC'1,4,2,3cb, and EAC'1,4,2,3cba by Various C' Components

Intermediate complex	Lysis after reaction with:			
	C'2 + C'3b + a + d*,‡	C'3a + C'3d‡	C'3d‡	Buffer with 0.1 per cent bovine albumin
	per cent	*per cent*	*per cent*	*per cent*
EAC'1,4,2,3c	99.0	0.3	0.2	0.6
EAC'1,4,2,3cb	95.7	86.7	0.6	0.8
EAC'1,4,2,3cba	99.1	84.9	84.0	86.0

* C'3b + C'3a + C'3d.

‡ In the usual buffer with 0.1 per cent gelatin.

thereafter. However, a heightened intrinsic fragility of these cells is indicated by the finding that EAC'1,4,2,3cba washed and resuspended in buffer without gelatin, then incubated 2 hours at 0° or 37°C, show 3 to 4 times as much lysis as similarly treated cells in a diluent containing 0.1 per cent gelatin as a stabilizer.

Table VII summarizes the reactivities of EAC'1,4,2,3c, EAC'1,4,2,3cb, and EAC'1,4,2,3cba, and indicates the components required for lysis of each complex. The lysis resulting when EAC'1,4,2,3cba were incubated with buffer containing 0.1 per cent bovine albumin indicates a significant contamination of

commercial bovine albumin (Armour fraction V) by C'3d. This finding emphasizes the necessity for using gelatin rather than bovine albumin as a stabilizer in the diluent.

The Reaction between EAC'1,4,2,3cba and C'3d.—Fig. 3 shows the effect of temperature on the reaction between C'3d and EAC'1,4,2,3cba. There is a sharp difference between the results at 0°C and those at 37°C, resulting from

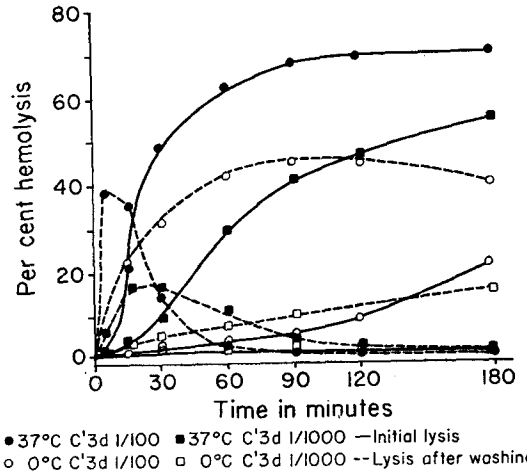


FIG. 3. Reaction between EAC'1,4,2,3cba and bovine C'3d at 0° and 37°C. A suspension of EAC'1,4,2,3cba was divided into aliquots and brought to reaction temperature. At zero time different concentrations of cold or warm C'3d in buffer with EDTA were added. Samples were removed at intervals during incubation, quickly centrifuged, and the supernatant fluids saved for a determination of the amount of "initial lysis" occurring before separation of the cells from the C'3d. They were then washed twice (the wash fluids also being saved for spectrophotometric examination), resuspended in buffer with EDTA, and incubated 1 hour at 37°C, followed by centrifugation. The amount of lysis in this final supernate was added to that in the wash fluids for an estimate of the amount of "lysis after washing" following removal of C'3d from the system.

the combined effects of slower E* lysis and slower C'3d reactivity at the lower temperature. After 60 minutes, for example, there was very little initial lysis at 0°C. After these cells were washed and brought to 37°C, there was considerable lysis of E* which could only have formed during the 0°C incubation; still, the total amount of lysis (sum of "initial lysis" curve and "lysis after washing" curve) was markedly lower than that for the samples originally incubated at 37°C, indicating that C'3d reacts more slowly at 0°C than it does at 37°C. A 10-fold reduction in the C'3d concentration resulted in a much greater decrease in lysis at 0°C than at 37°C.

It seems likely that the terms S* and SAC'1,4,2,3cbad are synonymous,

since lysis proceeds rapidly following treatment of EA with whole C′, or treatment of EAC′1,4,2,3cba with C′3d, regardless of whether the cells are then washed repeatedly or not. Because C′3d of bovine origin was employed for most of these studies, it seemed advisable to compare lysis due to bovine C′3d with that due to guinea pig C′3d. This was made possible by the very recent development of techniques for the separation of C′3d from whole guinea pig serum (unpublished data). EAC′1,4,2,3cba was incubated briefly with C′3d of bovine or guinea pig origin, washed thoroughly, and resuspended in buffer with EDTA. During subsequent incubation at 37°C, samples were withdrawn at intervals and centrifuged immediately, hemoglobin determinations on the supernatant

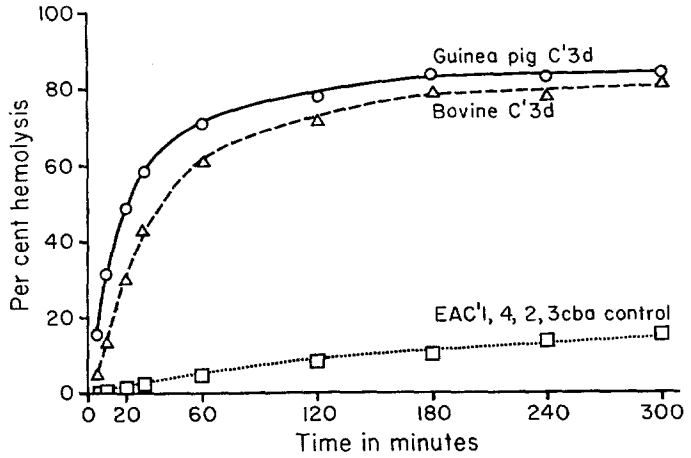


FIG. 4. Comparison of the rates of lysis at 37°C of untreated EAC′1,4,2,3cba and EAC′1,4,2,3cba treated with C′3d of bovine or guinea pig origin, and then washed.

fluids being made at 415 mμ. The rates of lysis of EAC′1,4,2,3cbad as compared with untreated EAC′1,4,2,3cba are shown in Fig. 4. The curves do not go through the origin because lysis was proceeding continuously during and after the washing process.

It can be seen that, in a functional sense, there was no apparent difference between guinea pig and bovine C′3d, as compared in this experiment. It is of interest that, after the first 180 minutes of incubation, the control EAC′1,4,2,3cba was lysing at a more rapid rate than the C′3d-treated cells. This may be accounted for if one assumes that the increased fragility of EAC′1,4,2,3cba, mentioned previously, is proportional to the number of SAC′1,4,2,3cba/cell. Those cells having the greatest number of SAC′1,4,2,3cba would be most likely to form SAC′1,4,2,3cbad (S*) during contact with C′3d, and hence to lyse. Consequently, after a population of EAC′1,4,2,3cba has been treated with C′3d, washed, and incubated 3 hours at 37°, the

only unlysed cells remaining are likely to be those with so few SAC'1,4,2,3cba that interaction with C'3d did not occur, and such cells are apparently less fragile on the average than the original EAC'1,4,2,3cba population.

C' Component Depletion Experiments.—Preliminary studies of the "fixation" of C' components by antigen-antibody precipitates clearly showed the depletion of C'3c and C'3b, but not of C'3a or C'3d, from whole guinea pig serum (1). Since separated components and the appropriate intermediate complexes were available as described above, a more detailed investigation of this problem was carried out. Individual C' fractions were incubated with erythrocytes in suitable intermediate states, and then both the washed absorbing cells and the supernatant fluids were assayed for changes in activity. It should be emphasized that

TABLE VIII

C'3c Depletion and Cell Reactivity Following Incubation of Washed EAC'1,4,2 with C'3c

Reaction mixture	Temperature	Time	C'3c remaining in supernate	C'3c depleted from supernate, loss	Lysis of resulting cells with:	
					C'2 + C'3b + a + d	C'3b + a + d
	°C	min.	$C'H_{50}$/ml	per cent	per cent	per cent
EAC'1,4,2 + C'3c	0	15	9.5	15	70.8	7.7
	0	120	6.0	46	75.3	21.2
EAC'1,4,2 + C'3c	30	15	3.1	73	97.0	23.5
	30	120	1.3	88	4.2	0.2
C'3c alone	30	120	11.0	—	—	—
EAC'1,4,2 alone	—	—	—	—	2.0	0.3

references to "$C'H_{50}$" of C'3b, C'3a, etc., are strictly relative, that these values depend on the precise conditions of each assay, and that they have meaning only in terms of these particular conditions.

The procedures employed have been described under Methods and Materials. Table VIII shows that C'3c was depleted from the fluid phase by EAC'1,4,2, and that EAC'1,4,2,3c activity appeared with the cells. Since EAC'1,4,2,3c decays rapidly at 37°C (the temperature at which the cell test was carried out) to a form which will not react with C'3b unless first "regenerated" with C'2, there is greater lysis in the presence of C'2 and C'3b +a +d than with C'3b +a +d alone. An irreversible inactivation of EAC'1,4,2,3c takes place when such cells are continously exposed to C'3c preparations (1). This inactivation is quite rapid at 37°C, but also takes place at 30°C as can be noted from the great loss of EAC'1,4,2,3c activity after 2 hours at 30°C as compared with 15 minutes (4.2 *vs.* 97.0 per cent lysis).

Initial studies showed only a slight depletion of C'3b under conditions similar

to those which resulted in marked C'3c depletion. It was thought likely that this was due primarily to the rapid decay of SAC'1,4,2,3c on the cells in the absence of free C'2, the resulting complex being unable to interact with C'3b. This was confirmed by the marked increase in C'3b depletion and increase in EAC'1,4,2,3cb formation when C'2 was added to the reaction mixture, as can be noted in Table IX. In the absence of free C'2, unheated EAC'1,4,2,3c did not react appreciably with C'3b after the first 15 minutes, whereas when C'3b and C'2 were present together, both depletion and EAC'1,4,2,3cb formation were markedly higher after 60 minutes. EAC'1,4,2,3c heated at 37°C before use did

TABLE IX

C'3b Depletion and Cell Reactivity Following Incubation of Washed EAC'1,4,2,3c with C'3b or C'2 + C'3b

Cells	Incubated 30°C with:	Incubation time	C'3b remaining in supernate	C'3b depleted from supernate, loss	Lysis of resulting cells with C'3a + d
		min.	*C'H$_{50}$/ml*	*per cent*	*per cent*
EAC'1,4,2,3c	C'3b	15	11.5	11.5	6.3
		60	11.0	15.4	5.3
Heated EAC'1,4,2,3c*	C'3b	15	14.0	0	0.8
		60	13.5	0	1.1
No cells	C'3b	60	13.0	—	—
EAC'1,4,2,3c	C'2 + C'3b	15	11.5	17.9	33.6
		60	6.8	51.4	53.2
Heated EAC'1,4,2,3c*	C'2 + C'3b	15	13.0	7.1	10.1
		60	11.1	21.4	29.3
No cells	C'2 + C'3b	60	14.0	—	—

* Heated at 37°C for 2 hours.

not react at all with C'3b alone, and even in the presence of C'2 there was appreciably less C'3b depletion and EAC'1,4,2,3cb formation than with unheated cells. This probably reflects the spontaneous irreversible inactivation of some SAC'1,4,2,3c at elevated temperatures, while other sites are only decayed in a reversible manner and are restored to their former reactive state after addition of fresh C'2 (1).

Depletion of C'3b from the fluid phase was clearly shown, while reactivity of the absorbing cells was altered. Depletion, however, was not as complete as observed with C'3c, these data paralleling the results obtained when whole C' was observed with an immune precipitate (1). Similar experiments showed that no depletion of C'3b and no alteration of EAC'1,4,2,3c cell activity occurred at 0°C. This was not surprising, inasmuch as this component reacts very slowly at 0°C, even at much higher concentrations than were used for these studies.

Initial efforts to deplete C'3a from the fluid phase by absorption with EAC'1,4,2,3cb were unsuccessful, although EAC'1,4,2,3cba formation was readily demonstrated. Consequently, a 4-fold increase in the number of absorbing cells and a 6-fold decrease in the concentration of C'3a were adopted in an effort to show removal of C'3a from the fluid phase. The results agreed with those obtained when whole guinea pig C' was absorbed with an immune precipitate (1), in that the very slight loss of C'3a observed was felt to be of doubtful significance. We have concluded that depletion of C'3a was not clearly shown, although the test conditions were probably more favorable for removal of this component than was true for the C'3c and C'3b studies. Nevertheless, very pronounced EAC'1,4,2,3cba activity was acquired by the cells, indicating that interaction with C'3a must have occurred.

No effort was made to investigate the depletion of C'3d by EAC'1,4,2,3cba, because of the numerous complications which would have been introduced by cell lysis. Studies with whole guinea pig C' absorbed with an immune precipitate indicated no detectable removal of C'3d (1).

C' Component Activity of a Beta$_{1C}$ Globulin from Fresh Human Serum.— Müller-Eberhard and Nilsson have recently described a substance isolated from fresh human serum, which has some of the properties of "C'3." Since this material appears to be fixed from whole serum by antigen-antibody complexes and is inactivated by hydrazine (4), it seemed possible that it might contain C'3c. Its ability to prevent decay of EAC'1,4,2 suggested the presence of C'3b as well.

Through the kindness of Dr. Kozo Inoue, a beta$_{1C}$ globulin fraction prepared by Dr. Müller-Eberhard was made available to us for investigation. This material was analyzed for C' components by the methods which have been described in the present publication. It was found to have a high C'3c activity as measured both by I-A and by immune hemolysis, and relatively high C'3b activity as well. There was very little C'3a or C'3d activity.

DISCUSSION

Within the last few years, it has become recognized that guinea pig "whole C'3" consists of more than one, and probably three, different components (5,6)[1]. Taylor and Leon have isolated three "whole C'3" components from human serum (7). However, there have been no reports concerning the properties or reaction characteristics of these individual components, and no information has been available regarding the formation of intermediate complexes after reaction of these various factors with sensitized erythrocytes treated with the initial three components of complement (EAC'1,4,2). The only evidence concerning the sequence of component reaction after the C'2 step in the guinea pig system

[1] "Whole C'3" is used to indicate the sum of those C' components which react after C'1, C'4, and C'2, and which do not require divalent cations.

(3, p. 186) appears to be in conflict with similar data for the human C′ system (7,8), but this problem may be one of nomenclature only. Additional confusion has been contributed by the lack of agreement concerning whether "whole C′3" is "fixed" by immune complexes, and until the work of Nelson (9) there was some doubt whether "whole C′3" "fixation" required the prior participation of C′1, C′4 and C′2.

This publication and the preceding one (1) concern the identification of four different C′ components (C′3c, C′3b, C′3a, and C′3d), which react sequentially with EAC′1,4,2 in the absence of divalent cations, to cause lysis of the cell. Some of the data presented here reinforce the findings reported in (1) concerning the labile nature of EAC′1,4,2,3c at 30–37°, as determined in hemolytic assays. Table IX shows that EAC′1,4,2,3c reacted much more extensively with C′3b when fresh C′2 was present in the reaction mixture, while heating these cells at 37°C prior to admixture with C′3b prevented any detectable reaction, unless C′2 was also present. Apparently, although C′2 is required for C′3c to react with EAC′1,4, the resulting EAC′1,4,2,3c complex is still able to "decay" at elevated temperatures. That this "decay" at 30–37°C affects primarily the C′2 rather than the C′3c moiety of the complex is shown by the observations that the I-A reactivity of "decayed" EAC′1,4,2,3c is unimpaired (1), and that fresh C′2 but no additional C′3c is necessary for "regeneration" of the complex to a form once again reactive with C′3b.

Tables IV and VI show that this very pronounced, C′2-reversible thermal lability of EAC′1,4,2,3c is not shared by EAC′1,4,2,3cb or EAC′1,4,2,3cba. Although some activity was lost from these complexes after prolonged heating at 30–37°C, this loss was only a fraction of the total reactivity, and subsequent reaction with C′3a and C′3d did not require C′2.

The data in Figs. 3 and 4, and unpublished experiments comparing the rate of lysis of EAC′1,4,2,3cbad with that of E* formed by brief treatment of EA with whole guinea pig C′, strongly suggest that C′3d is the last component to react before lysis begins, and that SAC′1,4,2,3cbad and S* are synonymous, as are EAC′1,4,2,3cbad and E*. These findings may facilitate investigation of the terminal step in immune hemolysis.

The heightened innate fragility observed with EAC′1,4,2,3cba is of considerable interest. Although the evidence is good that these cells interact rapidly with C′3d, resulting in the prompt initiation of E* formation and the onset of lysis, one would prefer better cell stability in the absence of C′3d. It appears as though interaction of SAC′1,4,2,3cb with C′3a might increase cell fragility by disrupting certain outer components of the cell wall, without effecting a complete breach of the integrity of this structure. The latter perhaps is then accomplished by subsequent interaction with C′3d. This may be similar to the way that antibody and C′ are able to modify the cell walls of certain strains of bacteria so that lysozyme can then induce protoplast formation (10). Wardlaw has recently

advanced a similar concept with regard to the lysis of bacteria by C′ and lysozyme (11), in which he suggests that the effect of C′ may be to disrupt or dissociate cell wall lipid or lipoprotein components, thus allowing access of lysozyme to its substrate. The temperature dependence of the C′3d reaction and the fact that this component apparently is not depleted from whole serum by absorption with an immune precipitate (1) suggest that C′3d may well be enzymic in nature.

The presence of C′3d in C′2 preparations is not unreasonable, since continuous gradient elution studies have shown that these two factors are eluted from DEAE cellulose at almost the same molarity. Presumably most of the C′3d is eliminated either by the preliminary dialysis at low pH or the later passage through CM cellulose required in C′2 preparation, because the C′3d activity of the various lots of C′2 which were tested was only a fraction of a per cent of that of whole serum. However, since this component is not depleted by antigen-antibody complexes, only a trace seems to be required for a very marked effect on EAC′1,4,2,3cba.

Investigation has revealed that there is appreciable C′3d activity in human saliva. For this reason, it is essential to use care while pipetting cells and reagents when carrying out studies of this component.

There is remarkable agreement between the depletion studies carried out on whole guinea pig serum (1), and those conducted with purified C′ fractions and the appropriate intermediate cell complexes. C′3c is the most readily depleted component studied, 85 per cent or more of the initial activity having been removed by both methods. Considerable C′3c depletion took place even at 0°C, although the reaction was more rapid at 30°.

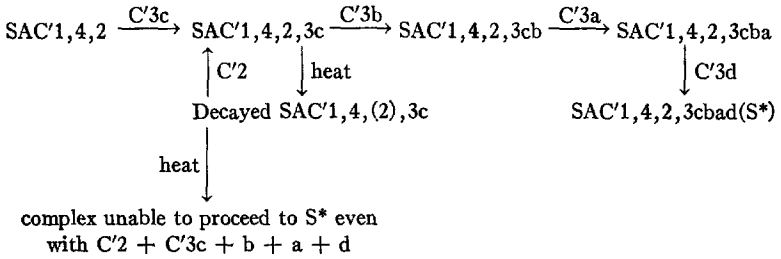
Appreciable amounts of C′3b could be removed both from whole serum and from purified C′3b. In the latter case, measurable depletion did not take place at 0°C.

Experiments with C′3a showed that while this component reacts very rapidly with EAC′1,4,2,3cb, even at 0°C, depletion could not clearly be shown, although there was a suggestion of slight loss of this component from the fluid phase. Using whole C′ absorbed with an immune precipitate, only about a 6 per cent loss of C′3a activity from the supernate was found (1).

Although the use of guinea pig components to measure the human C′ components in Müller-Eberhard's preparation is probably a less than optimal procedure, the results are of considerable interest. They clearly link the $beta_{1C}$ globulin prepared from fresh human serum with two separate components of "whole C′3", one of which (C′3c) is necessary for I-A, and both of which are "fixed" by immune complexes. The fact that decay of EAC′1,4,2 is blocked by this $beta_{1C}$ fraction (12) correlates well with data presented here and in the preceding paper, showing that after reaction with C′3c, EAC′1,4,2 still decays

rapidly, while after reaction with both C'3c and C'3b to form EAC'1,4,2,3cb, decay no longer takes place.

The reactions leading from SAC'1,4,2 to S* can perhaps be summarized best in diagrammatic form, as follows:



Reactions involving C'3b seem to proceed more slowly than the others, and are very strongly inhibited at 0°C. Reactions involving C'3c and C'3d proceed most rapidly at 30-37°C, but are only partially inhibited at 0°C, while reactions involving C'3a seem to take place almost as rapidly at 0°C as at 30-37°C.

SUMMARY

The elution characteristics from DEAE cellulose are presented for four components of guinea pig serum, which are capable of interacting sequentially with sheep erythrocytes sensitized with antibody and the first, fourth, and second components of complement (EAC'1,4,2) to cause immune hemolysis, and information is given regarding some of the properties of these components, termed C'3c, C'3b, C'3a, and C'3d. All can react in the presence of ethylenediaminetetraacetate, and are non-dialyzable. C'3c is quite stable at 56°C, but is rapidly inactivated at low pH or by contact with hydrazine or ammonium hydroxide. C'3b is moderately heat-stable, quite susceptible to low pH, and less readily destroyed by hydrazine. C'3a is very heat-labile, but relatively stable at low pH, while C'3d is heat-labile, sensitive to low pH, and insensitive to hydrazine.

EAC'1,4,2 reacts with C'3c to form EAC'1,4,2,3c, which reacts then with C'3b to give the intermediate, EAC'1,4,2,3cb. The following reaction with C'3a yields EAC'1,4,2,3cba, which reacts finally with C'3d to give EAC'1,4,2,3cbad (E*). The first and last reactions proceed moderately well at 0°C, but more rapidly at 30-37°C. The reaction with C'3b is almost completely inhibited at 0°C, while that involving C'3a proceeds almost as rapidly at 0°C as at higher temperatures. EAC'1,4,2,3cba cells have an increased fragility as compared with the other intermediate forms.

Depletion studies with purified fractions and appropriate intermediate complexes showed a high degree of depletion of C'3c, somewhat less of C'3b, and little or no depletion of C'3a from the fluid phase.

Examination of a $beta_{1C}$ globulin prepared from fresh human serum revealed high C'3c and C'3b activity, and very little C'3a or C'3d.

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