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Studies on the Mode of Action of the Fifth, Sixth and Seventh Component of Human Complement in Immune Haemolysis*

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Summary. The sixth (C'_6), seventh (C'_7) and eighth (C'_8) components of human complement were separated from each other by chromatography on hydroxyl apatite and obtained in functionally pure form. Density gradient ultracentrifugation revealed that C'_6 and C'_7 interact with the fifth component of complement (C'_5) to form a reversible protein–protein complex. Evidence was obtained that this complex occurs also in unfractionated, fresh human serum. Kinetic analysis of the reaction sequence in immune haemolysis disclosed that C'_5, C'_6 and C'_7 act, in this order, subsequent to the third component (C'_3) and prior to C'_8. Exploration of the mode of action of C'_5, C'_6 and C'_7 led to the formulation of two alternative hypotheses. The three components act either sequentially or as one functional unit. Their fluid phase interaction, the shape of dose–response curves and the encountered difficulty to isolate intermediate reaction products favour the concept that they act as a functional unit.

INTRODUCTION

Work in this laboratory (Müller-Eberhard, Nilsson, Dalmasso, Polley and Calcott, 1966; Nilsson and Müller-Eberhard, 1965, 1966; Hadding, Müller-Eberhard and Dalmasso, 1966) and in others (Nelson, Jensen, Gigli and Tamura, 1966; Klein and Wellensiek, 1965; Wellensiek and Klein, 1965) has revealed that the classical third component of complement consists of at least six different serum factors. When derived from human serum they are designated C'_3, C'_5, C'_6, C'_7, C'_8 and C'_9 (Complement Workshop, 1966). The reaction of these six components with the complement–erythrocyte complex which constitutes their 'substrate' may be conveniently subdivided into two principal steps. The first, involving C'_3, C'_5, C'_6 and C'_7, leads to the formation of a thermostable intermediate product (Nilsson and Müller-Eberhard, 1966); the second, requiring C'_8 and C'_9, results in the production of characteristic ultrastructural membrane lesions (Humphrey and Dourmashkin, 1965). The present paper is exclusively concerned with the first principal step of this reaction and, in particular, with the mode of action of C'_5, C'_6 and C'_7. Interest in these three components was aroused when it was found that they interact with each other in cell-free solution forming reversible protein–protein

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complexes. Preliminary evidence suggests that they are functionally interdependent in their action in immune haemolysis. The question will be explored, therefore, as to whether the observed fluid phase interaction between the three proteins is of functional significance.

In a previous communication (Nilsson and Müller-Eberhard, 1965), it was reported that the formation of a thermostable complex could be achieved with highly purified C'_5 and partially purified C'_6. Further work led to the recognition of an additional factor required in this reaction and to the resolution of partially purified C'_6 into C'_6 and C'_7. The complement–erythrocyte complex with which these three components react is designated systematically $EAC'_{1a,4,2a,3}$ and consists of sheep erythrocytes (E), antibody (A), the activated first (C'_{1a}), fourth (C'_4), activated second (C'_{2a}) and third (C'_3) components of complement. The reaction product will be termed systematically $EAC'_{1a,4,2a,3,5,6,7}$.

MATERIALS AND METHODS

C'_3 and C'_5

The methods for the isolation of these two components have been described previously (Nilsson and Müller-Eberhard, 1965). C'_3 obtained by the published procedure was found to be contaminated with C'_8; the contamination in terms of protein was less than 2 per cent. To remove C'_8 activity, purified C'_3 was subjected to preparative block electrophoresis in barbital buffer, pH 8·6, ionic strength 0·05. Under these conditions the more positively charged C'_8 is quantitatively separated from C'_3.

C'_6, C'_7 and C'_8

These components were obtained in highly purified form, although the degree of purification was considerably lower than that of C'_3 and C'_5. Preparations of C'_6, C'_7 and C'_8 are, therefore, termed 'functionally pure'. They were obtained by methods to be described in detail elsewhere (Nilsson, 1967). In brief, the following procedures were used. Euglobulin of fresh human serum was separated by chromatography on TEAE-cellulose utilizing a pH and ionic strength gradient elution procedure as described previously (Nilsson and Müller-Eberhard, 1965). Eluates falling into a pH range of 8·1–6·9 (corresponding to a specific conductance of 4·2–8 m-mho/cm) were pooled and applied to a hydroxyl apatite column equilibrated with phosphate buffer, pH 7·9, having a specific conductance of 6 m-mho/cm. Following application, C'_7, C'_6 and C'_8 were eluted, in this order, at a conductance of 11, 12 and 13 m-mho/cm, respectively (Fig. 1). The conductance of the buffers used for stepwise elution was adjusted by raising the phosphate concentration without changing the pH.

C'_9

C'_9 was prepared in highly purified form according to a method to be described (Hadding and Müller-Eberhard, 1967). The procedure consists of three preparative steps, including chromatography on TEAE-cellulose, Sephadex G-200 and preparative electrophoresis. The final product is entirely devoid of other complement component activities.

Fraction containing C'_6, C'_7 and C'_8

For some experiments, a crude column fraction was used which furnished C'_6, C'_7 and C'_8 but no other components. This material was derived from the above described TEAE-chromatography pool prior to its application to hydroxyl apatite.

Fraction containing C'_7 and C'_8

This fraction was collected from eluates of the above mentioned TEAE-cellulose chromatogram of euglobulin. Eluates within a pH range of 8·1–7·0 (corresponding to a specific conductance of 4·2–7 m-mho/cm) were pooled.

Preparation of $EAC'_{1a,4,2a}$ and $EAC'_{1a,4,2a,3}$

Intermediate complexes consisting of sheep erythrocytes, rabbit anti-sheep erythrocyte antibody, the first, the fourth and the second component of complement ($EAC'_{1a,4,2a}$) were prepared either by treating sensitized sheep erythrocytes with whole human serum in the presence of phloridzin (Nilsson and Müller-Eberhard, 1965), or by exposing sensitized sheep erythrocytes to partially purified $C'_{1q,r,s}$ (Lepow, Naff, Todd, Pensky and Hinz, 1963), purified C'_4 (Müller-Eberhard and Biro, 1963) and partially purified $^{oxy}C'_2$ (Polley and Müller-Eberhard, 1966) according to a previously described method (Müller-Eberhard, Dalmasso and Calcott, 1966). $EAC'_{1a,4,2a,3}$ were prepared by exposing $EAC'_{1a,4,2a}$ or $EAC'_{1a,4,}{}^{oxy}{}_{2a}$ to purified C'_3 for 5–7 minutes at 32° using 25 μg C'_3 per 1×10^8 cells.

Haemolytic assays of C'_5, C'_6, C'_7 and C'_8

1×10^8 $EAC'_{1a,4,2a,3}$ were exposed to mixtures of C'_5, C'_6 and C'_7 in 0·45 ml at 37° for 20 minutes, after which time 0·05 ml fresh, undiluted human serum or functionally pure C'_8 and C'_9 were added, and the cells were then held for 30 minutes at 37°. 0·01 M EDTA was present throughout the reaction. Assays for each one of the three components were performed in reaction mixtures which supplied only the other two components. Usually, 5 μg of C'_5 and a moderate excess of C'_6 and C'_7 were used. C'_8 was assayed employing 1×10^8 $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells, 1·5 μg C'_5 and a moderate excess of C'_6, C'_7 and C'_9, in a total volume of 1 ml containing 0·01 M EDTA.

Alternatively, C'_5 was quantified using KCNS-treated human serum (Dalmasso and Müller-Eberhard, 1966) and C'_6 using C'_6 deficient rabbit serum (Rother, Rother, Müller-Eberhard and Nilsson, 1966).

Kinetic analysis of the formation of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$

Four flasks, A, B, C and D, were placed in an ice bath and were charged with $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells. Flask A received C'_5, flask B, C'_6 and flask C, C'_7. Flask D served as control. All four reaction mixtures were made up to the same volume. After 12 minutes incubation at 18·5°, C'_6 plus C'_7 were added to A, C'_5 plus C'_7 to B, C'_5 plus C'_6 to C and C'_5, C'_6 plus C'_7 to D. One millilitre of the final reaction mixtures contained 5×10^7 cells, 0·75 μg C'_5, C'_6 and C'_7 in amounts to obtain 70 per cent yield of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ in 15 minutes. At timed intervals, samples containing $2·5 \times 10^7$ cells were withdrawn and pipetted into 5 ml ice cold veronal buffer. All samples were centrifuged in the cold and the supernatants were discarded. To each cell button was added 0·5 ml veronal buffer containing C'_8 and C'_9 in 0·01 M EDTA.

In another experiment flask A received C'_5 plus C'_6, B, C'_5 plus C'_7, C, C'_6 plus C'_7 and D, C'_5 only. After 12 minutes at 18·5°, C'_7, C'_6, C'_5 and C'_6 plus C'_7 were added to flasks A, B, C and D, respectively.

Dose–response measurements for C'_5, C'_6 and C'_7

$2·5 \times 10^7$ $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells were incubated with C'_5, C'_6 and C'_7 at 37° for 30 minutes. Following washing, they were exposed for 30 minutes at 32° to C'_8 and C'_9. In

the first set of experiments the three components were utilized in constant concentration ratios. The highest concentration caused conversion of 60–95 per cent of the cells present. The amount of C'_5 in the undiluted mixture of C'_5, C'_6 and C'_7 was 3·75 μg/2·5 × 10^7 cells in one experiment and 18·75 μg in another. In the second set of experiments the concentration of C'_5 was kept constant (1·5 μg/2·5 × 10^7 cells) and that of C'_6 and C'_7 was varied.

Quantification of specific C'_5 uptake during immune haemolysis

The reaction mixtures consisted of 1×10^9 EA, 1:40 human serum [previously depleted of C'_3, C'_4 and C'_5 by treatment with 1 M KCNS (Dalmasso and Müller-Eberhard, 1966)], 100 μg C'_3, 10 μg C'_4 and varying amounts of ^{125}I-labelled C'_5 in 1 per cent human serum albumin solution (Behringwerke, reinst). C'_5 was labelled according to a method described by McConahey and Dixon (1966). The specific radioactivity of C'_5-^{125}I was $1{\cdot}3 \times 10^5$ counts/min/μg and its haemolytic activity was unimpaired. To diminish non-specific uptake of C'_5-^{125}I, 70 μg of KCNS-treated, haemolytically inactive C'_5 was added to each reaction mixture. The total reaction volume was 4 ml and the reaction time at 37° was 30 minutes. Controls contained 0·01 M EDTA instead of optimal amounts of Ca^{++} and Mg^{++}. At the end of the incubation period, the tubes were transferred to an ice bath and 6 ml cold veronal buffer was added. They were centrifuged at 26,400 ***g*** and 1° for 20 minutes in a Spinco L-2 ultracentrifuge using a No. 30 rotor. The degree of haemolysis was determined on the supernatants and the specific C'_5 uptake on the three times washed sediments by subtracting the counts of the control cells (EDTA sample) from those of the partially lysed cell population (sample containing Ca^{++} plus Mg^{++}).

Estimation of loss of activity of C'_5, C'_6 and C'_7 after exposure to $EAC'_{1a,4,}{}^{oxy}{}_{2a}$ or $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$

Amounts of C'_5 (0·125 μg), C'_6 and C'_7 were selected, which in 30 minutes at 37° convert 70 per cent of $2{\cdot}5 \times 10^7$ $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ to $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$. These amounts were incubated with $7{\cdot}5 \times 10^7$ $EAC'_{1a,4,}{}^{oxy}{}_{2a}$ or $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells for 30 minutes at 37°. The cells were then removed by centrifugation and the supernatant tested for total $C'_{5,6,7}$ activity by renewed incubation with a fresh batch of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ containing $2{\cdot}5 \times 10^7$ cells. After 30 minutes at 37° the cells were washed and lysis was initiated by addition of C'_8 and C'_9. Depletion of C'_5, C'_6 or C'_7 activity was also estimated after exposure of only one or two of the components to $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$cells.

Sucrose density gradient ultracentrifugation

Sucrose density gradient ultracentrifugation was performed at 35,000 revs/min for 20 hours at 4° using a Spinco rotor SW39 and a Spinco L-2 ultracentrifuge.

RESULTS

DEFINITION OF C'_5, C'_6 AND C'_7 IN TERMS OF POSITION IN THE COMPLEMENT REACTION SEQUENCE

It was previously found that conversion of $EAC'_{1a,4,2a}$ to a thermostable intermediate complex required C'_3, C'_5 and C'_6 (Nilsson and Müller-Eberhard, 1965). Subsequently, it was recognized that partially purified C'_6, which was used in the earlier experiments, contained C'_6, C'_7 and C'_8 which could be separated from each other by hydroxyl apatite chromatography (Fig. 1). It became necessary, therefore, to define the position of the

newly recognized components in the entire complement reaction sequence and to re-examine the component requirements for the formation of the thermostable intermediate form into which $EAC'_{1a,4,2a}$ can be converted. Since in exploratory experiments it was found impossible to form a reaction product unless C'_5, C'_6 and C'_7 were supplied

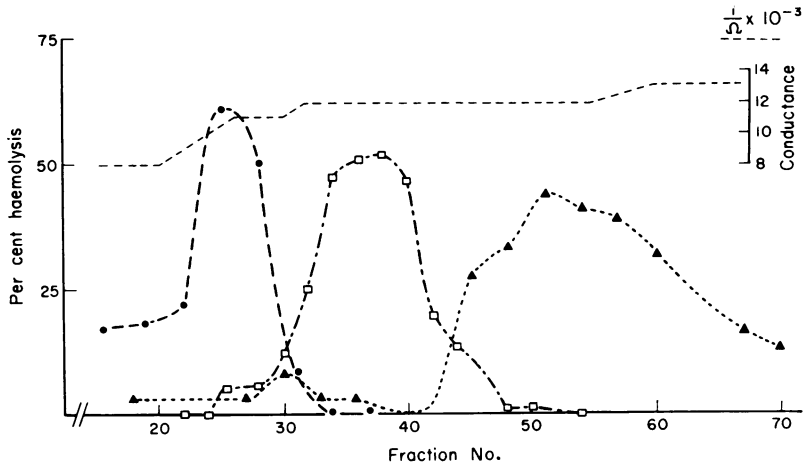

FIG. 1. Separation of C'_6 (□), C'_7 (●)and C'_8 (▲) by means of hydroxyl apatite chromatography. Starting material: a chromatographic fraction obtained by separation of euglobulin on TEAE-cellulose. The material contains C'_6, C'_7, C'_8 and γ-globulin and is devoid of C'_3, C'_5 and C'_9. Starting buffer: phosphate, pH 7·9; conductance, 8 m-mho/cm. Stepwise elution: phosphate, pH 7·9 and conductance of 11, 12 and 13 m-mho/cm, respectively.

TABLE 1

DEFINITION OF C'_5, C'_6 AND C'_7 IN TERMS OF POSITION IN THE COMPLEMENT REACTION SEQUENCE

Treatment of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ with:				
Step I		Step II		Haemolysis (%)
$C'_{5,6,7}$	C'_8	C'_8	C'_9	
+	−	−	−	0
+	−	+	−	0
+	−	−	+	3
+	−	+	+	42
+	+	−	−	0
+	+	−	+	46

Conditions: Number of cells, $2{\cdot}5 \times 10^7$; reaction volume, 0·5 ml; Step I, 8 minutes at 37°, followed by washing of cells; Step II, 1 hour at 37°; 0·01 M EDTA was present throughout the procedure.

together, these three components will preliminarily be treated as a group. Table 1 records the results of an experiment in which $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ were reacted with a mixture of C'_5, C'_6 and C'_7 in the presence or absence of C'_8. Following interaction with C'_5, C'_6 and C'_7 alone, a non-lytic intermediate product was formed, which for lysis required two additional factors, C'_8 and C'_9. This product will be referred to as $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$. Interaction with C'_5, C'_6, C'_7 and C'_8 also resulted in the formation of a non-lytic intermediate complex which, however, differed from $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ in that it

required for lysis only C'_9. This intermediate may be described as $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7,8}$. It was concluded that C'_5, C'_6 and C'_7, as a group, are preceded by C'_1, C'_4, C'_2 and C'_3 in the haemolytic reaction sequence and followed by C'_8 and C'_9.

CHARACTERIZATION OF A THERMOSTABLE INTERMEDIATE COMPLEX

The thermolabile complex $EAC'_{1a,4,2a}$ was incubated at 37° in the presence of C'_3 and of C'_5, C'_6 and C'_7. The latter three components were offered as a complete group of three, in pairs and individually. After certain intervals of time, samples were withdrawn and the cells were tested for residual reactivity with EDTA serum. As illustrated in Fig. 2,

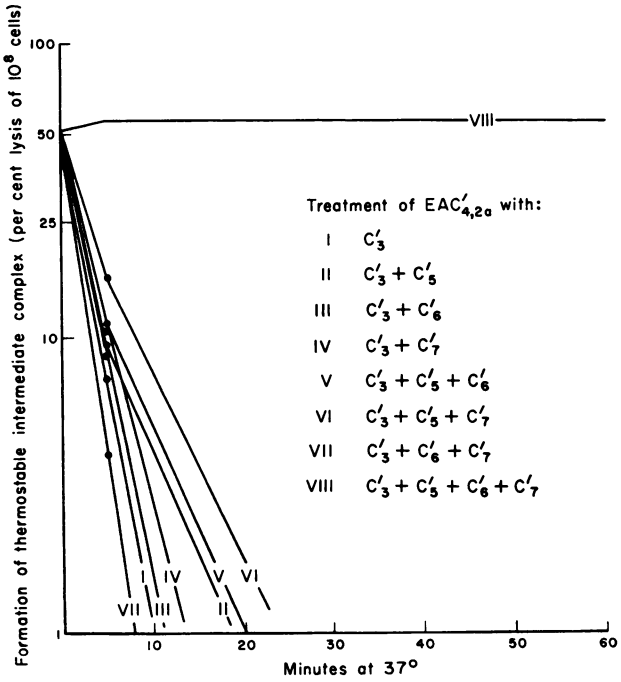

FIG. 2. Component requirement for the formation of a thermostable intermediate complex from $EAC'_{1a,4,2a}$. Eight reaction mixtures containing 10^9 cells were made up as indicated and held at 37°. Samples were withdrawn after 5, 10, 20 and 60 minutes and tested with EDTA-serum (1:20).

thermostable, haemolytically active cells were found only in mixtures containing C'_3, C'_5, C'_6 and C'_7. The thermostable intermediate complex may, therefore, be defined as $EAC'_{1a,4,2a,3,5,6,7}$. The subsequent intermediate complex, resulting from an interaction with C'_8, showed a similar thermostability.

The effect of pH on the formation of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ from $EAC'_{1a,4,}{}^{oxy}{}_{2a}$ was studied using isotonic phosphate buffers of pH 5·5–8·0. The results are depicted in Fig. 3 which shows a bell-shaped curve and a reaction optimum between pH 6 and 7. Fig. 4 shows the effect of temperature at pH 7·4. The reaction proceeds most rapidly at temperatures between 21° and 37°, and is virtually stopped at temperatures below 10°.

FATE OF C'_5, C'_6 AND C'_7 IN COMPLEMENT REACTION

Theoretically, a component may become inactivated in the process, attached to the cell surface, or it may remain unaffected. In the case of C'_2 (Mayer and Miller, 1965), C'_3

(Müller-Eberhard *et al.*, 1966) and C'_4 (Müller-Eberhard and Biro, 1963), it has been demonstrated that these components fulfill their function in immune haemolysis by

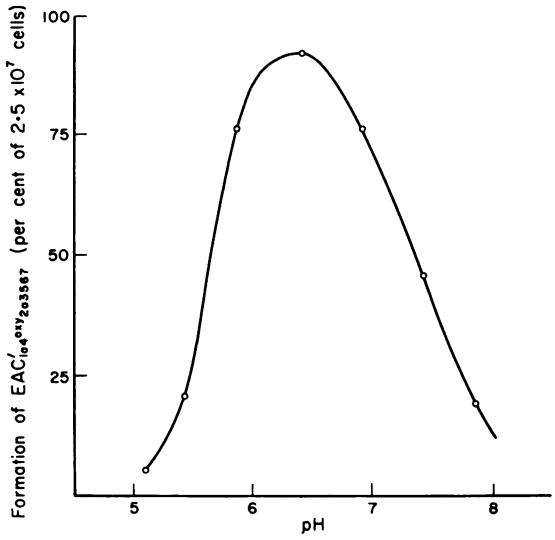

FIG. 3. Formation of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ cells as a function of pH. Diluent: isotonic phosphate buffered saline. Reaction time: 5 minutes at 37°.

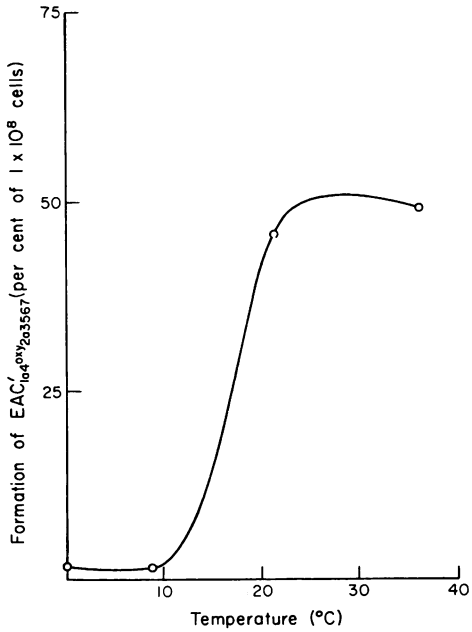

FIG. 4. Formation of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ as a function of temperature. Diluent: veronal buffered saline, pH 7·4. Reaction time: 5 minutes.

becoming physically attached to the erythrocyte surface. Previous attempts to demonstrate uptake of C'_5 by $EAC'_{1a,4,2a,3}$ cells and by immune precipitates in the presence of other complement components had yielded negative results (Nilsson and Müller-Eberhard,

1965). Lately, it was possible to obtain labelled C'_5 with a specific radioactivity that was 100 times that of the previously used batches. Since the haemolytic activity was unimpaired by introduction of the much more potent label, the question of physical uptake of C'_5 during haemolysis was re-investigated.

Human serum treated with 1 M KCNS (Dalmasso and Müller-Eberhard, 1966) was used as a source of all complement components, except for C'_3, C'_4 and C'_5. C'_3 and C'_4 were supplied by addition of purified components. $[^{125}I]C'_5$ was added in increasing amounts. After incubation with sensitized sheep erythrocytes, the degree of haemolysis was correlated with the degree of specific C'_5 uptake. The results are depicted in Fig. 5.

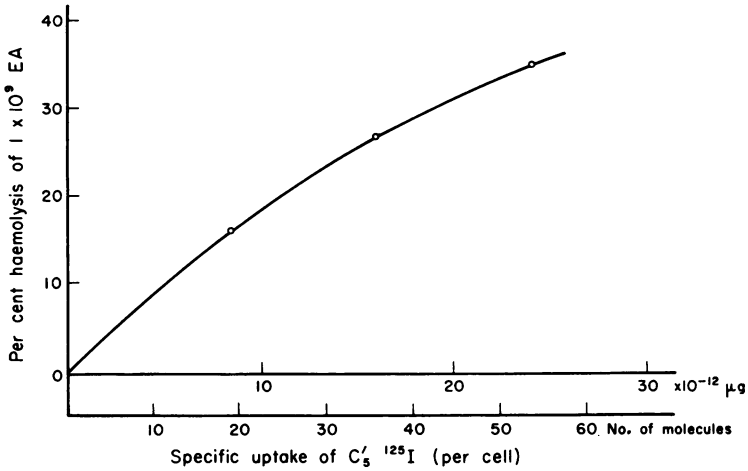

FIG. 5. Haemolysis as a function of cell bound C'_5. Increasing amounts (1·88, 3·75 and 7·5 μg) of haemolytically active ^{125}I labelled C'_5 were added to reaction mixtures containing 10^9 EA, KCNS-treated human complement (1:40), 100 μg C'_3 and 10 μg C'_4. Diluent, veronal buffered saline containing Ca^{++} and Mg^{++}; volume, 4 ml; time at 37°, 30 minutes. Controls: as above but containing 0·01 M EDTA. Specific radioactivity of C'_5-^{125}I: $1{\cdot}3 \times 10^5$ counts/min/μg.

Haemolysis increased with the amount of C'_5-^{125}I input and with the amount of cell bound C'_5-^{125}I. The specific uptake of C'_5-^{125}I did not exceed 0·5 per cent of the input. Thus, to achieve 45 per cent lysis of 10^9 cells, 7·5 μg of C'_5 were required, of which only 0·035 μg were specifically bound to the cells. The present data do not allow any conclusion as to whether the bound radioactive material represents intact C'_5 molecules or only fragments thereof. Uptake expressed in number of molecules, therefore, represents a minimal estimate. Density gradient ultracentrifugation of the supernatant after termination of the reaction failed to reveal slowly sedimenting, radioactively labelled split products.

In view of the low degree of C'_5 uptake, residual activity in the supernants was determined following interaction of C'_5, C'_6 and C'_7 with $EAC'_{1a,4,}{}^{oxy}{}_{2a}$ or $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$. Total $C'_{5,6,7}$-activity was found to be reduced to 6 per cent of input after interaction with $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$. By contrast, exposure to $EAC'_{1a,4,}{}^{oxy}{}_{2a}$ left 93 per cent of the activity in the supernatant. Table 2 shows the results obtained when C'_5, C'_6 and C'_7 were allowed to react with $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ either together, or in pairs or individually. When single components were exposed to the cells, a significant loss of activity was noticed only with C'_5, but not with C'_6 or C'_7. C'_6 was found to be inactivated only in the presence of C'_5; and C'_7 only in the presence of C'_5 and C'_6. The sequence of inactivation during interaction with $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ thus appears to be: C'_5, C'_6, C'_7.

REACTION SEQUENCE OF C'_5, C'_6 AND C'_7

To determine the sequence of action of C'_5, C'_6 and C'_7 kinetic analyses were performed. Three batches of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ were exposed to either C'_5, C'_6 or C'_7 at 18·5°. After 12 minutes incubation, each reaction mixture was supplied with the pair of components that was lacking during the initial period of incubation. Samples were then taken and $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ formation was measured as a function of time at 18·5°. A fourth batch of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ was treated similarly, except that all three components were supplied in the second period of incubation, while none were present initially. As illustrated

TABLE 2

C'_5, C'_6 AND C'_7 ACTIVITY IN THE FLUID PHASE AFTER INCUBATION OF THESE COMPONENTS WITH $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ CELLS

Components present during incubation			Loss of activity (% input)
C'_5			30
	C'_6		7
		C'_7	8
C'_5	C'_6		67
C'_5		C'_7	30
	C'_6	C'_7	0
C'_5	C'_6	C'_7	95

Conditions: (1) Components indicated were treated with $7{\cdot}5 \times 10^7$ $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells. (2) The supernatants were incubated with $2{\cdot}5 \times 10^9$ fresh $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells after addition of components omitted in initial incubation period. (3) Cells were washed and set up with C'_8 and C'_9. Temperature, 37°; time of each incubation period, 30 minutes.

in Fig. 6, the most rapid onset of the reaction occurred when the cells were first offered C'_5 and then C'_6 and C'_7. All of the other combinations resulted in a delayed onset of the reaction. This finding is in agreement with earlier observations (Nilsson and Müller-Eberhard, 1965) and indicates that C'_5 follows C'_3 in the haemolytic reaction sequence.

A second kinetic experiment was designed to determine the reaction sequence of C'_6 and C'_7. The conditions were similar to those of the previous experiment, with the exception that in the initial incubation period pairs of the three components were allowed to interact with $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$. After 12 minutes at 18·5° the lacking third factor was added and formation of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3,5,6,7}$ was followed as a function of time. The control in this experiment consisted of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ first exposed to C'_5 and then to C'_6 and C'_7. The results are depicted in Fig. 7 and show that addition of C'_7 following pre-incubation with C'_5 and C'_6 resulted in immediate onset of the reaction without any measurable lag phase. All other combinations caused a lag in the onset of the reaction which was most pronounced when the cells were first treated with C'_6 and C'_7, and then with C'_5. It was, therefore, concluded that the sequence of action is: C'_5, C'_6, C'_7.

In view of the outcome of the kinetic analysis, it seemed probable that the intermediate products $EAC'_{1a,4,2a,3,5}$ and $EAC'_{1a,4,2a,3,5,6}$ occur during the formation of the thermostable complex. Earlier attempts to isolate $EAC'_{1a,4,2a,3,5}$ had failed (Nilsson and Müller-Eberhard, 1965). As C'_5 is now available in chemically pure form, and C'_6 and C'_7 in

functionally pure form, attempts to demonstrate the postulated intermediate complexes were continued. In a number of experiments, some of which are recorded in Table 3, it

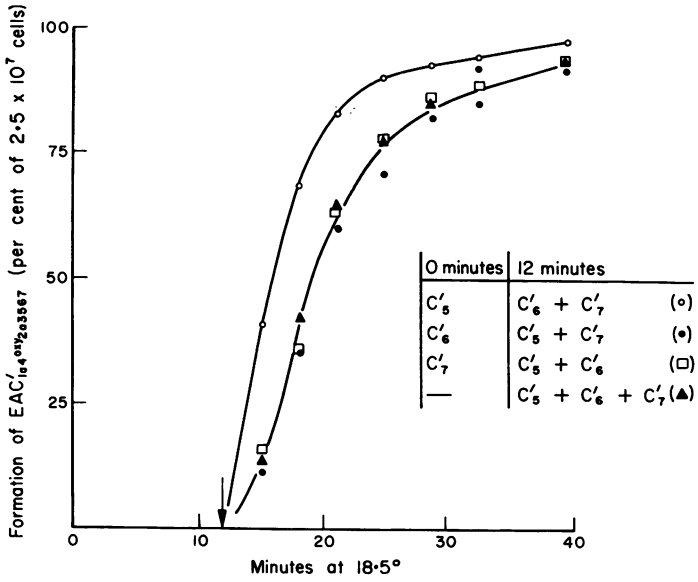

FIG. 6. Kinetic analysis of the reaction sequence of C'_5, C'_6 and C'_7: determination of the first reacting component. Three batches of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells were sequentially reacted with C'_5, C'_6 and C'_7 as indicated. Following a 12-minute reaction period with one component alone, the other two components were added (arrow). A fourth batch of cells was incubated first in the absence of any of the components and at 12 minutes it was supplied with all three components simultaneously (control).

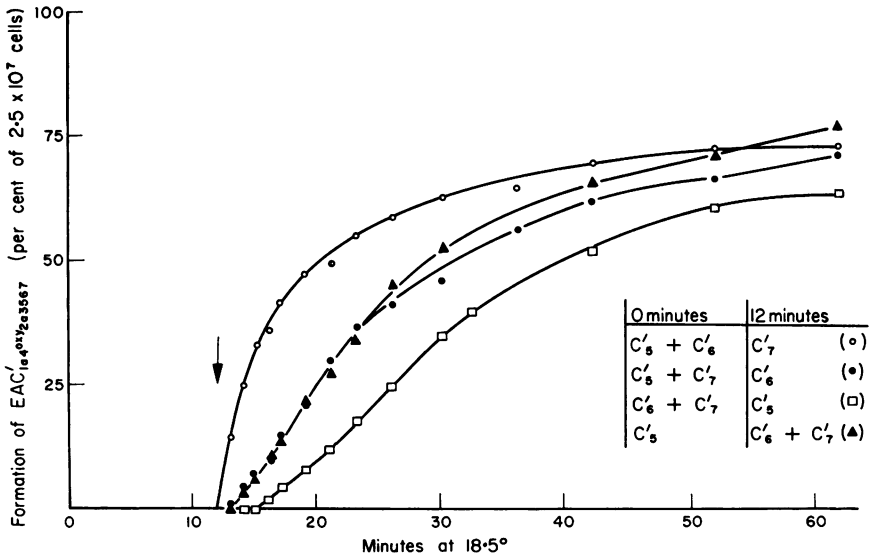

FIG. 7. Kinetic analysis of the reaction sequence of C'_6 and C'_7. Four batches of $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells were sequentially reacted with C'_5, C'_6 and C'_7 as indicated.

was found that at normal ionic strength formation of $EAC'_{1a,4,2a,3,5}$ was either minimal or did not occur at all. Demonstration of $EAC'_{1a,4,2a,3,5,6}$ was found equally difficult. By contrast, when C'_5, C'_6 and C'_7 were added simultaneously to $EAC'_{1a,4,2a,3}$, the product

$EAC'_{1a,4,2a,3,5,6,7}$ was recovered in relatively high yield. At half normal ionic strength $EAC'_{1a,4,2a,3,5}$ or $EAC'_{1a,4,2a,3,5,6}$ were more readily formed, but in much lower yield than $EAC'_{1a,4,2a,3,5,6,7}$ prepared in the presence of all three components. Moreover, $EAC'_{1a,4,2a,3,5}$ formed at half physiological ionic strength reverted to $EAC'_{1a,4,2a,3}$ when exposed at 0° to veronal buffer of physiological ionic strength.

These findings may be interpreted in two ways: (a) the intermediate products $EAC'_{1a,4,2a,3,5}$ and $EAC'_{1a,4,2a,3,5,6}$ do occur, but are of such labile nature that they are inactivated in the process of washing the cells, and (b) they do not occur, and the

TABLE 3

EFFICIENCY OF FORMATION OF INTERMEDIATE PRODUCTS IN THE REACTION BETWEEN $EAC'_{1a,4,2a,3}$ AND $C'_{5,6,7}$

Step I			Step II			Formation of $EAC'_{1a,4,2a,3,5,6,7}$ (%)					
C'_5	C'_6	C'_7	C'_5	C'_6	C'_7	Expt 1	Expt 2	Expt 3	Expt 4a	Expt 4b	Expt 5
+	−	−	−	+	+	0	10	19	3	31	26
−	+	−	+	−	+	−	−	−	−	−	12
−	−	+	+	+	−	−	−	−	−	−	6
+	+	−	−	−	+	0	0	15	2	8	12
+	−	+	−	+	−	−	−	−	−	−	12
−	+	+	+	−	−	−	−	−	−	−	0
+	+	+	−	−	−	33	63	57	49	66	83
−	−	−	+	+	+	−	47	33	Lost	69	90

Experiment No. 1:
1×10^8 $EAC'_{1a,4,2a,3}$; veronal buffered saline, $\Gamma/2 = 0{\cdot}15$.

Experiment Nos. 2 *and* 3:
As in No. 1; isotonic mixture of glucose and veronal buffered saline of $\Gamma/2 = 0{\cdot}075$.

Experiment No. 4:
1×10^8 $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$; No. 4a, $\Gamma/2 = 0{\cdot}15$; No. 4b, isotonic mixture of glucose and veronal buffered saline, $\Gamma/2 = 0{\cdot}075$.

Experiment No. 5:
$2{\cdot}5 \times 10^8$ $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$; veronal buffered saline, $\Gamma/2 = 0{\cdot}15$.

Conditions:
$EAC'_{1a,4,2a,3}$ cells were exposed in two consecutive steps to C'_5, C'_6 and C'_7 as indicated. Following each step (5 minutes, 30°) the cells were washed and then incubated with C'_8 and C'_9 for 30 minutes at 37°. Amount of C'_5, 5–10 μg; C'_6, C'_7, moderate excess. Cells treated with $C'_{5,6,7}$ simultaneously served as positive controls.

sequence of action as demonstrated by kinetic analysis is a reflection of a sequential 'activation' of C'_5, C'_6 and C'_7 in the fluid phase. According to the latter hypothesis, C'_5, C'_6 and C'_7 act on the cell as one functional unit, in which case an interaction between these components in cell free solution should occur, possibly in physico-chemically demonstrable form. Evidence for the occurrence of a protein–protein type interaction between the three components will be presented next.

INTERACTION OF C'_5, C'_6 AND C'_7 IN CELL FREE SOLUTION

In a number of experiments the three components were subjected to density gradient ultracentrifugation individually or in mixtures of two or three. The following observations were made: (a) In absence of C'_5, purified C'_6 and purified C'_7 sedimented with an *s*-rate of 5–6S. (b) Both C'_6 and C'_7 retained this sedimentation rate when examined together in the same gradient. (c) C'_6 in mixtures with C'_5 or with C'_5 and C'_7 sedimented

markedly faster than in the isolated state. (d) C'_7 in mixtures with C'_5 or with C'_5 and C'_6 also sedimented considerably faster than alone. When C'_5, C'_6 and C'_7 were centrifuged

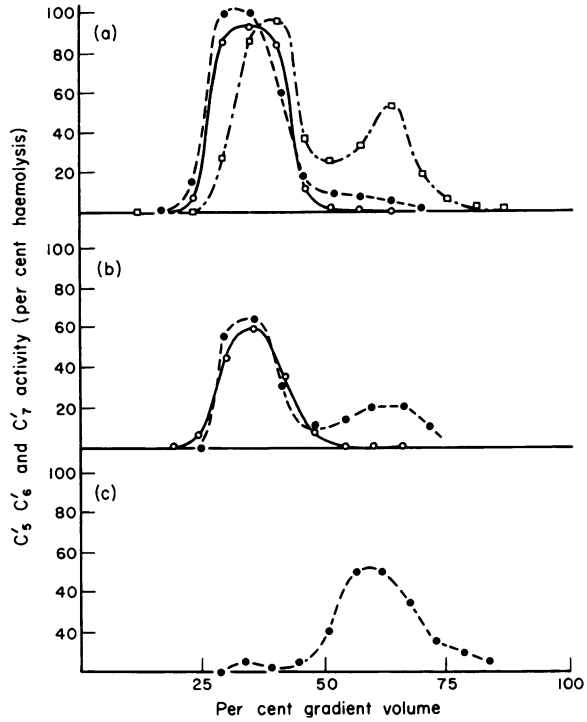

FIG. 8. Influence of C'_5 (○) on the sedimentation behaviour of C'_6 (□) and C'_7 (●) upon zone ultracentrifugation. (c). Functionally pure C'_7. (b) The same preparation of C'_7 in a mixture with 100 μg of purified C'_5. (a) Mixture of functionally pure C'_6 and C'_7 with 100 μg purified C'_5. Ultracentrifugation was carried out at 35,000 revs/min for 20 hours in a 5–25 per cent sucrose density gradient in veronal buffered saline, pH 7·5, $\Gamma/2 = 0{\cdot}15$. The direction of sedimentation was to the left.

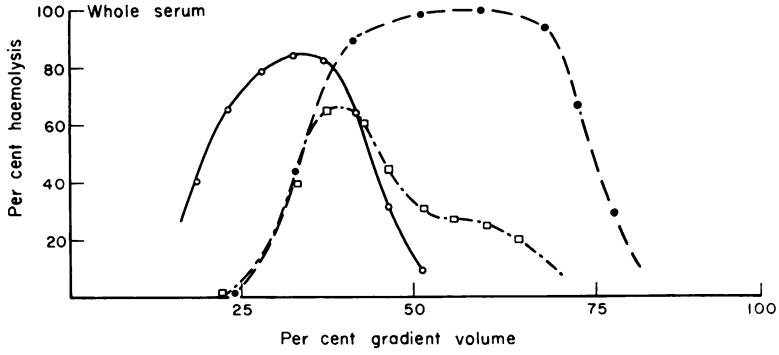

FIG. 9. Sedimentation behaviour of C'_5 (○), C'_6 (□) and C'_7 (●) in whole human serum upon zone ultracentrifugation. Conditions as described in Fig. 8.

together, both C'_6 and C'_7 sedimented with C'_5. In the 'complex', C'_7 activity was consistently of somewhat higher sedimentation rate than C'_6 activity. Representative examples of these experiments are depicted in Fig. 8. It was concluded that C'_6 and C'_7

form a reversible complex with C'_5 in the fluid phase. Evidence for the occurrence of the C'_5–C'_6–C'_7 complex in fresh human serum is shown in Fig. 9. C'_5 is present in the lower half of the gradient, and C'_6 and C'_7 are distributed between the peak of C'_5 (9S) and the 5–6S region, indicating a bimodal distribution and separation into free and 'bound' C'_6 and C'_7.

The different relative distribution of C'_5, C'_6 and C'_7 activity in ultracentrifugally separated serum as compared to recombined purified components could be related to the amounts of C'_5 present. In the case of serum, a maximal amount of approximately 8 μg

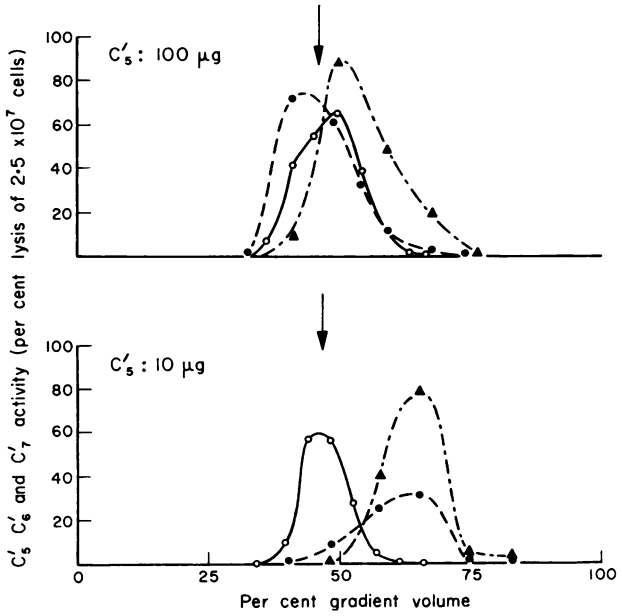

FIG. 10. Influence of the concentration of C'_5 (○) on the sedimentation behaviour of C'_6 (▲) and C'_7 (●) upon zone ultracentrifugation. Experimental conditions were similar to those described in Fig. 8.

C'_5 was present, whereas the recombined purified components contained 100 μg C'_5. Reduction of C'_5 to 10 μg in the mixture with purified C'_6 and C'_7 was followed by a markedly weaker interaction, permitting C'_6 and C'_7 to sediment at rates only slightly greater than those of the free components (Fig. 10).

ANALYSIS OF MODE OF ACTION BY DOSE–RESPONSE DETERMINATIONS

To analyse further the mode of action of C'_5, C'_6 and C'_7, dose–response determinations were carried out. This was done to obtain additional information as to whether the C'_5, C'_6, C'_7 reaction constitutes a one-step or multiple-step process. A dose–response curve obtained with C'_5, C'_6 and C'_7 present in constant relative proportions was of sigmoidal shape (Fig. 11), and thus indicative of a multiple-step reaction. In view of the fact that the interaction of the three components is strongly dependent on the concentration of C'_5, an alternative interpretation offered itself. If the reaction were dependent on the fluid phase interaction of the three components, then it should slow down and stop in a concentration range in which the complex was largely dissociated. To test this hypothesis, dose–response measurements were performed with C'_6 and C'_7 in the presence of constant amounts of

C'_5. The resulting curve was concave towards the abscissa and a completely linear relation was found between $C'_{6,7}$-concentration and the average number of hits per cell (Fig. 12).

This result prompted a repeat of the first dose–response experiment under slightly

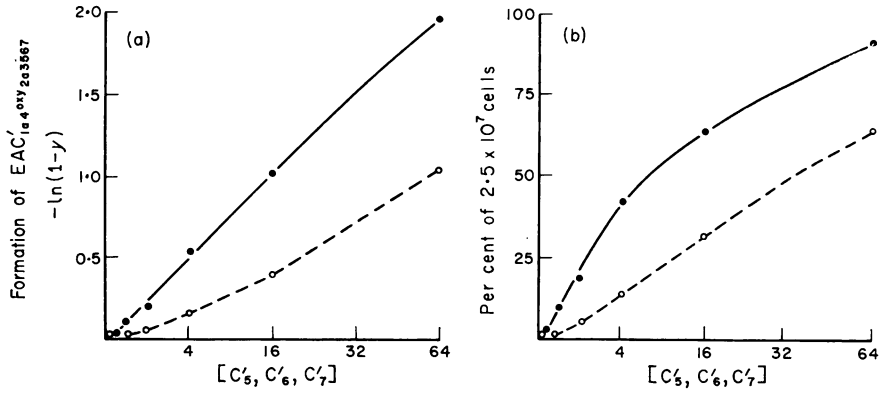

FIG. 11. Dose–response of C'_5, C'_6 and C'_7 at constant ratios. $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ cells in 0·5 ml were reacted for 30 minutes at 37° with mixtures of C'_5, C'_6 and C'_7, diluted in two-fold steps. Two experiments were performed simultaneously, with identical amounts of C'_6 and C'_7, but with a five-fold difference in C'_5 concentration. The largest dose of C'_5 utilized was 3·75 μg/tube in one experiment (○) and 18·75 μg in the other (●). Haemolysis was expressed as per cent lysis (b) and as average number of hits per cell (a).

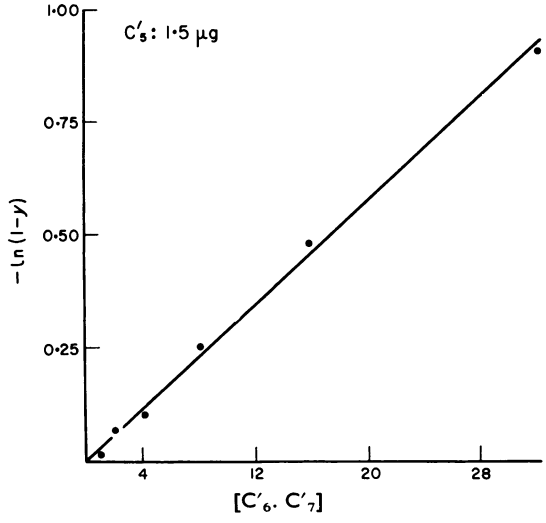

FIG. 12. Dose–response of C'_6 and C'_7 at constant ratios in the presence of a constant amount of C'_5. $2{\cdot}5 \times 10^7$ $EAC'_{1a,4,}{}^{oxy}{}_{2a,3}$ in 0·5 ml were exposed to 1·5 μg C'_5 and two-fold dilutions of a mixture of C'_6 and C'_7. After 30 minutes incubation at 37°, the cells were washed and subsequently exposed to C'_8 and C'_9 for 30 minutes at 37°. Haemolysis was expressed as average number of hits per cell.

modified conditions. The relative C'_5 concentration in the mixture of C'_5, C'_6 and C'_7 was increased five-fold (from 3·75 to 18·75 μg/0·5 ml). All other conditions were identical with those employed in the initial experiment. As seen in Fig. 11, conditions favouring $C'_{5,6,7}$ association led to a modified dose–response curve, approaching a concave

shape and proportionality between $C'_{5,6,7}$ concentration and the average number of hits per cell.

DISCUSSION

The work presented deals with the analysis of a well defined portion of the immune haemolytic reaction, namely the conversion of the thermolabile intermediate product, $EAC'_{1a,4,2a}$, to a thermostable complex. While the phenomenon is simple, the underlying mechanism of this reaction step is rather complex. In addition to C'_3, three further complement components were found to be required, C'_5, C'_6 and C'_7. In fact, the existence of the latter three components was discovered through their requirement in this conversion, C'_5 and C'_6 previously (Nilsson and Müller-Eberhard, 1965) and C'_7 in the course of the present study.* C'_5 has been isolated from serum by a method described earlier (Nilsson and Müller-Eberhard, 1965) and, like C'_3, it was available for these studies as chemically pure protein. C'_6 and C'_7 were purified from serum approximately 1000- to 2000-fold chiefly by the use of hydroxyl apatite chromatography. Although the degree of their purity is lower than that of C'_3 and C'_5 and although they have not been strictly identified with discrete proteins, both components were obtained free of other complement component activities.

As four components were found to be required for the reaction under investigation, it was initially assumed that they act sequentially. That C'_3 is the first acting of the four components was readily determined, since it converts $EAC'_{1a,4,2a}$ to the next intermediate product, $EAC'_{1a,4,2a,3}$ (Müller-Eberhard *et al.*, 1966). A series of kinetic experiments indicated a definite order of action for the other three which is reflected in their numerical symbols: $C'_{5,6,7}$. The hypothesis of sequential action prompted a number of experiments, the outcome of which cast doubt on its correctness. Severe difficulties were encountered in trying to isolate or to prepare an intermediate product resulting from the action of C'_5 without involving C'_6 and C'_7. Only in unphysiological conditions (low ionic strength) could the product $EAC'_{1a,4,2a,3,5}$ be obtained in fair yield. Upon re-establishing physiological ionic strength, however, this complex reverted to the preceding state. Even more frustrating were attempts to make the complex $EAC'_{1a,4,2a,3,5,6}$, that is to say to cause C'_5 and C'_6 to react with $EAC'_{1a,4,2a,3}$ in the absence of C'_7. The possibility that technical factors interfered with the successful preparation of these intermediate products cannot be ruled out completely. At present, the impression prevails, however, that they cannot be prepared with any reasonable degree of efficiency. By contrast, the simultaneous presence of all three components, $C'_{5,6,7}$, in the reaction mixture leads to the formation of the thermostable product with considerable efficiency. These observations have given rise to an alternative hypothesis of the mode of action of C'_5, C'_6 and C'_7. It proposes, in essence, that C'_5, C'_6 and C'_7 act as a functional unit.

If C'_5, C'_6 and C'_7 represent a functional unit, they should constitute an interacting multiple component system. Evidence for physico-chemical interaction between them could, indeed, be obtained by zone ultracentrifugation. While C'_6 and C'_7 have an *s*-rate of 5–6S when examined in their purified form, both activities sediment at a faster rate if analysed in the presence of C'_5. Provided the concentration of C'_5 is relatively

* For guinea-pig complement Wellensiek and Klein (1965) demonstrated the requirement of three components (a, b and β) and Inoue and Nelson (1966) of four components (C'_{3c}, C'_{3b}, C'_{3e}, and C'_{3f}) for the formation of a similarly thermostable complex from $EAC'_{1a,4,2a}$.

large, both C'_6 and C'_7 sediment within the C'_5 zone at a rate of approximately 8–10S. The phenomenon is interpreted to indicate that C'_6 and C'_7 possess an affinity for C'_5, which, however, is not strong enough to result in a completely stable protein–protein complex. A stable complex would be expected to sediment ahead of the excess reactant (C'_5). If the equilibrium position is changed such that dissociation is favoured, C'_6 and C'_7 can be found to sediment at rates intermediate to that of C'_5 and of unbound C'_6 and C'_7. The transport experiments indicate that C'_6 and C'_7 enter into reversible complexes with C'_5, the proportion of bound and of free reactants being dependent on the concentration of the reactants and upon environmental conditions. It is emphasized that this type of interaction was not only observed with the purified components but also in whole human serum. An analogous type of interaction has recently been observed with the second and the fourth components of human complement (Müller-Eberhard, Polley and Calcott, 1967).

If C'_5, C'_6 and C'_7 function as a unit, their interdependence should be reflected in the shape of the dose–response curve. Initial determinations revealed a sigmoidal shape of the curve, which ordinarily is indicative of a multiple step reaction. In exceptional cases, however, a sigmoidal dose–response curve may arise from dissociation of the active principle into inactive subunits upon dilution. This has been demonstrated for tryptophane synthetase which consists of a reversible complex of three subunits, two A protein molecules and one B protein molecule (Creighton and Yanofsky, 1966). Dissociation of the enzyme at low concentration has also been cited as the explanation for sigmoidal dose–response curves in the case of β-glucuronidase (Bernfeld, Bernfeld, Nisselbaum and Fishman, 1954) and hyaluronidase (Bernfeld, Tuttle and Hubbard, 1961). In view of the demonstrated reversible complex formation between C'_5, C'_6 and C'_7, the dose–response determinations were repeated under conditions favouring association of the components even upon dilution. This was achieved by increasing the relative C'_5 concentration. This measure resulted in a marked change of the shape of the dose–response curve, which now appeared concave towards the abscissa. Upon transformation to a plot of average number of hits per cell versus relative $C'_{5,6,7}$-concentration (Mayer, 1961), a linear relation was obtained. These results are compatible with the 'functional unit' hypothesis.

In conclusion, this study on the reaction mechanism of C'_5, C'_6 and C'_7 has led to the formulation of two hypotheses, which may be referred to as the 'sequential action' and the 'functional unit' hypotheses. The former is essentially substantiated by the results of kinetic experiments which have revealed a sequential order of action but is put in question by the difficulty of demonstrating intermediate products. The second hypothesis is chiefly supported by the finding that C'_5, C'_6 and C'_7 are capable of interacting physico-chemically. The kinetic data and the sigmoidal shape of recorded dose–response curves would tend to refute the second hypothesis. However, the possibility exists that the kinetic data reveal sequence of activation of the components rather than sequence of action. Similarly, the sigmoidal dose–response curve need not necessarily indicate a multiple-step reaction, but rather progressive dissociation of the $C'_{5,6,7}$ complex with dilution. Further work will show which of the two working hypotheses is more compatible with experimental evidence.

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