*Immunology*, 1972, **23**, 267.

# Bypass-Activation of the Complement System Starting with C3

## I. GENERATION AND FUNCTION OF AN ENZYME FROM A FACTOR OF GUINEA-PIG SERUM AND COBRA VENOM

D. BITTER-SUERMANN, M. DIERICH, W. KÖNIG AND U. HADDING

*Institut für Medizinische Mikrobiologie, der Johannes-Gutenberg-Universität, 65 Mainz, Germany*

(*Received 14th September* 1971)

**Summary.** Antibody independent activation of the complement system starting with C3 can be achieved by means of a purified factor from cobra venom (VF), which interacts with a purified serum factor (SF). The latter is a normal constituent of guinea-pig and human serum (C3-proactivator).

The interaction between VF and SF is $Mg^{++}$ dependent and leads to the formation of a complex. Immunological analysis reveals that both VF- and SF-antigens are contained in the complex. The VF–SF complex activates enzymatically isolated C3, which in the presence of the subsequent components yields all effects of the normal complement sequence.

Purified C5 is not affected by the complex. Its activation is mediated by activated C3.

The VF–SF system represents a model for direct activation of C3 to C9 independent of antibody, C1, C4 and C2. An analogous pathway of alternate complement activation might be used by other substances, e.g. endotoxin, guinea-pig $\gamma$1-immune aggregates and zymosan. The corresponding serum factors are under investigation.

## INTRODUCTION

Sensitized erythrocytes (EA) can be lysed by complement. Specifically bound antibodies activate C1; this subsequently leads to the consecutive activation of C4, C2, C3, C5, C6, C7, C8 and C9. The final event is the lysis of the cell. This type of reaction sequence is referred to as immune cytolysis, and serves as reference point for the definition and nomenclature of the complement system (WHO, 1968).

In addition some alternate complement activating mechanisms have been described in the recent literature. Thompson and Rowe (1968), Thompson and Lachmann (1970), and Lachmann and Thompson (1970) were able to demonstrate a complement activation leading to lysis of unsensitized erythrocytes (E). In their system an active $\overline{C5,6}$–complex was generated by Zymosan; this complex induced lysis of E in the presence of C7 to C9. The whole phenomenon was called 'reactive lysis'. Pickering, Wolfson, Good and Gewurz (1969) and Ballow and Cochrane (1969) depicted another type of antibody independent activation of complement by using cobra venom. They found that red cells are lysed to a certain extent in serum if cobra venom is present. The investiga-

tions of Flexner and Noguchi (1903) and of Ritz (1912) have shown that a crude preparation of cobra venom inactivates complement; later it was demonstrated that this effect was due to a depletion of the components C3 and C5 (Klein and Wellensiek, 1965; Wellensiek and Klein, 1965). It was shown by Müller-Eberhard (1967) that a factor of the cobra venom (VF) was able to combine with a $\beta$-globulin of human serum, called C3-proinactivator. The resulting complex caused the depletion of C3. Neither VF nor the serum factor (SF) alone was able to deplete serum from C3.

The observations concerning the lysis of unsensitized cells gave a stimulus in this laboratory to take up again earlier work on the action of cobra toxin upon C3 and C5. This had been conceived as an inactivation process (Klein *et al.*, 1965). However, in the light of the lysis of E, it could represent an activation of C3 (Dierich, Bitter-Suermann, König and Hadding, 1971). With regard to this problem experimental work was focused on the following points:

1. Kinetics and ion requirements of the complex formation between purified VF and SF (guinea-pig).
2. Kinetics of the C3-turnover as mediated by the VF–SF complex.
3. The nature of the connecting events that occur between the C3-turnover on the one hand and the lysis of unsensitized cells on the other hand.

Evidence will be presented in this paper to show that the VF–SF complex acts upon C3 like an enzyme; this reaction yields a product which is identical with activated C3.

## MATERIALS AND METHODS

*Conductivity*

The conductivity expressed as milli-Siemens (mS) was measured by an instrument of Radiometer (Copenhagen). A 0·01 M NaCl-solution corresponds to 1 mS at 20°, 1 mS is equal to 1 mmho.

*Buffer for complement assay*

A veronal buffer was used as general diluent containing $Ca^{++}$ and $Mg^{++}$ and 0·1 per cent gelatine (VBSG) (Mayer, 1961).

*Purified complement components*

The components C3 and C5 have been purified according to Bitter-Suermann, Hadding, Melchert and Wellensiek (1970).

*C1-free serum*

C1 is removed from the guinea-pig serum in a two-step procedure: (a) according to Nelson (1965) C1 is precipitated from the serum. For that purpose serum is dialysed for 12 hours against 0·01 M phosphate buffer, pH 7·5. After dialysis the serum is centrifuged for 20 minutes at 5000 rev/min and 4°. The C1 is found as precipitate and discarded. (b) The supernatant is dialysed against a barbital-buffer, 1·01 g/litre (0·0042 M), pH 7·35, to which 0·065 M NaCl is added. Under these conditions C1 is found as a 19S-macromolecule; therefore it can be separated from the serum by ultracentrifugation (Colten, Borsos and Rapp, 1968). The centrifugation was performed in a preparative zonal rotor (Ti 14) at 39,000 rev/min for 18 hours in a 8–30 per cent sucrose-gradient in the mentioned barbital-buffer (ultracentrifuge: Spinco model $L_2$65B).

*Site-forming units (SFU)*

In the immune haemolysis test system complement components are measured on the basis of their haemolytic effectiveness. The amount of the respective components is expressed as site forming units (SFU) (Bitter-Suermann *et al.*, 1970).

*Test cells*

EAC142 and EAC1423 were prepared as described earlier (Bitter-Suermann *et al.*, 1970).

*Determination of single complement components*

All experiments were performed in the microlitre-system as described earlier (Ringelmann, Opferkuch, Röllinghoff and Loos, 1969). Each value is the average of a duplicate determination. The following controls were always included:

(1) 0·1 ml of the test cell suspension + 1·2 ml $H_2O$ for 100 per cent lysis.

(2) 0·1 ml of the test cell suspension + 1·2 ml VBSG as cell control.

(3) 0·1 ml of the cell suspension + 0·2 ml VBSG + 1 ml of the respective completing reagent, containing the final complement components as control for the test system.

After incubation the haemoglobin in the supernatant is measured at 412 nm with a Zeiss-spectralphotometer PM4-unit. (a) Test system for C1: as described by Borsos and Rapp (1963). (b) The test system for C3 and C5 have been described earlier (Bitter-Suermann *et al.*, 1970). For C3 it is in brief as follows: 0·1 ml VBSG + 0·1 ml of a $1{\cdot}3 \times 10^8$ EAC142-suspension + 0·1-ml sample + 1·0 ml of a reagent containing C5 to C9 (Klein and Wellensiek, 1965) in an appropriate dilution are mixed and incubated for 60 minutes at 37°.

*Test system for the cobra venom factor (VF)*

(a) Screening test: 0·2 ml of a 1:8 diluted guinea-pig serum and a 0·1-ml sample are incubated at 37° for 60 minutes. Thereafter 0·1 ml of the mixture is tested for C3.

(b) Test system for kinetic experiments: 0·1 ml VBSG containing SF + 0·1 ml VBSG containing C3 in a concentration to yield 80 per cent lysis of the test cells + 0·1-ml sample are incubated and treated as under (a).

*Test system for the serum factor (SF)*

0·1 ml VBSG containing C3 as above + 0·1 ml VF (15 μg purified VF/ml VBSG) + 0·1-ml sample are incubated at 37° for 60 minutes. Afterwards 0·1 ml of the mixture is tested for C3. For screening tests the VF-solution was prepared using lyophilized crude cobra venom (3 mg/ml VBSG).

*Purification of cobra venom factor (VF)*

Lyophilized cobra venom (Naja Naja) was purchased from Miami Serpentarium Laboratories (Miami, Florida 33156, U.S.A.). For the purification of VF we took advantage of the data provided by Slegers and Simon (1968). These authors showed that by chromatography on SE Sephadex-C25 the commercial preparation of crude cobra venom can be separated into more than ten different protein fractions using a 0·05 M sodium phosphate buffer, pH 6·0 and a NaCl gradient. (We found that in this separation procedure VF is eluted in the first peak with the starting buffer.) The VF-pool was dialysed overnight against 0·02 M Tris-HCl buffer, pH 7·5, which served also as starting buffer for the next

chromatographic step on DEAE cellulose (DE 52 Whatmann). The VF is eluted by a NaCl-gradient at about 18 mS. The VF-pool is dialysed against a potassium phosphate buffer of 0·02 M, pH 8·0. The next purification step consists of chromatography on CaOH-apatite (Bitter-Suermann *et al.*, 1970). VF is eluted by a phosphate-gradient at about 8 mS. The final product was found to give a single band when subjected to analytical polyacrylamide gel electrophoresis. The elution pattern of VF is shown in Fig. 1.

*Purification of the serum factor (SF)*

All steps of the purification procedure have been carried out in a K-$PO_4$-buffer 0·02 M, pH 7·5 plus an amount of NaCl to give a conductivity of 7 mS. The preparation was started with gel-filtration of 40 ml guinea-pig serum on a Sephadex G-200 column (5 × 100 cm). The SF was eluted within the ascending part of the 4S protein peak. This portion was transferred on to a DEAE-cellulose column. The SF was eluted by starting buffer. Afterwards the SF-pool was passed through a CaOH-apatite column. For the elution of SF a phosphate-gradient was applied; SF emerges from the column at about 10 mS. Finally a gel-filtration on Sephadex G-200 completed the purification procedure. The typical elution pattern with reference to the first three steps is shown in Fig. 2.

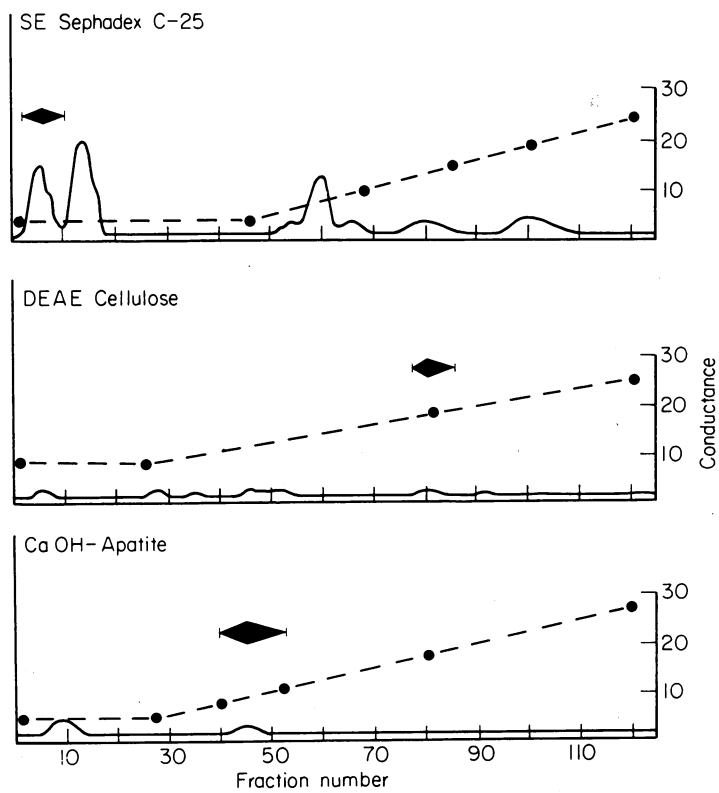


FIG. 1. Elution pattern of VF-preparation achieved by gel filtration and ion exchange-chromatography. The protein concentration was measured at 256 nm. It is represented by the continuous line without marks. ●, Conductance in mS. The black bars indicate the position of VF.

*Physicochemical characterization of VF, SF and of the VF–SF complex*

(a) Determination of molecular weight was performed as described by König, Bitter-Suermann, Dierich and Hadding (1971).

(b) Measuring of the isoelectric point was performed, using a LKB 8100 Ampholine Electrofocusing Equipment and carrier ampholytes of the pH range from 6–8.

## RESULTS

### MODE OF INTERACTION BETWEEN VF AND SF; ROLE OF $Mg^{++}$

It was shown that EDTA inhibited the effect of cobra venom upon C3 in human serum, suggesting that bivalent cations were essential (Müller-Eberhard, 1967). Bivalent ions were removed from a SF preparation by gel filtration on a Sephadex G-25 column equilibrated with Tris-HCl buffer, and from the VF preparation by dialysis against distilled water. Both preparations were mixed and tested for their activity upon C3. Under these circumstances no effect upon C3 could be observed. The activity against C3 was immediately restored when $Mg^{++}$ were added to the system (Fig. 3). However, no effect was observed when one of the following ions were added: $Ca^{++}$, $Mn^{++}$, $Fe^{++}$, $Co^{++}$, $Zn^{++}$, $Sn^{++}$.

Since the binding of $Mg^{++}$ by EDTA is a very rapid process, this ligand can be used to stop effectively the interaction of VF and SF. This principle provides a tool to study the kinetics of the complex formation from VF and SF. The basis of its use is the fact that, once formed, the VF–SF complex cannot be inhibited by EDTA with respect to its

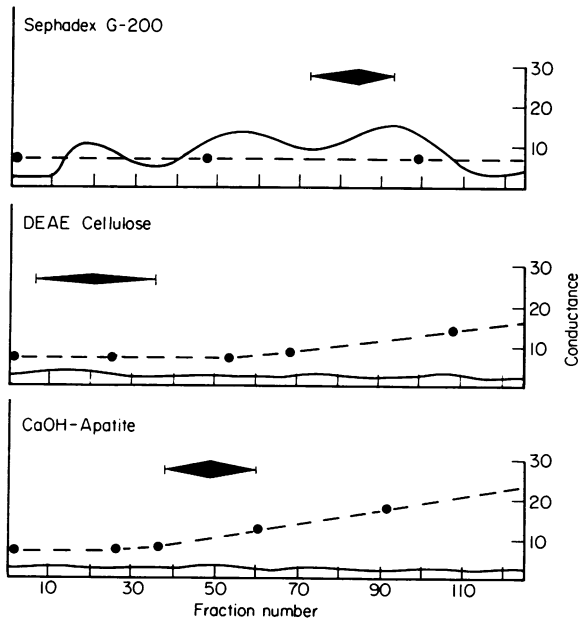


FIG. 2. Elution pattern of SF-preparation achieved by gel filtration and ion exchange-chromatography. The protein concentration was measured at 256 nm. It is represented by the continuous line without marks. ●, Conductance in mS. The black bars indicate the position of SF.

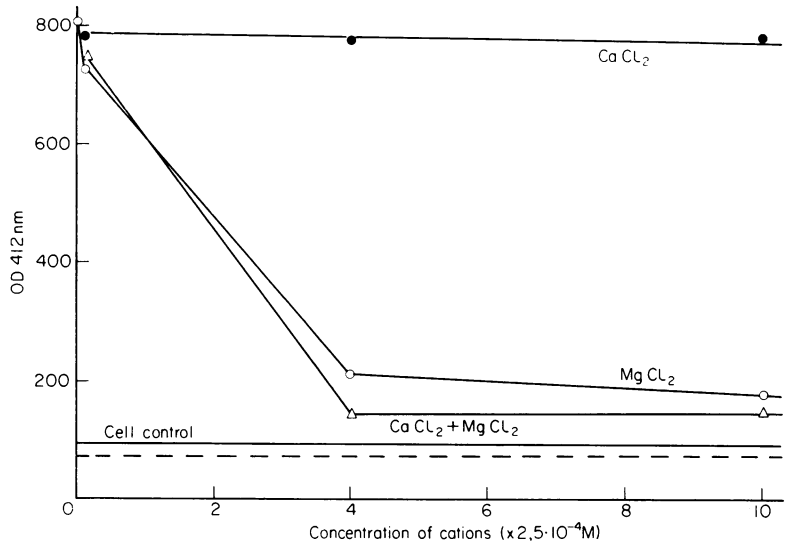


FIG. 3. Influence of $Mg^{++}$ and $Ca^{++}$ on the complex formation. The ordinate gives Hb-values, expressed as optical density at 412 nm, which are directly proportional to the amount of haemolytically active C3. The degree of haemolysis is inversely proportional to the activity of the complex. The origin of the curves at 800 OD represents the C3 amount in the unreactive system free of cations. Cell controls: continuous line: test cells and completing reagent; dotted line: test cells in VBSG.

activity against C3. In order to determine the kinetic data of the VF–SF complex formation the interaction of these two factors was stopped by adding EDTA at different time intervals during the incubation period. Afterwards the capacity of all samples to convert native C3 was tested. For this test an incubation time of 90 minutes at 37° was chosen to detect even small quantities of VF–SF. By this technique it could be demonstrated that the

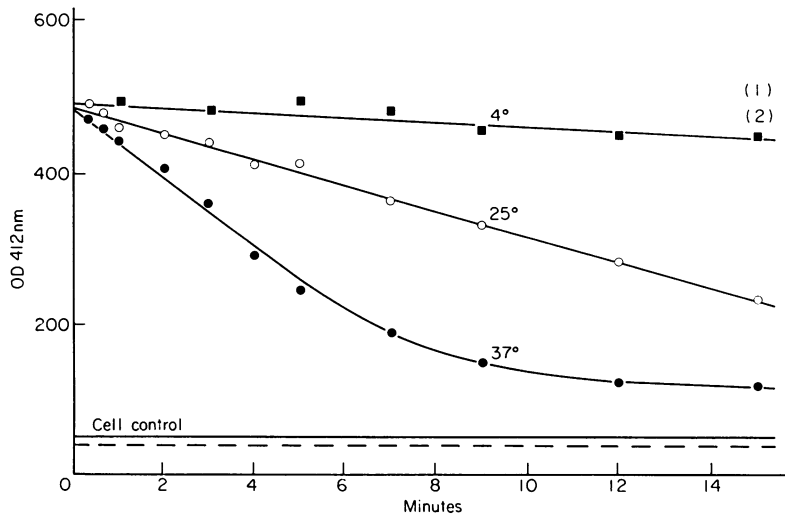


FIG. 4. Temperature dependence of the complex formation between VF and SF. Ordinate and cell controls as in Fig. 3. (1) Hb-value of 100 per cent lysis; (2) amount of C3 available.

formation of VF–SF complex begins without a detectable lag-phase as delineated in Fig. 4. The study of the complex formation at different temperatures revealed a clear temperature dependence of this process.

### CHARACTERIZATION OF VF, OF SF AND OF THE VF–SF COMPLEX

(a) *VF*: VF, purified as described under methods, represented 0·5 per cent w/w of the lyophilized whole venom. VF had a molecular weight of about 160000 and an isoelectric point at pH 6·8. VF could be dialysed against distilled water for at least 2 days and thereafter lyophilized without notable loss of activity. It was stable at 4° for at least 10 days.

(b) *SF*: A four step purification procedure resulted in a protein of high purity but considerable lability. Therefore purification was performed at 4°. This protein behaved as a $\beta$-globulin. The concentration of SF in normal guinea-pig serum is limited; with an excess of VF, guinea-pig serum could not be diluted more than 1:100 to yield a complex

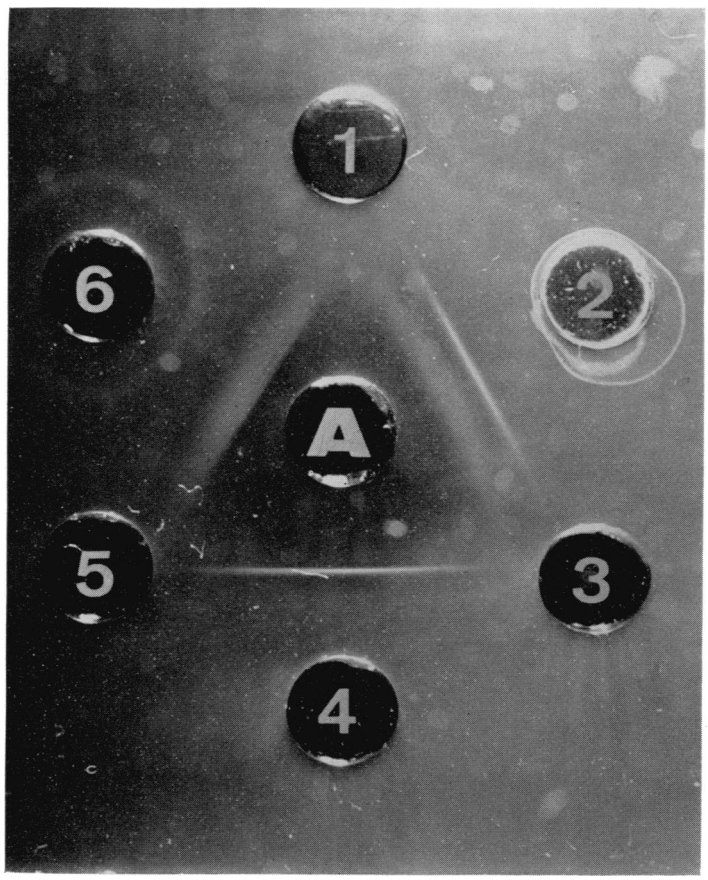


FIG. 5. Immunological analysis of VF (4), SF (6) and the VF–SF complex (2) in an Ouchterlony test. (A) Antiserum. The holes 1, 3, 5 are empty.

formation. SF had a molecular weight of 80000 and an isoelectric point at pH 6·1. It was heat labile; by exposing guinea-pig serum for 30 minutes at 45° the SF-activity was lost. Exposure to a 0·035 M solution of hydrazine for 30 minutes had no effect on its activity.

(c) *VF–SF complex*: Neither VF nor SF showed any activity against purified C3 when applied alone. On the other hand, the VF–SF complex, once formed was highly active. The complex had a molecular weight of about 230000 and an isoelectric point at pH 6·4. Its stability was notable; it could be dialysed against distilled water for several hours and lyophilized without loss of activity.

In additional experiments the complex was compared with the factors VF and SF with respect to their behaviour in purification procedures. It was found that in ion exchange chromatography the complex exhibits nearly the same traits as the factor VF alone. However by gel filtration it can be separated easily from VF and from SF. Two methods were used to obtain a purified VF–SF preparation.

The first method consisted in preparing a VF–SF complex by mixing purified preparations of VF and of SF in the presence of $Mg^{++}$. The resulting VF–SF complex was then

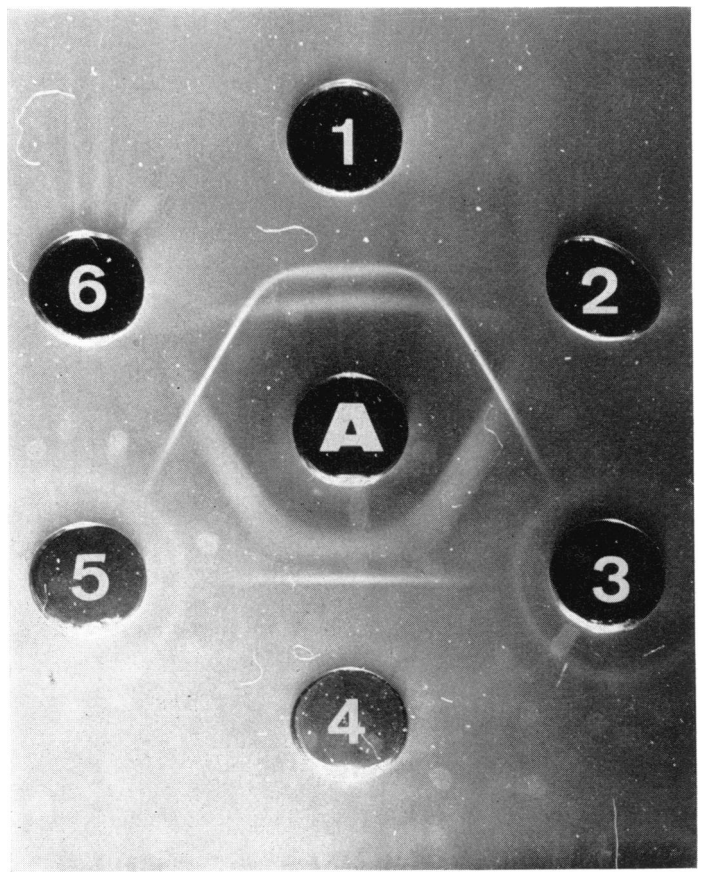


FIG. 6. Demonstration of two distinct antigens in the VF–SF complex. 1 and 4, VF–SF complex; 2 and 6, VF; 3 and 5, SF; (A) antiserum.

isolated by ion exchange chromatography and gel filtration. A VF–SF complex preparation of high homogeneity and purity was obtained. This complex preparation was lyophilized and adjusted to a concentration of 25 μg/ml VBSG.

The second method consisted in preparing a VF–SF complex by interacting guinea-pig serum with an excess of purified VF, for 2 hours at 37°. By this way a maximal amount of VF–SF complex was formed. The complex was isolated by a series of steps on Sephadex G-200, DEAE, Ca-Apatite and again Sephadex G-200, and was very active.

By using an active complex, purified according to the first method we were able to induce the formation of antibodies in rabbits. Because of the pathological effects exerted by the complex *in vivo* it was repeatedly applied in small amounts to the animals. The resulting rabbit antiserum was tested by the gel-diffusion technique in 1 per cent agar against the isolated preparations of SF, of VF and of VF–SF. As seen in Fig. 5 the antiserum against the VF–SF complex contained antibodies with two different specificities directed towards the VF and the SF component respectively. Against the complex two precipitation lines appeared in the gel. As shown in Fig. 6, the lines of the VF–SF complex fuse completely with the line of the isolated VF or SF. If the complex behaved as a single molecular species, a single line of precipitation would be expected between the complex and the antiserum. Furthermore this line should spur over both free components. Even though all physicochemical data support the existence of VF–SF complex we have been unable in various experiments to demonstrate the expected precipitation pattern. The two lines obtained might be interpreted as due to dissociation of the complex under the conditions of diffusion in agar. However, antibody against the VF–SF complex was able to block completely the activity directed against C3.

## TURNOVER OF C3 BY THE VF–SF COMPLEX

The VF–SF complex depleted a C3-solution of haemolytically active C3. This was demonstrated not only for purified C3 but also for C3 in the serum of different species, e.g. guinea-pig, rabbit or man. The depletion of C3 started without a lag-phase when preformed VF–SF complex was used (Fig. 7). In several experiments a clear temperature dependence of C3-turnover was found. For the temperature range between 10° and 30° a $Q_{10}$ of 2·21 was calculated. This corresponds to an activation energy of 13·1 kcal/M. At 37° the C3-turnover was found to be maximal. A further increase beyond this temperature did not result in an additional increase of the turnover. The temperature dependence of the C3-turnover is compatible with the assumption that the VF–SF complex acts upon C3 as an enzyme.

In order to characterize the VF–SF complex as an enzyme the C3-turnover was further investigated. The C3-concentration was plotted against the reaction velocity of the C3-turnover according to Michaelis-Menten. To obtain these data, the complex was incubated at 37° with a variety of C3-concentrations (Fig. 8). The incubation time was restricted to 40 seconds in order to secure an excess of substrate even for the lowest C3-concentrations. The typical curve as demonstrated in Fig. 8 was transformed into a Lineweaver-Burk plot as shown in Fig. 9. From several experiments a km-value of about $10^{-13}$ M × f/l was determined. Since in this system C3 was measured as haemolytically active molecules (SFU) the calculation of the number of physicochemically defined native C3 molecules required the introduction of a correctional factor (f). This factor was determined approximately on the basis of the molecular weight of C3 and of the ratio

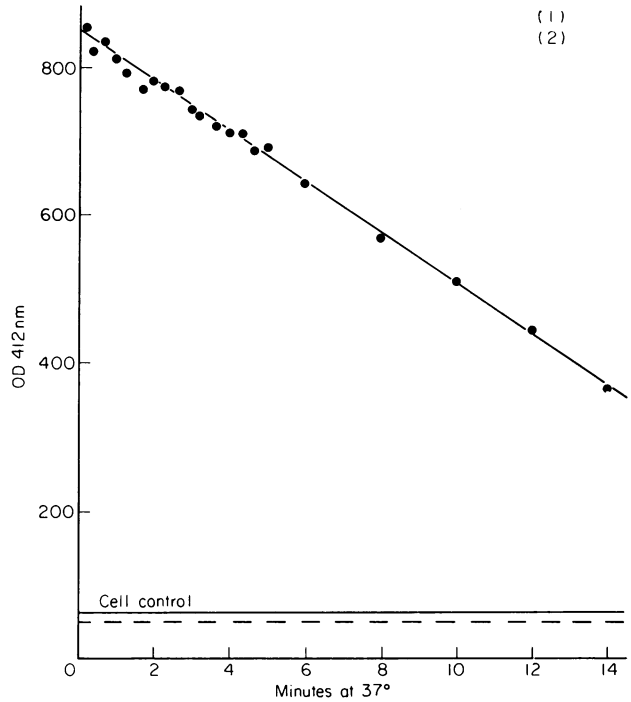


FIG. 7. Depletion of C3 by preformed VF–SF complex with special regard to the initial phase of the reaction. Ordinate and cell controls as in Fig. 3. (1), Hb-value of 100 per cent lysis. (2), Amount of C3 available.

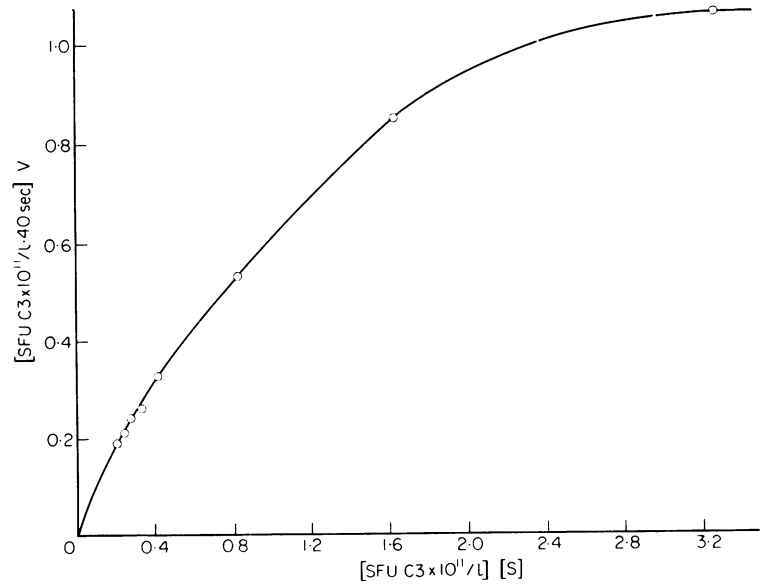


FIG. 8. Turnover of C3 by the VF–SF enzyme plotted according to Michaelis-Menten. Ordinate (V), reaction velocity. Abscissa (S), substrate concentration.

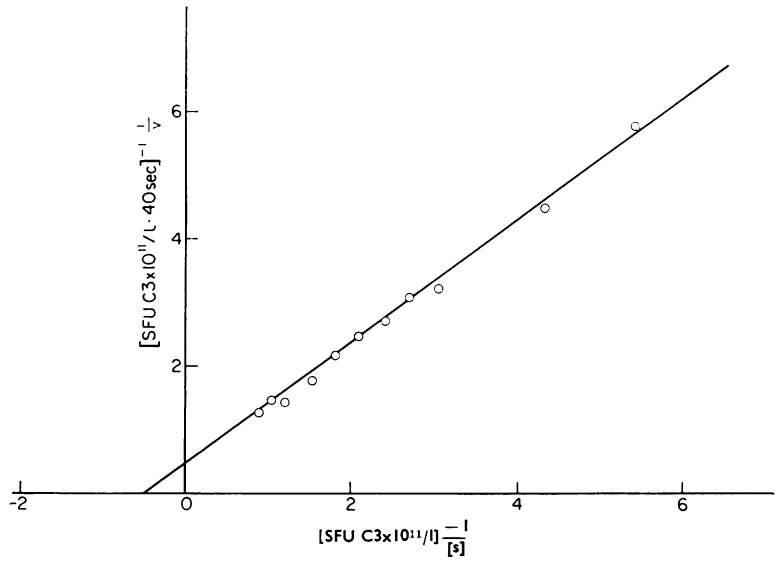


FIG. 9. Transformation of the data from Fig. 8 according to Lineweaver-Burk.

SFU/μg of purified C3. The factor (f) varied from species to species and had to be determined for each C3 preparation; usually it could be found within the range of $10^2$ to $10^4$. For the reported experiments the corrected km-value for the VF–SF enzyme is about $10^{-9}$ M C3/1.

In an additional series of experiments the action of the VF–SF complex on cell-fixed C3, i.e. against C3-sites was studied. No site-inactivation was observed in these experiments. In experiments with partially purified VF–SF complex an activity directed against cell-bound C3 was found; this activity could be separated from the described VF–SF enzyme by further purification. Its properties are under investigation.

### IDENTIFICATION OF THE C3-TURNOVER AS AN ACTIVATION PROCESS

The question of whether the inactivation of C3 by the VF–SF complex was an activation with subsequent decay of the product to a haemolytically inert state was answered by the following experiment. Two test systems were set up and treated in a parallel manner. The first contained 1 volume guinea-pig serum, 1 volume of VBS, 2 volumes suspension of preformed VF–SF enzyme and EDTA. The final concentration of EDTA amounted to 0·01 M, while the serum was present in a 1:8 dilution. The second system was composed in an analogous way, but it contained instead of VBS 1 volume of a suspension of unsensitized erythrocytes (E) to yield a final concentration of $1{\cdot}3 \times 10^8$ E/ml.

In a kinetic experiment performed at 37°, 0·1-ml samples from both incubation mixtures were taken at different time intervals and tested in the first case for C3- and C5-turnover. In the second case the Hb-value of the supernatant was determined in order to evaluate the degree of haemolysis. The results are summarized in Fig. 10 and Fig. 11. These show that the C3-turnover was almost complete while C5 was only diminished to about 50 per cent. In other experiments it was found that the turnover of C3 was always

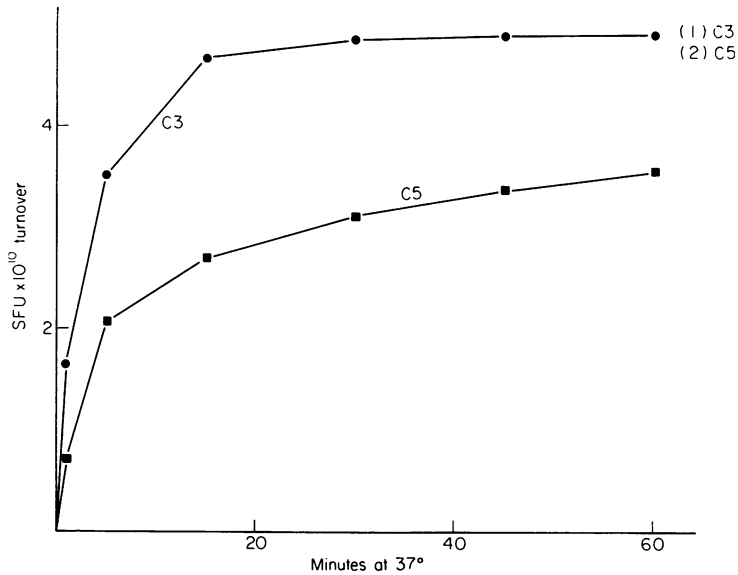


FIG. 10. Turnover of C3 and C5 by the preformed VF–SF enzyme in serum containing EDTA. (1) and (2), amount of C3 and of C5 originally available. The loss of C5 activity from 20–60 minutes is due to the normal decay of C5 as found in controls and is not caused by turnover.

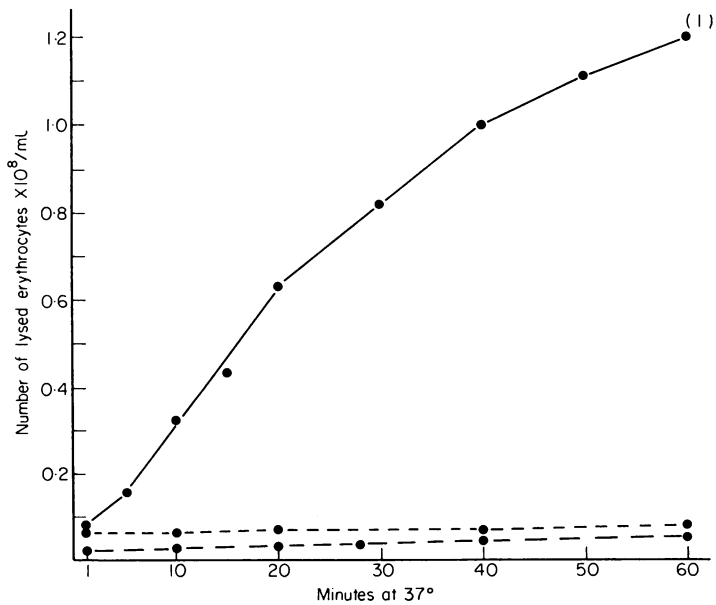


FIG. 11. Lysis of unsensitized sheep-erythrocytes (E) induced by preformed VF–SF enzyme in a serum containing EDTA. (1), Input of cells 0·1-ml sample containing $1{\cdot}3 \times 10^8$ E/ml in 1·2 ml $H_2O$. Cell controls, upper dotted line; E in serum containing EDTA, lower dotted line; E and VF–SF enzyme in buffer.

complete regardless of the concentration of this component. On the other hand, C5-consumption only occurred if C3 was present in large excess. A strict correlation existed between the values of the C3-turnover and the amount of C5-consumption, and might explain why the demonstration of C5-turnover was difficult when isolated C3 and isolated C5 were used; the usual preparations of C3 were not concentrated enough to induce significant C5-consumption. Fig. 10 shows that C5 was consumed only as long as C3-turnover took place. Thus while the VF–SF enzyme did not directly influence isolated C5, it activated C5 and all the following components in the serum via C3, as demonstrated by lysis of E (Fig. 11). While C3 and C5 inactivation was virtually complete within the first 20 minutes, the lysis of E continued gradually up to 60 minutes after the start of the experiment. This delay was presumably due to the time for the action of the later stages (C6 to C9) leading to cell lysis and the release of haemoglobin.

## DISCUSSION

The described observations on the physicochemical characteristics of VF, of SF and of the VF–SF complex are in accordance with the data found for the human system. (Müller-Eberhard, 1967; Müller-Eberhard, Hadding and Calcott, 1967; Ballow and Cochrane, 1969). The immunological analysis of the VF–SF complex reveals that it contains not only VF-material but also SF-material. Previous reports have shown that the interaction of VF and SF leads to a heavier molecule which contains VF-material. Our findings, that antigenic determinants not only characteristic for VF but also for SF can be demonstrated are in accordance with the concept of complex formation, although the proportions of the constituents in the complex are unknown.

The experiments reported above show that $Mg^{++}$ is an essential element in the formation of the complex and cannot be replaced by other bivalent cations. From the available data it is not clear whether the $Mg^{++}$ plays only a role in the complex formation, or whether the ion is tightly bound to the VF–SF enzyme. In the first case the interaction between VF and SF would be an enzymatic process, which requires $Mg^{++}$-ions (metal activated enzyme); in the latter case $Mg^{++}$ would be incorporated into the structure of the resulting enzyme (metallo enzyme) (Mildvan, 1970).

Preliminary experiments carried out using atomic absorption spectroscopy indicate that $Mg^{++}$ is incorporated into the complex, although it is surprising that the complex once formed cannot be functionally altered by EDTA.

Whether the formation of the complex is an enzymatic process is not clear, although temperature dependence was demonstrated (Fig. 6). The enzymatic nature of the action of the VF–SF complex upon C3 has been suggested by several authors, since the action of cobra toxin upon serum leads to the appearance of C3 fragments and also of C5 fragments. These substances exhibit anaphylatoxic and chemotactic activities. In addition the cobra venom mediated lysis of unsensitized erythrocytes suggested that this process was comparable to the activation of C3 as induced by the C3-convertase. The experiments described in this paper present affirmative evidence that the VF–SF complex acts as an enzyme upon the substrate C3.

The experiments also show that the generation of this enzyme is independent of antibody, and of C1, C4 and C2. Indeed, VF is able to induce C3 activation when added to a serum which previously had been completely deprived of C1.

Another point of distinction between the C3 convertase and the VF–SF complex is that the activity of the latter is stable over a period of several hours at 37° whereas the former undergoes a rapid decay at this temperature.

No inactivation of cell-bound, haemolytically active C3 was observed by VF–SF complex preparations of high purity, although partially purified preparations acted not only against native C3 but also against C3-sites. The latter activity could be separated from the activity against native C3 by gelfiltration on Sephadex G-200. The C3-site inactivating principle had a lower molecular weight than the VF–SF complex but a higher one than SF and also than VF, and is the subject of further investigation.

The activity range of the VF–SF enzyme is not limited to guinea-pig C3, but also includes rabbit and human C3. However, it is ineffective against purified C5, although if added to a mixture of C3 and C5 both components are inactivated. The degree of C5 conversion is related to the amount of native C3 initially present in the reaction mixture. Moreover C5 is only consumed as long as C3-turnover takes place. This suggests that an active but rapidly decaying C3-fragment is responsible for the C5-turnover.

The effect of cobra toxin upon C3 and C5 has been regarded conventionally as an inactivating process (Flexner and Noguchi, 1903; Klein and Wellensiek, 1965; Müller-Eberhard, 1967, Cochrane, Müller-Eberhard and Aikin, 1970). However, in the light of present knowledge, this is because a secondary decay follows the primary process of activation.

The biological implications of the data presented in this paper reach beyond the special properties of cobra venom as an activating agent for C3. There is substantial evidence that the cobra toxin stands only as an example of a whole class of agents like endotoxin, polysaccharides and immune aggregates of γ1-globulins (guinea-pig). The common property of these agents seems to be their capacity to interact with one or more serum factors other than C1, C4 and C2 leading to the formation of an enzymatic activity directed against C3. These problems are under investigation.

## ACKNOWLEDGMENTS

This work was supported by grants from the DFG. The authors wish to thank Mrs K. Boenisch, Mrs B. Eschenhof and Mrs U. Heinemann for excellent technical assistance.

Part of this work was presented at the Fourth Complement Workshop, Baltimore, January 1971.

## REFERENCES

Ballow, M. and Cochrane, C. G. (1969). 'Two anticomplementary factors in the cobra venom: hemolysis of guinea pig erythrocytes by one of them.' *J. Immunol.*, **103**, 944.

Bitter-Suermann, D., Hadding, U., Melchert, F. and Wellensiek, H. J. (1970). 'Independent and consecutive action of C5, C6 and C7 in immune hemolysis. I. Preparation of EAC1-5 with purified guinea pig C3 and C5.' *Immunochemistry*, **7**, 955.

Borsos, T. and Rapp, H. J. (1963). 'Chromatographic separation of the first component of complement and its assay on a molecular basis.' *J. Immunol.*, **91**, 851.

Cochrane, C. G., Müller-Eberhard, H. J. and Aikin, B. S. (1970). 'Depletion of plasma complement *in vivo* by a protein of cobra venom: its effect on various immunologic reactions.' *J. Immunol.*, **105**, 55.

Colten, H. R., Borsos, T. and Rapp, H. J. (1968). 'Ultracentrifugation of the first component of complement: Effects of ionic strength.' *J. Immunol.* **100**, 808.

Dierich, M., Bitter-Suermann, D., König, W. and Hadding, U. (1971). 'Formation and function of a complement activating enzyme generated from factors of guinea pig serum and cobra venom. (Short communication).' *Eur. J. Immunol.*, **1**, 309.

Flexner, S. and Noguchi, H. (1903). 'Snake venom in relation to hemolysis, bacteriolysis and toxicity.' *J. exp. Med.*, **6**, 277.

KLEIN, P. G. and WELLENSIEK, H. J. (1965). 'Multiple nature of the third component of guinea-pig complement. I. Separation and characterization of three factors a, b and c, essential for haemolysis.' *Immunology*, **8**, 590.

KÖNIG, W., BITTER-SUERMANN, D., DIERICH, M. and HADDING, U. (1971). 'Physiochemical characterization of the fifth (C5), sixth (C6), seventh (C7), eighth (C3) and ninth (C9) component of guinea pig complement.' *Eur. J. Immunol.*, **1**, 372.

LACHMANN, P. J. and THOMPSON, R. A. (1970). 'Reactive lysis: The complement-mediated lysis of unsensitized cells. II. The characterization of activated reactor as C56 and the participation of C8 and C9.' *J. exp. Med.*, **131**, 643.

MAYER, M. M. (1961). 'Complement and complement fixation.' In: *Experimental Immunochemistry* (Ed. by E. A. Kabat and M. M. Mayer) ed. 2, p. 133. C. C. Thomas, Springfield, Ill.

MILDVAN, A. S. (1970). 'Metals in enzyme catalysis.' In: *The Enzymes*, Boyer Vol. II, 3rd edn. p. 463. Academic Press, New York, London.

MÜLLER-EBERHARD, H. J. (1967). 'Mechanism of inactivation of the third component of human complement (C3) by cobra venom.' *Fed. Proc.*, **26**, 744.

MÜLLER-EBERHARD, H. J., HADDING, U. and CALCOTT, M. A. (1967). 'Current problems in complement research'. In: *Immunopathology, Fifth International Symposium* (Ed. by P., Grabar P. A. Miescher), p. 179. Schwabe u. Co., Basel, Stuttgart.

NELSON, R. A. (1965). 'The role of complement in immune phenomena'. In: *The Inflammatory Process* p. 819 (Ed. by B. W. Zweifach, R. T. McCluskey, and L. H. Grant). Academic Press, New York, London.

PICKERING, R. J., WOLFSON, M. R., GOOD, R. A. and GEWURZ, H. (1969). 'Passive hemolysis by serum and cobra venom factor: A new mechanism inducing membrane damage by complement.' *Proc. nat. Acad. Sci.*, **62**, 521.

RINGELMANN, R., OPFERKUCH, W., RÖLLINGHOFF, M. and LOOS, M. (1969). 'Komplement-Messungen mit Hilfe des Mikrolitersystems.' *Z. Med. Mikrobiol. u. Immunol.*, **154**, 329.

RITZ, H. (1912). 'Über die Wirkung des Cobragiftes auf die Komplemente.' *Z. Immunitätsforsch.*, **13**, 62.

SLEGERS, J. and SIMON, J. (1968). 'Utilisation des dextranes modifiés pour fractionment du venin de cobra.' *J. Chromatog.*, **36**, 241.

THOMPSON, R. A. and ROWE, D. S. (1968). 'Reactive hemolysis—a distinctive form of red cell lysis.' *Immunology*, **14**, 745.

THOMPSON, R. A. and LACHMANN, P. J. (1970). 'Reactive Lysis: The complement-mediated lysis of unsensitized cells. I. The characterization of the indicator factor and its identification as C7.' *J. exp. Med.*, **131**, 629.

WELLENSIEK, H. J. and KLEIN, P. G. (1965). 'Multiple nature of the third component of guinea-pig complement. II. Separation and description of two additional factors $\beta$ and d; preparation and characterization of four intermediate products.' *Immunology*, **8**, 604.

WHO (1968) *Bull. Wld Hlth Org.* **39**, 935.