

MODULATION OF FUNCTION OF THE ACTIVATED FIRST  
COMPONENT OF COMPLEMENT BY A FRAGMENT DERIVED  
FROM SERUM

I. EFFECT ON EARLY COMPONENTS OF COMPLEMENT\*

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It has been recently demonstrated that activated Hageman factor is digested by plasmin to yield prealbumin fragments (1) which convert prekallikrein to kallikrein, the enzyme that cleaves kininogen to yield bradykinin (2, 3). A relation between this kinin-forming system and the complement sequence exists in terms of both the inhibitor of the first component of complement ( $\bar{C}1\text{INH}$ ),<sup>1</sup> which inhibits both the first component ( $\bar{C}1$ )<sup>2</sup> and kallikrein (4, 5), as well as plasmin (6) and the active Hageman factor (7), and the anaphylatoxin inacti-

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<sup>1</sup> *Abbreviations used in this paper:* AAMe, *N*-acetyl-L-arginine methyl ester hydrochloride; AGLMe, *N*- $\alpha$ -acetylglycyl-L-lysine methyl ester acetate; ATMe, *N*-acetyl-L-tyrosine methyl ester; TAME, para-tosyl-L-arginine methyl ester hydrochloride;  $\bar{C}1\text{INH}$ , inhibitor of the first component of complement; DGVB<sup>++</sup>, dextrose-Veronal-buffered saline with Ca<sup>++</sup> and Mg<sup>++</sup>; EA, sheep erythrocytes coated with anti-sheep hemolysin made in rabbits; GPC-EDTA, guinea pig sera diluted in EDTA; GVB<sup>++</sup>, Veronal-buffered saline with Ca<sup>++</sup> and Mg<sup>++</sup>; HAE, hereditary angioedema; PTA, plasma thromboplastin antecedent; SBTI, soybean trypsin inhibitor.

<sup>2</sup> The nomenclature used conforms to that agreed upon by the World Health Organization (1968. *Bull. World Health Organ.* **38**:935.). Sheep erythrocytes (E), sensitized with rabbit antibody (A), react with the components of complement (C) in the sequence C1, 4, 2, 3, 5, 6, 7, 8, 9. The activated state of a component is signified by a bar above the component number. In addition to this convention, the species of origin of the components is human unless indicated otherwise by a superscript (guinea pig, gp). SAC $\bar{1}$ , SAC $\bar{1}^4$ , and SAC $\bar{1}^2$  refer to the proportion of hemolytically active sites formed per erythrocyte during the interaction of EA with  $\bar{C}1$ , EAC $\bar{1}$  with C4, and EAC $\bar{1}^4$  with C2, respectively.

vator, an enzyme which removes the C terminal arginine residue from the C3a and C5a fragments of the third (C3) and fifth (C5) components of complement as well as from bradykinin (8). Kallikrein not only cleaves the permeability factor bradykinin from kininogen but also is itself specifically chemotactic for human neutrophils (9), thus affording two of the essential ingredients of the inflammatory response. An additional function of the kinin-forming system is to yield a fragment which profoundly alters the function of fully activated C1. Fragment treatment of isolated, fully activated C1, or partially activated C1, in whole human serum markedly enhances the hemolytic efficiency of C1, in the latter instance without additional activation. Further, fragment-treated C1 interacts with the fourth (C4) and the second (C2) components of complement to achieve a much greater inactivation of the third component than is accomplished by C1, C4, and C2 in the absence of the fragment.

#### Materials and Methods

The sources and methods of handling of sheep erythrocytes, guinea pig sera, and human sera were as described (10). Veronal-buffered saline, pH 7.5, ionic strength 0.145, containing 0.1% gelatin, 0.00015 M  $\text{Ca}^{++}$ , and 0.0005 M  $\text{Mg}^{++}$  (GVB<sup>++</sup>), dextrose-Veronal-buffered saline, ionic strength 0.075 (DGVB<sup>++</sup>), and 0.01 M or 0.04 M disodium ethylenediaminetetraacetate (EDTA) were prepared as described (11). The sources and methods of preparation of diethylaminoethyl- (DEAE-) and carboxymethyl- (CM-) cellulose, Sephadex G-200 and G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), hydroxylapatite, and the buffers used for chromatography have previously been reported (11-13). Para-tosyl-L-arginine methyl ester hydrochloride (TAMe) and *N*-acetyl-L-tyrosine methyl ester (ATMe) were purchased from Mann Research Labs, Inc., New York; *N*- $\alpha$ -acetylglucyl-L-lysine methyl ester acetate (AGLMe) and *N*-acetyl-L-arginine methyl ester hydrochloride (AAMe) were obtained from Cyclo Chemical Corp., Los Angeles, Calif. Soybean trypsin inhibitor (SBTI) was obtained from Worthington Biochemical Corp., Freehold, N. J.

*Preparation and Assay of Human Complement Components.*—C1 was prepared from pooled human serum by low ionic strength precipitation at pH 7.5 (11, 14) with the addition of  $\text{Ca}^{++}$ , 0.00015 M; further purification was achieved by Sephadex G-200 gel filtration (12). The C1 preparation eluted with the 19S protein peak and it was found to be fully activated (14). C4 and C2 were prepared by DEAE- and CM-cellulose chromatography (5, 11). C3 and C5 were isolated from DEAE-cellulose chromatography (11) and further purified by hydroxylapatite column chromatography (15, 16). The sixth (C6), seventh (C7), eighth (C8), and ninth (C9) components of human complement were obtained from Cordis Corp., Miami, Fla. C1<sup>SP</sup> and C2<sup>SP</sup> were prepared as described (11).

Sheep erythrocytes were coated with commercial anti-sheep hemolysin made in rabbits (EA). EAC1<sup>SP</sup> cells were prepared by mixing EA in DGVB<sup>++</sup> with an equal volume of activated first component of guinea pig complement (C1<sup>SP</sup>), diluted to provide 250 effective molecules/cell in the fluid phase; after incubation at 30°C for 45 min, the cellular intermediate was washed three times in DGVB<sup>++</sup> and stored in the same buffer with penicillin and streptomycin (11). EAC4 were prepared from the EAC1<sup>SP</sup> cells (17) and further incubated in 0.01 M EDTA-GVB (18). EAC14 cells were prepared by exposing EAC4 cells to C1<sup>SP</sup> (18). The procedures for the effective molecule titrations of C1 (19), C1INH (20), C4 (18), C2 (21), and C3 (22) were performed as described. The number of C1 molecules bound to a cell (EAC1) was determined by C1 transfer (23).

*Purification and Assay of the Components of the Kinin-Forming System.*—35 ml of serum were dialyzed overnight at 4°C in 0.01 M phosphate buffer at pH 7.8, and the precipitate formed was removed by centrifugation. The supernatant was applied to a 3.5 × 30 cm DEAE-cellulose column equilibrated with 0.01 M phosphate buffer, pH 7.8, followed by washing with the equilibrating buffer and elution with a linear salt gradient to 0.3 M NaCl. The initial protein peak (OD 280  $m\mu$ ) containing IgG, kallikrein, and plasma thromboplastin antecedent (PTA) was concentrated by ultrafiltration using a UM-10 membrane (Amicon Corp., Lexington, Mass.) to 5–10 ml, and was then further concentrated by wall vacuum using collodion bags, number 100, (Schleicher & Schuell, Inc., Keene, N. H.) to a final volume of 2 ml. This material was applied to a 2.5 × 150 cm Sephadex G-150 superfine column equilibrated with 0.02 M tris (hydroxymethyl) aminomethane (Tris) chloride pH 7.6. The pattern was developed by upward flow at 5 ml/hr and 2.5 ml fractions were collected. Heat-inactivated plasma prepared by heating fresh plasma at 61°C for 2 hr and dialyzing overnight against 0.15 M NaCl was used as a substrate for kallikrein (24). Kallikrein activity was determined by incubating between 0.05 and 0.2 ml of enzyme source with 0.2 ml of substrate for 2 min at 37°C and measuring the bradykinin generated by adding the mixture to the organ bath containing the guinea pig ileum used for bioassay. Control tubes containing the enzyme source or the substrate were evaluated separately for bradykinin generation. The kallikrein peak was pooled, concentrated, and its purity assessed by disc gel electrophoresis at pH 9.3 (Buchler Instruments, Inc., Fort Lee, N. J.) (25) and by electro-focusing in 4% polyacrylamide gels (26). In each instance a single broad band was obtained representing a mixture of kallikrein, IgG, and trace contamination with PTA.

The shortening of the partial thromboplastin time of PTA-deficient plasma was used to assay for PTA. 0.05 M ml of PTA source was incubated for 2 min at 37°C with 0.05 ml cephalin (6 mg/ml) and 0.05 ml PTA-deficient plasma in siliconized glass tubes. 0.05 ml 0.05 M  $CaCl_2$  was added, and the clotting time determined at room temperature. The tubes were tilted each minute and the end point defined as the time required for the clot to adhere to the glass tube.

Fibrinogen was measured by digestion of azocasein as described by Hummel et al. (27). Kininogen was prepared as previously described by Spragg et al. (3).

For the esterolytic assay of kallikrein, each synthetic substrate was dissolved in 0.08 M phosphate buffer, pH 7.8, ionic strength 0.14. Esterolytic activity was measured by a modification of the colorimetric method described by Siegelman et al. (28). 0.2 ml of enzyme was incubated with 0.8 ml of substrate in phosphate buffer at a concentration of 10 nmoles/ml. At 1 min and 31 min at 37°C, 0.4 ml of enzyme-substrate mixtures were removed and transferred to small tubes containing 0.25 ml of 15% trichloroacetic acid (TCA), and the precipitate formed was removed by centrifugation. 0.05 ml of 2% potassium permanganate ( $KMnO_4$ ), 0.05 ml of 10% sodium sulfite ( $Na_2SO_3$ ), and 2 ml of chromotropic acid were added in sequence to 0.25 ml of supernatants, followed by incubation in a boiling water bath for 15 min. The purple color developed by the interaction of the methanol, derived from the splitting of the ester substrates with chromotropic acid, was measured in the spectrophotometer at 580  $m\mu$ . The values were compared with those of a standard curve of methanol and the results calculated as millimoles per liter of methanol liberated in each reaction mixture during the 30 min incubation at 37°C.

## RESULTS

### *Enhancement of the $\bar{C}I$ Hemolytic Activity by Fractions with Kinin-Generating Capacity*

Aliquots of 0.5 ml of  $\bar{C}I$  containing 1000 units/ml were incubated with 0.1 ml DGVB<sup>++</sup> or 0.1 ml of five individual pools of fractions eluted from a DEAE-cellulose column during the

purification of the enzyme kallikrein. Control samples of 0.5 ml DGVB<sup>++</sup> received 0.1 ml of each of the pools. The mixtures were incubated at 30°C for 30 min and the C $\bar{I}$  hemolytic activity of each sample measured.

Five areas capable of generating bradykinin with fresh plasma were observed as previously described (29) (Fig. 1). The first peak, eluted with the starting buffer, contains IgG globulin, PTA, and releases bradykinin from either fresh plasma, heat-inactivated plasma, or purified kininogen (29) and therefore contains kallikrein activity. The second peak contains activated Hageman factor, and peaks three, four, and five represent fragments of active Hageman factor

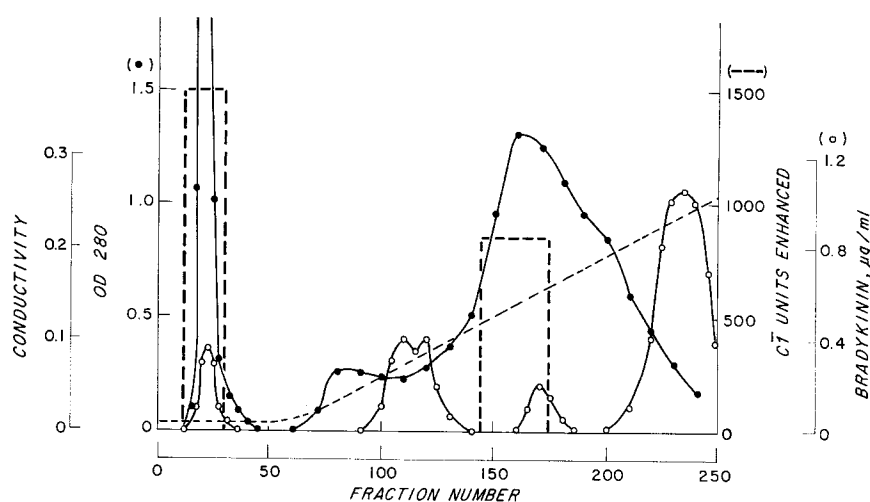


FIG. 1. DEAE-cellulose chromatography of human sera with assessment of protein concentration (●), kinin generation from fresh plasma (○), and C $\bar{I}$  enhancement (---). The salt gradient used for elution of the column is expressed as the corresponding molarity of NaCl.

of progressively smaller size and net negative charge (29). Plasminogen and plasmin overlap peaks two and three. Enhancement of the C $\bar{I}$  hemolytic activity was observed with the first and fourth peaks, while peaks two, three, and five were negative in this regard. C $\bar{I}$  was precipitated in the step which preceded chromatography of the supernatant and no C $\bar{I}$  hemolytic activity was demonstrable in any of the five kinin-generating peaks.

The C $\bar{I}$  enhancement produced by the kallikrein-rich peak 1 was further analyzed in terms of its dose response effect.

Aliquots of 0.5 ml of C $\bar{I}$  containing 1000 units/ml were incubated with DGVB<sup>++</sup> or seven increasing amounts (0.01–0.1 ml) of peak 1. The final volume of all the mixtures was brought to 0.6 ml. A control sample of 0.5 ml DGVB<sup>++</sup> received 0.1 ml of peak 1. The mixtures were incubated at 30°C for 30 min and the C $\bar{I}$  hemolytic activity measured in each sample.

As shown in Fig. 2, the C $\bar{I}$  activity increased from 1000 units/ml in the control sample to 2500 units/ml with increasing amounts of peak 1, at which point a plateau of C $\bar{I}$  enhancement was reached. The kallikrein peak incubated with buffer alone was free of C $\bar{I}$  hemolytic activity.

Five 0.5 ml samples of C $\bar{I}$  diluted to contain 1000 units/ml received different additions during a total incubation time of 40 min. The respective additions were 0.01 ml of a concentrated peak 1, 0.01 ml SBTI 0.1 mg/ml, peak 1 followed by SBTI for 30 min, SBTI followed by peak 1 for 10 min, or buffer. In a sixth sample 0.01 ml peak 1 and 0.01 ml SBTI were incubated for 10 min before receiving 0.5 ml C $\bar{I}$ . The final volume of all samples was brought to 0.62 ml with buffer.

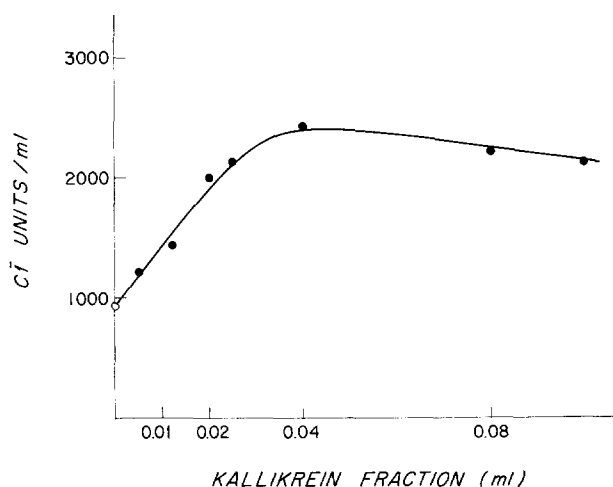


FIG. 2. Enhancement of C $\bar{I}$  hemolytic activity by incubation with different amounts of peak 1 (Fig. 1) (●) as compared with buffer (○).

The results are depicted in Fig. 3. SBTI did not influence the C $\bar{I}$  hemolytic activity alone (B). Peak 1 increased the C $\bar{I}$  hemolytic activity 300% (C); the increase was limited to 125% when peak 1 was previously incubated with SBTI (D). The SBTI has a lesser effect when its preincubation with kallikrein was omitted (E), and has virtually no effect when added after the interaction of C $\bar{I}$  with peak 1 (F). This suggests that the enhancement was due to a SBTI-sensitive proteolytic enzyme such as kallikrein.

Accordingly, in a separate experiment the dose response of the effect of SBTI on TAME hydrolysis, and kinin formation as well as C $\bar{I}$  enhancement, was examined.

Five duplicate samples of SBTI containing 10, 1, 0.1, 0.01, and 0.001 mg/ml in saline were incubated with an equal volume of buffer or peak 1. A control sample of peak 1 received buffer alone. After 10 min at 30°C the samples were analyzed.

Inhibition of kinin generation was slightly more sensitive to SBTI than the splitting of the synthetic substrate; 50% inhibition was achieved with 0.0050 mg and 0.0075 mg of SBTI respectively. In contrast almost 100 times as much SBTI (0.5 mg) was required to achieve 50% suppression of C $\bar{I}$  enhancement, suggesting that the enhancing activity may reside in a structure other than the active enzymatic site of a TAME esterase such as kallikrein. Attention was therefore focused on the possibility that the C $\bar{I}$ -enhancing material in peak 1 was somehow related to the more negatively charged material observed in peak 4 (Fig. 1).

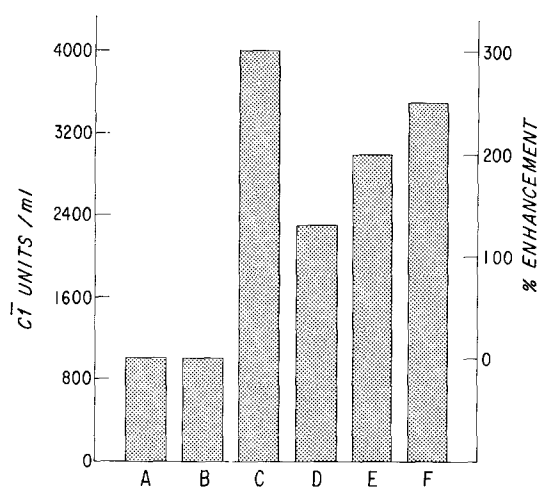


FIG. 3. Inhibition by SBTI of the C $\bar{I}$  enhancement produced by peak 1. C $\bar{I}$  incubated with DGVB<sup>++</sup> (A), C $\bar{I}$  incubated with SBTI (B), C $\bar{I}$  incubated with peak 1 (C), peak 1 incubated with SBTI before the addition of C $\bar{I}$  (D), C $\bar{I}$  incubated with SBTI before the addition of peak 1 (E), and C $\bar{I}$  incubated with peak 1 before the addition of SBTI (F).

#### *Recognition of a C $\bar{I}$ -Enhancing Material Apparently Derived from the Kallikrein-Containing Fraction*

Peak 1 of the DEAE-cellulose column was subjected to Sephadex G-150 gel filtration. The fractions were combined into eight pools, concentrated by pressure dialysis to a final volume of 10 ml, and tested for kinin formation on heated plasma and for C $\bar{I}$  enhancement by their effect on C $\bar{I}$ , 1000 units/ml.

As shown in Fig. 4, a protein peak produced largely by IgG was found in fractions 120-160; superimposed on the descending portion of this peak was bradykinin-generating activity, attributable entirely to kallikrein at approximately 45% of the column bed volume. A peak of C $\bar{I}$ -enhancing activity was observed in the same position. A second peak of C $\bar{I}$  enhancement was present at approximately 80% bed volume. This material designated Kf was devoid

of kinin-generating activity from either fresh or heat-inactivated plasma, indicating that neither kallikrein nor the active Hageman factor fragments was present. Rechromatography of the material corresponding to 45% of the bed volume again yielded C $\bar{I}$ -enhancing activity at 45 and 80% bed volume, with the same characteristics as observed in the initial chromatogram.

In order to determine whether or not Kf represents some portion of the C $\bar{I}$  molecule, it was examined for the esterolytic and proteolytic characteristics of C $\bar{I}$ . The ability of Kf to hydrolyze synthetic substrates was studied in an experiment in which 0.2 ml samples of Kf were incubated with 0.8 ml of TAME,

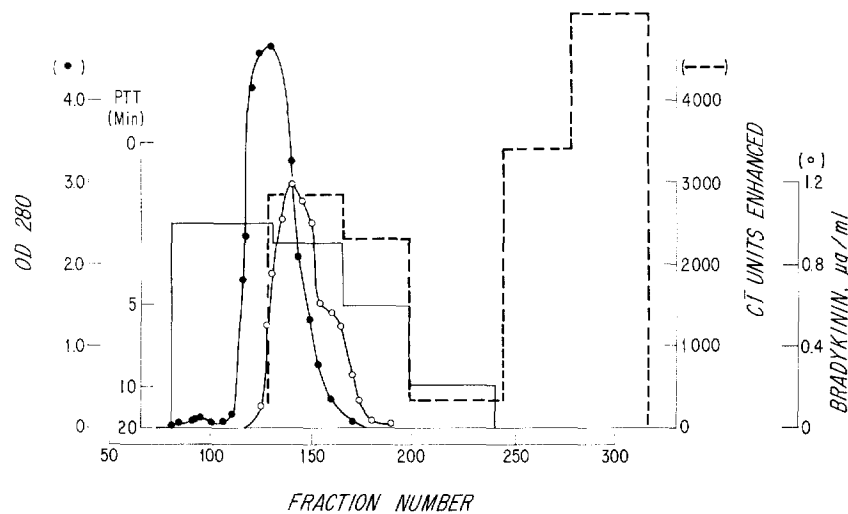


FIG. 4. Gel filtration on Sephadex G-150 of peak 1 (Fig. 1) with assessment of protein concentration (●), kinin generation from fresh plasma (○), C $\bar{I}$  enhancement (---), and partial thromboplastin time (PTT) (—).

ATMe, AAME, and AGLMe containing 10 mmoles of substrate/ml. Kf did not hydrolyze any of the synthetic substrates studied. The capacity of Kf to destroy C4 and C2 in the fluid phase was determined by incubating 0.1 ml samples of Kf with 0.5 ml of C4 containing 1000 units/ml, or C2 containing 100 units/ml, or with buffer. After 30 min at 30°C the residual C4 and C2 were measured in each sample. 90 and 30% of C4 and C2 respectively were destroyed by C $\bar{I}$  while Kf failed to inactivate these two components in the fluid phase.

The heat stability of Kf at 56°C was evaluated in terms of C $\bar{I}$  enhancement. When plotted logarithmically there was a linear loss of activity with a half-life of 35 min. The pH stability of Kf was examined in a range of 4–9 by adding 0.1 M HCl or 0.1 M NaOH to the Tris chloride buffer. After 1 hr the pH in

each sample was brought to 7.5 and the final volume adjusted to 1 ml with buffer. At pH's 4, 5, and 6 there was a 20% loss in C $\bar{I}$ -enhancing capacity, while there was no loss between pH 7 and 9.

*Effect of Kf on Cell-Bound Functions of C $\bar{I}$*

*Utilization of C2 by EAC $\bar{I}4$  and EAC $\bar{I}Kf4$ .—*

Two 0.5 ml samples of C $\bar{I}$  containing 1800 units/ml were incubated with 0.1 ml of either buffer or Kf. A control sample of Kf received 0.5 ml buffer. After 30 min at 30°C, the C $\bar{I}$  hemolytic activity was measured in all samples. C $\bar{I}$  incubated with buffer contained 1800 C $\bar{I}$  units, that incubated with Kf exhibited 4300 units, and Kf alone was devoid of any hemolytic activity. These three samples were diluted to the same final dilution (1:18). Two aliquots of EAC $\bar{I}4$  cells  $1 \times 10^8$ /ml were incubated with an equal volume of the diluted C $\bar{I}$  and the third aliquot of cells with C $\bar{I}Kf$ . After 15 min at 30°C, the samples were centrifuged and washed in DGVB $^{++}$ . One of the EAC $\bar{I}4$  preparations and the EAC $\bar{I}Kf4$  intermediate were resuspended in buffer, while the other EAC $\bar{I}4$  was resuspended in the diluted Kf. All samples were incubated 15 min at 30°C, centrifuged, washed once in buffer, and resuspended to the original cell concentration. 10 ml of each cellular intermediate prewarmed at 30°C received 10 ml of C2, 1.2 units/ml, and the incubation was continued. 1 ml samples were removed at time intervals up to 15 min, added to 1.5 ml of guinea pig sera diluted 1:22.5 in 0.04 M EDTA (GPC-EDTA), and the reaction developed for 60 min at 37°C.

The results (Fig. 5) show that the EAC $\bar{I}4$  generated with untreated C $\bar{I}$  followed by treatment with buffer or Kf had a  $T_{max}$  of 4.5 min with a maximal hemolytic activity ( $Z_{max}$ ) with C2 of 0.85 and 0.95 SAC $\bar{I}42$  respectively, while EAC $\bar{I}Kf4$  had a  $T_{max}$  of 3 min and a  $Z_{max}$  of 1.98 SAC $\bar{I}Kf42$ . The results were similar in a subsequent experiment even when the C $\bar{I}Kf$  was diluted more than the C $\bar{I}$  so as to achieve a comparable number of C $\bar{I}$  hemolytic units, that is Kf shifted the  $T_{max}$  from 3.5 min to 1.75 min and the  $Z_{max}$  from 0.85 to 1.59 sites.

*Interaction of C $\bar{I}$  and C $\bar{I}Kf$  with EAC $\bar{I}42$ .—*

An EAC $\bar{I}42$  intermediate was generated to provide cells with bound unactivated C2. EAC $\bar{I}4$  cells,  $1 \times 10^9$ /ml, in DGVB $^{++}$  at pH 8.5, with a final Mg $^{++}$  concentration of 0.001 M, were incubated with an equal volume of C2 diluted in the same buffer to contain less than 1 C2 unit when tested with routinely prepared EAC $\bar{I}4$  cells. The mixture was incubated at 30°C for 30 min, centrifuged at room temperature, washed once, and resuspended in the same buffer to the original volume. The buffers used for washing and resuspension were prewarmed at 30°C. Activation of these EAC $\bar{I}42$  cells was obtained by incubating these cells with aliquots of C $\bar{I}$  or C $\bar{I}Kf$  described above diluted to contain 5, 20, and 50 C $\bar{I}$  units/ml. 9 ml of C $\bar{I}$  and C $\bar{I}Kf$  dilutions were prewarmed at 30°C, and each received 1 ml of EAC $\bar{I}42$  cells,  $1 \times 10^9$ /ml. A sample of EAC $\bar{I}42$  was incubated with Kf without C $\bar{I}$ . Upon mixing and at time intervals up to 20 min, 1 ml samples were removed and added to 1.5 ml of GPC-EDTA. The reaction was developed at 37°C for 60 min.

Fig. 6 shows that untreated C $\bar{I}$  produced 0.42, 0.57, and 0.65 SAC $\bar{I}42$  with a  $T_{max}$  of 7, 3, and 2.75 min respectively with the three C $\bar{I}$  concentrations used,



while  $\overline{C1Kf}$  at the same concentrations formed 1.35, 1.5, and 1.6  $\overline{SAC1Kf42}$ , with a  $T_{max}$  of 3.25, 1.75, and 1.75 min respectively. Thus for the same input of hemolytic units,  $\overline{C1Kf}$  generated cells with a shorter  $T_{max}$  and a higher  $Z_{max}$  than untreated  $\overline{C1}$ , even when the unactivated C2 was already bound to the cell.

*Effect of Kf on C1 Binding.—*

Two aliquots of  $\overline{C1}$  and  $\overline{C1Kf}$  were diluted in either  $DGVB^{++}$  or  $GVB^{++}$  to contain 20 units of  $\overline{C1}$  each. 9 ml of these four  $\overline{C1}$  preparations, prewarmed at 30°C, received 1 ml of

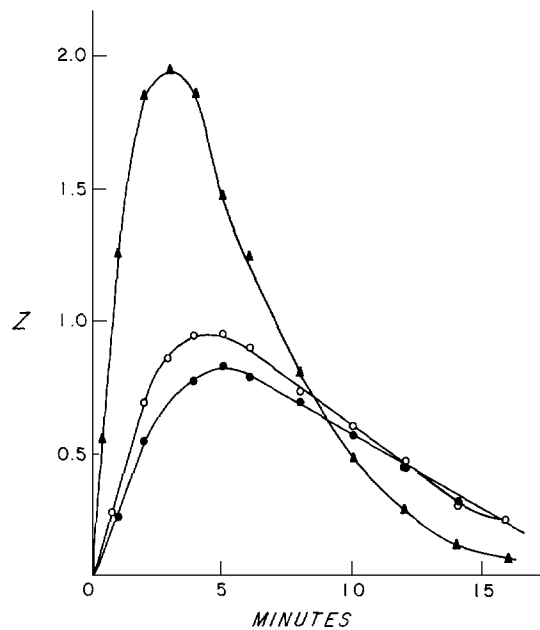


FIG. 5.  $T_{max}$  of cells generated with  $\overline{C1}$  and  $EAC\overline{14}$  ( $EAC\overline{14}$ , ●), the same  $EAC\overline{14}$  treated with Kf (○), and cells generated with  $\overline{C1Kf}$  and  $EAC\overline{14}$  ( $EAC\overline{14Kf}$ , ▲).  $Z$  refers to the number of hemolytically active sites generated by each cellular intermediate during the 16 min of incubation with C2.

$EAC\overline{14}$  cells,  $1 \times 10^9$ . The mixtures continued to be incubated at 30°C, and at time intervals up to 20 min 1 ml samples were removed and added to 1.5 ml of GPC-EDTA.

The results, Fig. 7, show that in  $DGVB^{++}$   $\overline{C1}$  formed 0.58  $\overline{SAC142}$ , with a  $T_{max}$  of 3.5 min; 0.85  $\overline{SAC142}$  were measured in  $GVB^{++}$  with a  $T_{max}$  of 5 min. In contrast  $\overline{C1Kf}$  in  $DGVB^{++}$  generated 1.7  $\overline{SAC1Kf42}$  with a  $T_{max}$  of 1.5 min, but only 1.1  $\overline{SAC1Kf42}$  in  $GVB^{++}$  with a  $T_{max}$  of 3.5 min, suggesting that the effect of Kf was not to create a form of  $\overline{C1}$  capable of moving about the cell.

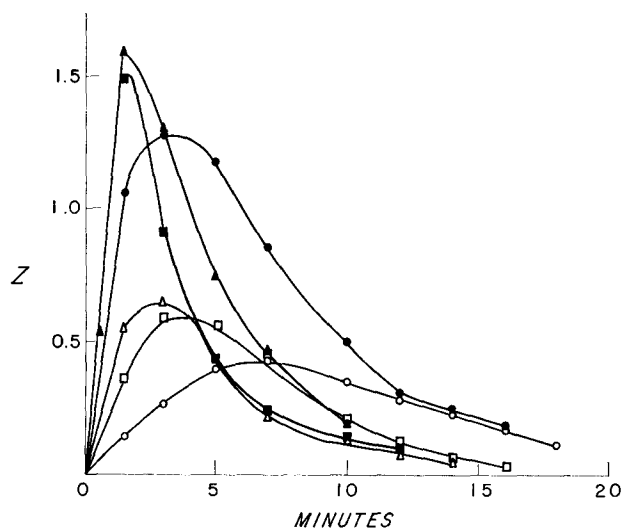


FIG. 6.  $T_{\max}$  developed by  $EAC\bar{4}2$  cells treated with three concentrations of  $C\bar{1}$  ( $\circ = 5$ ,  $\square = 20$ ,  $\triangle = 50$  units/ml) and  $C\bar{1}Kf$  ( $\bullet = 5$ ,  $\blacksquare = 20$ ,  $\blacktriangle = 50$  units/ml).  $Z$  refers to the number of hemolytically active sites generated.

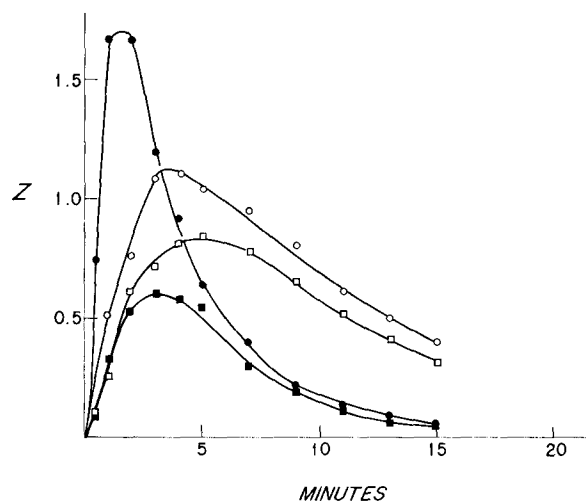


FIG. 7. Kinetics of  $EAC\bar{1}42$  and  $EAC\bar{1}Kf42$  formation by  $EAC\bar{4}2$  and  $C\bar{1}$  or  $C\bar{1}Kf$  in  $DGVB^{++}$  ( $EAC\bar{1}42$ ,  $\blacksquare$ ;  $EAC\bar{1}Kf42$ ,  $\bullet$ ) or  $GVB^{++}$  ( $EAC\bar{1}42$ ,  $\square$ ;  $EAC\bar{1}Kf42$ ,  $\circ$ ).

This point was further investigated in an experiment in which cell-bound  $C\bar{1}$  and  $C\bar{1}Kf$  were compared after washing the cells.

Two samples of  $C\bar{1}$  treated either with buffer or  $Kf$  were serially diluted in  $DGVB^{++}$ , 1/2000-1/32,000. Duplicate 0.5 ml samples of  $C\bar{1}$  or  $C\bar{1}Kf$  received 0.5 ml of  $EAC\bar{4}$  to generate

EAC $\bar{I}4$ . After 30 min at 30°C, one series of EAC $\bar{I}4$  and EAC $\bar{I}Kf4$  samples was centrifuged, washed once in DGVB $^{++}$ , and resuspended in 1 ml DGVB $^{++}$ ; the duplicate series of samples was kept unwashed. The reaction mixtures were developed with C2 and GPC-EDTA.

The results indicated that C $\bar{I}Kf$  generated significantly more hemolytically active sites (12,800 units/ml) than C $\bar{I}$  (1820 units/ml) and that these sites tolerated washing (8450 units/ml) at least as well as those generated without Kf (845 units/ml).

Attention was next directed to the capacity of the effect of Kf to be transferred.

C $\bar{I}$  and C $\bar{I}Kf$  containing 3850 and 20,000 units/ml respectively were both diluted in DGVB $^{++}$  to contain 20 units/ml. Aliquots of each sample were incubated with an equal volume of EA,  $1 \times 10^8$ /ml. After 30 min at 30°C and 15 min at 0°C, both reaction mixtures were centrifuged, and the C $\bar{I}$  present in both supernatants measured. The cells were resuspended to their original volume in DGVB $^{++}$  and the number of C $\bar{I}$  molecules bound to the EA determined by transfer of C $\bar{I}$  from dilutions of the EAC $\bar{I}$  and EAC $\bar{I}Kf$  in GVB $^{++}$  to EAC $\bar{I}4$  cells,  $1 \times 10^8$ . In addition, dissociation of C $\bar{I}$  or C $\bar{I}Kf$  at low ionic strength was also measured by transfer.

The results show that of the 20 C $\bar{I}$  units/ml added to the EA, 5 units were found in the supernatant and 14 units on the cells as assessed by transfer in GVB $^{++}$ . In contrast with Kf-treated C $\bar{I}$  only 0.6 units were found in the supernatant and 10 units by transfer. Although only half of the C $\bar{I}Kf$  input was recovered, the hemolytic activity observed must represent enhanced C $\bar{I}$  molecules since the initial dilution of C $\bar{I}Kf$  was five times greater than that of the starting C $\bar{I}$ ; if enhancement had disappeared during the transfer only 4 C $\bar{I}$  hemolytic units would have been expected. The undetected activity could have either been lost because of disappearance of the Kf effect, or not have been susceptible to transfer because of persistent binding to the donor cell. Neither C $\bar{I}$  nor C $\bar{I}Kf$  transferred well in DGVB $^{++}$ .

#### *Effect of Kf on C $\bar{I}$ Activation.—*

Two 2 ml aliquots of C1 prepared as previously described (14) and C $\bar{I}$  each containing 1000 units/ml were incubated with 0.1 ml Kf or buffer. At 0 time and 5 min intervals up to 60 min 0.01 ml samples were removed and diluted 1:1000 in DGVB $^{++}$ . 0.5 ml samples of each dilution were immediately added to 0.5 ml EAC $\bar{I}42$  cells,  $1 \times 10^8$ /ml. The samples were incubated for 15 min at 30°C and developed with GPC-EDTA. C $\bar{I}$  incubated with buffer produced the formation of 0.32 SAC $\bar{I}42$  at 0 time and 0.45 SAC $\bar{I}42$  at the end of 60 min incubation; incubation with Kf increased the hemolytic activity to 0.75 SAC $\bar{I}Kf42$  at 0 time and 1.02 SAC $\bar{I}Kf42$  at 60 min.

C1 incubated either with buffer or Kf failed to prepare the EAC $\bar{I}42$  intermediate for lysis. The lack of effect of Kf on C1 indicated that its action is restricted to already activated C $\bar{I}$  molecules.

*C3 Utilization by C3 Convertase Generated by  $\overline{C1}$  and  $\overline{C1Kf}$ .—*

Two batches of  $\overline{EAC4}$  cells were converted to  $\overline{EAC14}$  and  $\overline{EAC1Kf4}$  by incubation with either  $\overline{C1}$  or  $\overline{C1Kf}$  diluted to contain 15 units/ml. The cells were washed and resuspended in  $DGVB^{++}$ . 0.5 ml aliquots of C3 diluted to contain 50 units/ml were incubated with 0.5 ml of the intermediate  $1 \times 10^8$ /ml or with buffer. All samples received 0.5 ml C2 containing 100 units/ml. After 30 min at 30°C the cells were sedimented and the C3 in the supernatant fluid was measured.

The results, Fig. 8, show that of the 50 units of C3 added, 47 were present

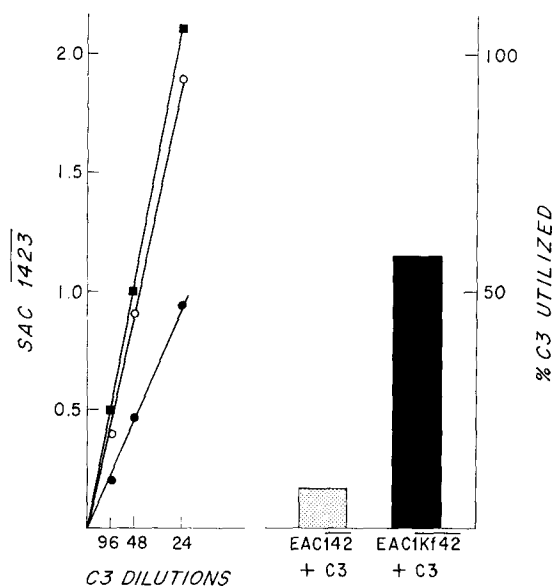


FIG. 8. C3 utilization by  $\overline{EAC142}$  (○) and  $\overline{EAC1Kf42}$  (●) as compared with incubation in buffer (■). The titrations for residual C3 are shown on the left, while the right depicts the per cent of C3 utilization.

in the supernatant after incubation with  $\overline{EAC142}$  cells, and only 22 were detected in the sample incubated with the  $\overline{EAC1Kf42}$  intermediate.

*Effect of Kf on Fluid Phase Functions of  $\overline{C1}$* *Fluid Phase Destruction of C4 by  $\overline{C1}$  and  $\overline{C1Kf}$ .—*

Two aliquots of  $\overline{C1}$  were incubated either with buffer or Kf and a sample of Kf was incubated with buffer alone. Untreated  $\overline{C1}$  containing 400 units/ml and Kf-treated  $\overline{C1}$  exhibiting 1900 units/ml were diluted to contain 50 units/ml. Four aliquots of a single dilution of C4 containing 1300 units/ml were incubated with either buffer, Kf,  $\overline{C1}$ , or  $\overline{C1Kf}$ . After 30 min at 30°C the residual C4 was measured in each reaction mixture.

Of the 1300 units present in the samples incubated with buffer or Kf at the end of the experiment (Fig. 9), 600 units were destroyed by  $\bar{C}1$ , while only 50 were destroyed by the same number of  $\bar{C}1$  hemolytic units present in the  $\bar{C}1$ Kf sample.

*Fluid Phase Destruction of C2 by  $\bar{C}1$ Kf in the Presence and Absence of C4.—*

Samples of  $\bar{C}1$  and  $\bar{C}1$ Kf were diluted to contain 100 units/ml each. Four duplicate 0.5 ml samples of C2 containing 20 units/ml were incubated with 0.5 ml of either buffer, Kf,  $\bar{C}1$ , or  $\bar{C}1$ Kf. One sample of each mixture received 0.5 ml C4, 100 units/ml, the other, 0.5 ml buffer. After 30 min at 30°C, the residual C2 in each sample was measured.

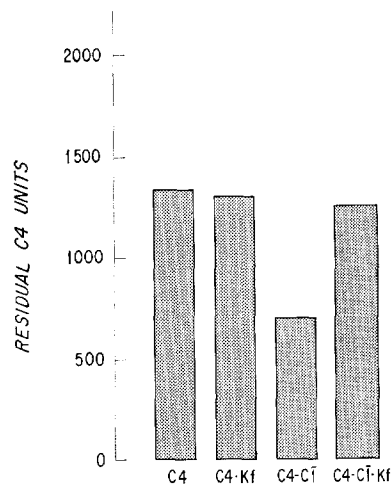


FIG. 9. C4 destruction by  $\bar{C}1$  and  $\bar{C}1$ Kf. Residual C4 in samples incubated with buffer, Kf,  $\bar{C}1$ , and  $\bar{C}1$ Kf.

11 units were destroyed by  $\bar{C}1$ , while  $\bar{C}1$ Kf inactivated 3 C2 units. Of the 18 units detected in the mixture of C2 and C4, 16 were destroyed when  $\bar{C}1$  was added and only 3 when  $\bar{C}1$ Kf was present (Fig. 10). Kf alone did not have an effect on either C4 or C2 alone or in combination.

*Effect of Kf on Human Sera*

In order to examine the effect of Kf on C1 and  $\bar{C}1$  which has not been subjected to chromatographic separation, studies were carried out in normal human sera and in serum from a patient with hereditary angioedema (HAE), deficient in the  $\bar{C}1$ INH.

Two 0.5 ml samples of serum from a patient with HAE, diluted 1:200 in DGVB<sup>++</sup>, were incubated with 0.1 ml Kf or 0.1 ml buffer. After 30 min at 30°C the C1 activity was measured

in dilutions of both samples. The C1 titer in the sample incubated with buffer was 146,000, while incubation with Kf enhanced the C1 activity to 212,000 units/ml.

Although this experiment clearly showed that Kf acted upon C1 in whole serum, it failed to demonstrate whether this effect involved activation of C1, or was only because of an effect on the already activated  $\overline{C1}$  molecules, and thus this point was further examined.

Normal human serum and HAE serum were diluted 1:100 in DGVB<sup>++</sup>. Two 2 ml samples of both diluted sera were incubated at 30°C; one sample of each received 0.1 ml Kf, and the other 0.1 ml buffer. At 0 time and 5 min intervals thereafter up to 60 min, 0.01 ml was removed

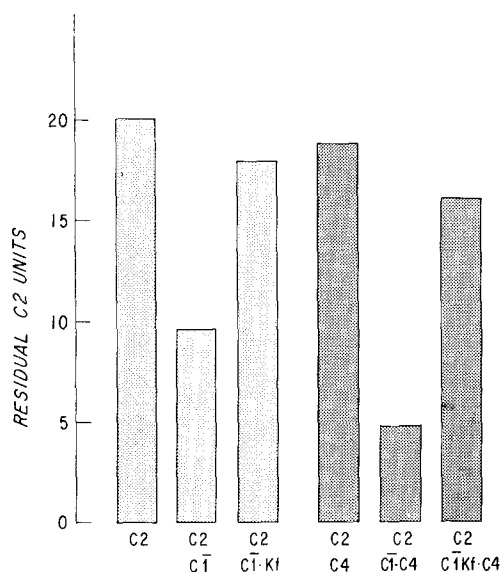


FIG. 10. C2 destruction by  $\overline{C1}$  and  $\overline{C1}$ Kf in the absence and presence of C4. Residual C2 in samples incubated with buffer,  $\overline{C1}$ ,  $\overline{C1}$ Kf, C4, C4 and  $\overline{C1}$ , and with C4 and  $\overline{C1}$ Kf.

and diluted 1:1000 in DGVB<sup>++</sup>. 0.5 ml of each dilution was immediately added to 0.5 ml EAC $\overline{42}$  cells  $1 \times 10^8$  prepared as previously described. The mixtures were incubated for 5 min at 30°C and the reaction developed by the addition of GPC-EDTA. The results are shown in Fig. 11. HAE serum incubated with buffer produced the formation of 0.7 SAC $\overline{142}$  at 0 time of incubation and 0.6 SAC $\overline{142}$  at the end of 60 min.

Incubation of HAE serum with Kf produced 0.94 SAC $\overline{1Kf42}$  at 0 time and 1.50 at the end of 60 min. Normal human serum incubated either with buffer or Kf failed to generate lytic intermediates during the 5 min incubation of diluted serum with EAC $\overline{42}$ . The kinetic data are most consistent with the enhancing effect of Kf being limited to active  $\overline{C1}$  molecules present in HAE sera.

## DISCUSSION

Several years ago a material associated with a partially purified preparation of human kallikrein (30) was recognized as being capable of enhancing the hemolytic activity of fully activated first component of human complement. In the present studies chromatography of human serum to yield the active enzymes of the kinin-forming sequence again revealed an association between the kallikrein-containing fraction and C $\bar{I}$  enhancement (Fig. 1). Peak 1 containing kallikrein also contains IgG and PTA (1, 31). PTA can be partially

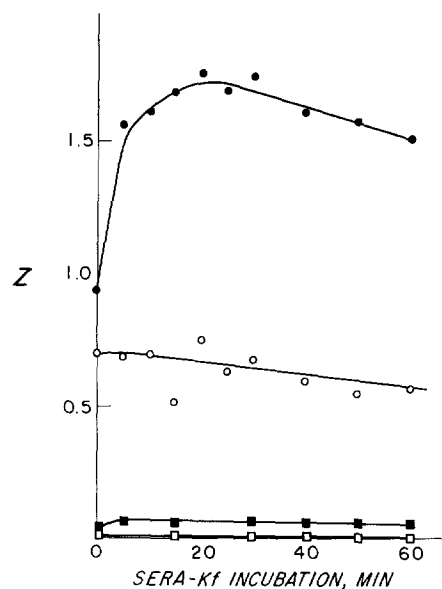


FIG. 11. Effect of Kf on human sera. Normal human serum (□, ■) and HAE serum (○, ●) were incubated either with buffer (□, ○) or Kf (■, ●).

separated from kallikrein and the C $\bar{I}$ -enhancing material by Sephadex G-150 gel filtration (Fig. 4). The kallikrein-rich fraction enhances C $\bar{I}$  hemolytic activity in a dose response fashion (Fig. 2) and its effect is reversed by pretreatment of the kallikrein with SBTI (Fig. 3). The concentration of SBTI required to prevent C $\bar{I}$  enhancement is 100 times greater than that necessary to suppress kinin generation or esterase activity; it may well be that the enhancement of C $\bar{I}$  activity by the kallikrein-rich peak 1 is not directly due to interaction with the active enzymatic site of kallikrein. SBTI does not inhibit PTA (32).

It is noteworthy that the DEAE-cellulose chromatography of serum (Fig. 1) revealed a second region of C $\bar{I}$ -enhancing activity in an area known to contain partially digested Hageman factor fragments but not kallikrein (1, 29). In

addition further chromatography and rechromatography of the kallikrein-rich material by Sephadex gel filtration revealed a continual shift of C $\bar{I}$ -enhancing capacity to material of a molecular weight considerably less than kallikrein (Fig. 4), designated Kf. Kf was distinguished from the Hageman factor fragments by its failure to generate bradykinin from fresh plasma, and from kallikrein by its inability to produce bradykinin from heat-inactivated plasma. The lack of activity against such synthetic amino acid esters as AAME, TAME, AGLMe, or ATMe not only further distinguishes Kf from kallikrein but also from the subunits of C $\bar{I}$ , C $\bar{I}$ r, and C $\bar{I}$ s (33). It is differentiated from C1q on the basis of gel filtration and chromatographic characteristics (34).

The enhancement of C $\bar{I}$  function by Kf could be because of activation of unrecognized precursor molecules, reactivation of active molecules which have somehow decayed, or an increased efficiency of already active C $\bar{I}$  molecules. There is no evidence that Kf will activate isolated precursor C1 or C1 in normal serum or serum from a patient with HAE devoid of C $\bar{I}$ INH (Fig. 11). An effect of Kf to reduce the binding of C $\bar{I}$  could create an apparent increase in efficiency by permitting the molecule to move about the cell so as to create multiple active  $\bar{42}$  sites; however in contrast to C $\bar{I}$ , C $\bar{I}$ Kf demonstrated a reduction of  $T_{\max}$  and  $Z_{\max}$  upon interaction with EAC $\bar{42}$  cells in GVB $^{++}$  (Fig. 7). Further, the EAC $\bar{I}$ Kf $\bar{4}$  intermediate tolerated washing in a manner similar to the EAC $\bar{I}$  $\bar{4}$  intermediate. C $\bar{I}$ Kf tolerated transfer and there was again no evidence of increased dissociability.

The effect of Kf on C $\bar{I}$  was demonstrable only when the interaction occurred in the fluid phase before interaction with the cellular intermediate. When C $\bar{I}$ Kf was interacted with an EAC $\bar{4}$  cell, and the reaction was brought to completion by the addition of C2 and the terminal components, the  $T_{\max}$  was shorter and the  $Z_{\max}$  greater than when C $\bar{I}$  was used either at a comparable dilution of starting C $\bar{I}$  (Fig. 5) or in terms of the same number of apparent hemolytic units. When C $\bar{I}$  and C $\bar{I}$ Kf of comparable hemolytic activity were examined for their capacity to activate a site on the EAC $\bar{42}$  intermediate, C $\bar{I}$ Kf again yielded a shorter  $T_{\max}$  and a greater  $Z_{\max}$  than was achieved by the introduction of C $\bar{I}$  (Fig. 6). The capacity of C $\bar{I}$ Kf to yield a greater  $Z_{\max}$  and a shorter  $T_{\max}$  than C $\bar{I}$  even when employed at a greater dilution so as to contain comparable hemolytic activity in a C $\bar{I}$  titration suggests that the effect of Kf is to yield a more efficient molecule and not reactivate molecules which have somehow lost their activity, unless such reactivated molecules are more efficient than those still in the starting state.

That Kf does indeed lead to a functionally altered form of C $\bar{I}$  is supported by finding that fluid phase C4 and C2 are spared from inactivation (Figs. 9 and 10). If failure to rapidly inactivate is synonymous with more efficient utilization of C4 and C2, then the capacity of C $\bar{I}$ Kf to activate cell-bound  $\bar{42}$  (Fig. 6) to give a shorter  $T_{\max}$  and a greater  $Z_{\max}$  than C $\bar{I}$  would be expected.



Similarly the EAC $\bar{1}$ Kf $\bar{4}$  intermediate would utilize fluid phase C2 to give a shorter  $T_{\max}$  and  $Z_{\max}$  than the EAC $\bar{1}$  $\bar{4}$  intermediate as was observed (Fig. 5). The view that C $\bar{1}$ Kf permits a more efficient utilization of C4 and C2 so as to generate more  $\bar{4}$  sites as recognized by a shorter  $T_{\max}$  and a greater  $Z_{\max}$  (Fig. 5 and 6) would be consistent with the finding of increased C3 inactivation by an EAC $\bar{1}$ Kf $\bar{4}$  cell (Fig. 8).

#### SUMMARY

An activity designated Kf can be separated from human serum and shown to give a 100–300% enhancement in the hemolytic activity of fully activated, fractionated C $\bar{1}$ . The enhancement of C $\bar{1}$  activity is not because of activation of precursor C1 and it is not attributable to an effect on C $\bar{1}$  binding. EAC $\bar{4}$ 2 or EAC $\bar{4}$  intermediates interacted with C $\bar{1}$ Kf exhibit a greater  $T_{\max}$  and shorter  $Z_{\max}$  than when such intermediates are reacted with the same number of hemolytic units of C $\bar{1}$ . C3 consumption by the EAC $\bar{1}$ Kf $\bar{4}$ 2 intermediate greatly exceeds that of the EAC $\bar{1}$  $\bar{4}$ 2 intermediate produced from the same EAC $\bar{4}$  cells by comparable inputs of the other two complement components. Taken together, these findings suggest that Kf-treated C $\bar{1}$  achieves more efficient utilization of C4 and C2 to create a larger number of  $\bar{4}$  sites as appreciated on the intermediates by shorter  $T_{\max}$  and a greater  $Z_{\max}$ , and an increased capacity to utilize C3. The capacity of Kf to enhance C $\bar{1}$  upon introduction into whole serum of a patient with hereditary angioedema (HAE) in a manner comparable to its effect on fractionated C $\bar{1}$  suggests that the effect of Kf may be pertinent to certain pathophysiologic conditions of man.

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