QUANTITATIVE STUDIES ON THE PROPERDIN-COMPLEMENT SYSTEM

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Preliminary studies indicate that properdin may play a significant role in maintaining the resistance of the body against bacteria (1, 2), viruses (3), and postradiation bacteriemia (4). Furthermore, Pillemer and coworkers have demonstrated (5, 6) that properdin is involved in the reaction between zymosan and the complement component, C'3, a system which is amenable to study *in vitro*. These workers have proposed an assay for properdin in which dilutions of test serum, zymosan, and RP[1] are incubated together, after which residual C'3 is determined. Since little information was available as to the nature of the reaction, it was explored, utilizing a new and more precise technique for determining residual C'3.

Materials and Methods

Human Complement (Hu C').[2]—The pooled serum from healthy human donors was distributed in small vials and kept frozen at −20°C.

Sensitized Sheep Cells (EA).[3]—Sheep cells[4] preserved with trisodium ethylenediaminetetraacetic acid,[5] (Na_3E.D.T.A.)[6] and penicillin (7), were standardized and sensitized by the method of (8).

Buffer.—Veronal buffer (9) containing 0.00015 M Ca^{++} and 0.0005 M Mg^{++} was used for all dilutions and washings.

Zymosan.—Preparation LE-1[4] was used throughout this work and was activated before use as described in reference 10.

EAhuC'A.—The EAhuC'A complex (11), the combination of EA with what are believed

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[1] RP is serum devoid of properdin but containing all the components of complement (C'1, C'2, C'3, and C'4).

[2] Hu C', human complement.

[3] EA, sensitized sheep cells.

[4] Kindly supplied by Dr. L. Pillemer.

[5] Kindly supplied as the 3x recrystallized disodium salt by Alrose Chemical Co., Providence.

[6] E.D.T.A., ethylenediaminetetraacetic acid.

to be C'1, C'2, and C'4, was prepared by addition of 20.0 ml. of a sublytic dilution of hu C', usually hu C'/100, to a mixture of 40.0 ml. of EA and 40.0 ml. of buffer at 37°C. After 2 to 3 minutes, 2 volumes of chilled saline were added, the mixture was rapidly cooled to 4–6°C. and aliquots of this standard suspension of $EAhuC'_A$ were added to chilled tubes. After centrifugation in the cold, the supernatants were poured off, the tubes were wiped with filter paper, and the $EAhuC'_A$ button broken up by tapping the tubes with the fingers. Once formed, $EAhuC'_A$ deteriorates rapidly unless precautions are taken to maintain the temperature at 4–6°C.

Kinetic Technique.—All reactions were carried out in baths controlled to ±0.1°C., using a wrist action shaker for mixing.

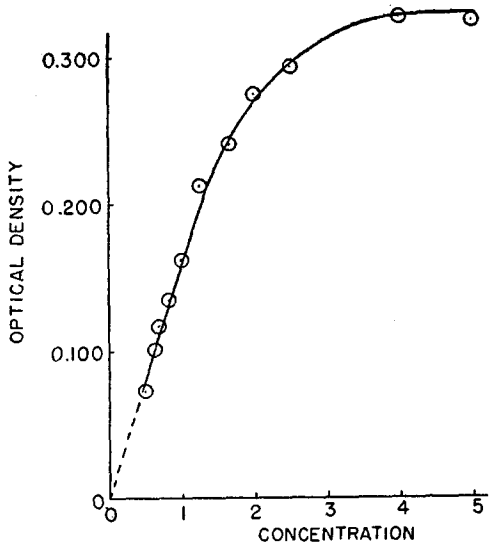

FIG. 1. The effect of varying quantities of serum 0.0075 M in $Na_3E.D.T.A.$ on a constant quantity of $EAhuC'_A$.

RESULTS

Effect of Varying C'3 on Lysis of $EAhuC'_A$.—It has already been shown (11) that hu C'3, in common with guinea pig C'3 (12) is active in the absence of divalent cations. Thus, human serum treated with $Na_3E.D.T.A.$ can provide C'3 whereas it cannot provide C'_A since the formation of $EAhuC'_A$ requires Ca^{++} (11).

$EAhuC'_A$ were prepared as described under Materials and Methods. In this particular experiment, 5.0 ml. aliquots of the diluted mixture were added to the chilled tubes. The packed $EAhuC'_A$ obtained were resuspended in the cold with 3.5 ml. of varying dilutions of hu C', 0.0075 M in $Na_3E.D.T.A.$ The mixtures were incubated for 1 hour at 32°C., the optimal temperature for lysis by hu C' (13), and then centrifuged. The optical densities of the supernatants are plotted (Fig. 1) against the relative concentration of C'3 added.

A direct relationship between added C'3 and lysis exists over a considerable range. Thus, by adding suitable dilutions of standard and unknown serum to EAhuC'$_A$ and comparing the slopes of the linear portions of the plots of lysis *vs.* concentration, it is possible to have a precise measure of C'3 content. If an accurate measure of variations in the C'3 content of a single pool, subjected to varying conditions, is all that is necessary, the standard serum may be omitted, provided dilutions are used that fall on the linear portion of the curve.

Kinetics of the Reaction between Properdin, Zymosan, and C'3 at Various Temperatures.—Having established a method for quantitative measurement of C'3, the interaction between properdin, zymosan, and C'3 was studied at 37°C., 32°C., 27°C., and 22°C. Two consecutive reactions are involved here:

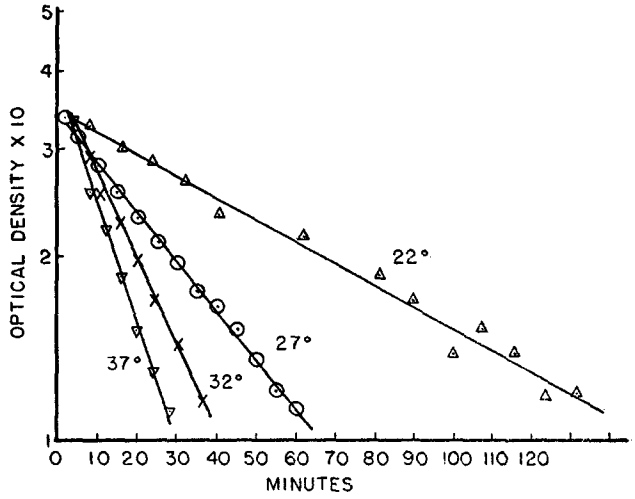

FIG. 2. The effect of temperature on the kinetics of the over-all reaction between zymosan, properdin and C'3.

first, the combination of properdin with zymosan to form the PZ complex; and second, the reaction of PZ with C'3.

10.0 ml. of human serum equilibrated at the temperature of the experiment were added at 0 time to a tube containing 10 mg. of zymosan. 0.5 ml. samples were withdrawn at intervals, mixed with 1.0 ml. of cold buffer, and centrifuged immediately. 1.0 ml. portions of the supernatants were diluted with 6.0 ml. of cold buffer and 0.3 ml. of 0.15 M Na_3E.D.T.A. 3.5 ml. aliquots of the cold, diluted supernatants were added to tubes containing packed EAhuC'$_A$ (from 5.0 ml. of standard EAhuC'$_A$ suspension) which were mixed, stoppered, and incubated at 32°C. for 1 hour. The optical densities obtained are plotted against the time of sampling on semilog paper (Fig. 2).

The data obtained fit first order kinetics quite well. To obtain the energy of activation of the over-all reaction, the logarithms of the rate constants (obtained from the data of Fig. 2) were plotted against the reciprocals of the ab-

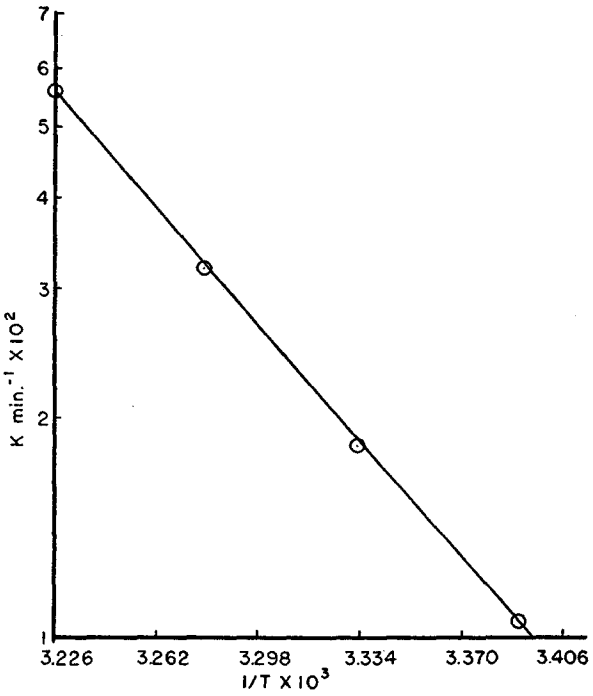

FIG. 3. The relationship between reaction rate and the reciprocal of the absolute temperature at which the reaction was run.

TABLE I

Protocol for Experiment 3. Varying Dilutions of Serum Set up with a Constant Concentration of Zymosan

Flask	Serum dilution	Serum	Zymosan	T_0	Sample volume	Buffer	0.15 M NaE.D.T.A.
		ml.	*mg.*	*min.*	*ml.*	*ml.*	*ml.*
A	1/16	40	40	0.0	4.0	2.0	0.3
B	1/8	20	20	0.5	2.0	4.0	0.3
C	1/4	10	10	0.9	1.0	5.0	0.3
D	1/2	5	5	1.3	0.5	5.5	0.3
E	Undiluted	5	5	1.6	0.5	11.5	0.6

solute temperatures (Fig. 3). A value of 20,500 calories per mole was found. This value represents the activation energy of the rate-limiting reaction. At present it is not known whether the formation of PZ or the reaction of PZ with C'3 is rate-limiting.

Effect of Varying Serum Concentration.—Varying dilutions of serum were incubated at 32°C. with zymosan at a final concentration of 1 mg./ml. as shown in Table I.

The volumes sampled from each series were chosen so that when added to buffer and Na_2E.D.T.A. the final dilution of serum, in all cases, would be 1 up to 25.2. The Na_2E.D.T.A. was used to bind Mg^{++} and thus block further reaction of zymosan, and of PZ (5). 3.5 ml.

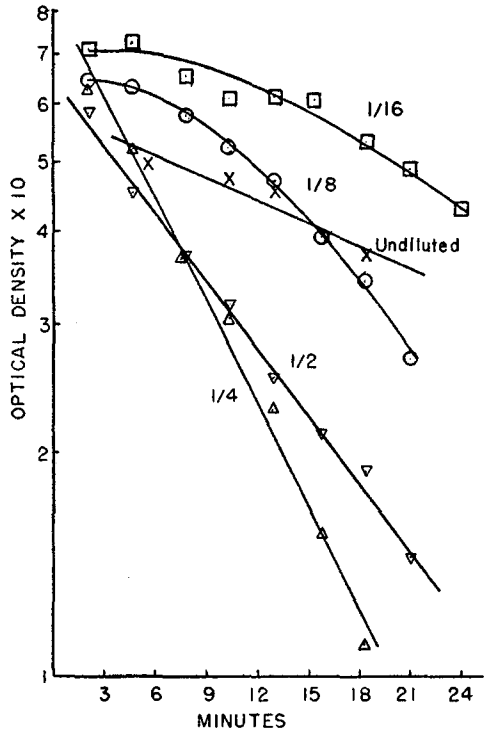

FIG. 4. The effect of serum dilution on the reaction of zymosan with properdin and C'3.

aliquots of the diluted samples were added to the packed cells obtained from 10.0 ml. of standard EAhuC'$_A$ suspension and incubated at 32°C. for 1 hour.

Inspection of the results, (Fig. 4), shows that the concentration of serum has a profound effect on the rate as well as on the order of the reaction. In dilute serum (1/8, 1/16) the rate of inactivation of C'3 does not become appreciable for some time and then rapidly increases. This suggests that the formation of the PZ complex may be slow at these serum dilutions. Once formed, the PZ rapidly inactivates the C'3 present. The decrease in reaction rate found with sera more concentrated than 1/4 does not appear to affect the first

order kinetics of the reaction. The cause of this phenomenon is currently under investigation.

Effect of Varying Zymosan Concentration.—

Varying concentrations of zymosan were incubated with 5.0 ml. of a 1/2 dilution of serum at 32°C. 0.5 ml. samples were pipetted into a mixture of 6.0 ml. of cold buffer and 0.3 ml. of 0.15 M $Na_3E.D.T.A.$ Analyses for relative C'3 content were carried out as in the previous expt.

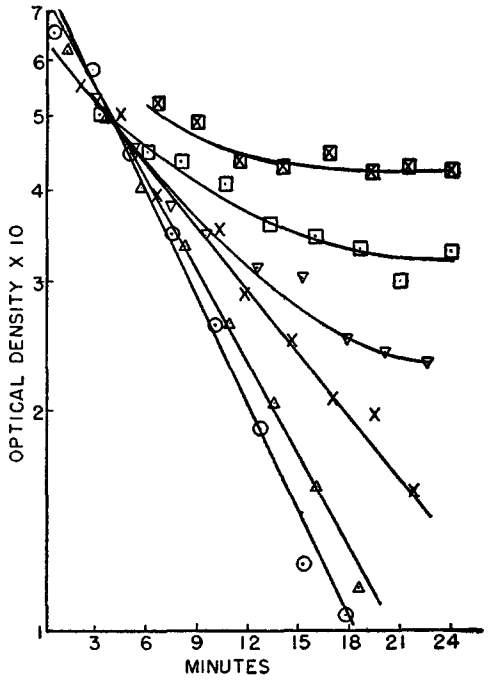

FIG. 5. The effect of zymosan concentration on the reaction between zymosan, properdin and C'3. The quantities of zymosan added were ⊙, 10 mg., △, 7.5 mg., ×, 5 mg., ▽, 3.75 mg., ⊡, 2.5 mg., ⊠, 1.25 mg.

From Fig. 5 it appears that the reaction follows first order kinetics provided an excess of zymosan is present. At low zymosan concentrations the reaction tapers off, possibly owing to saturation of PZ with C'3, or to inactivation of the small amount of PZ present in the course of reacting with excess C'3.

Kinetics of the Reaction between Dextran, Properdin, and C'3.—

1.0 ml. samples of dextran No. B1355 S4[7], 5 mg./ml., were added at zero time to 6.0 ml. of human serum at both 32 and 37°C. 0.5 ml. samples were mixed with 1.0 ml. of cold

[7] Obtained through the courtesy of Dr. E. A. Kabat.

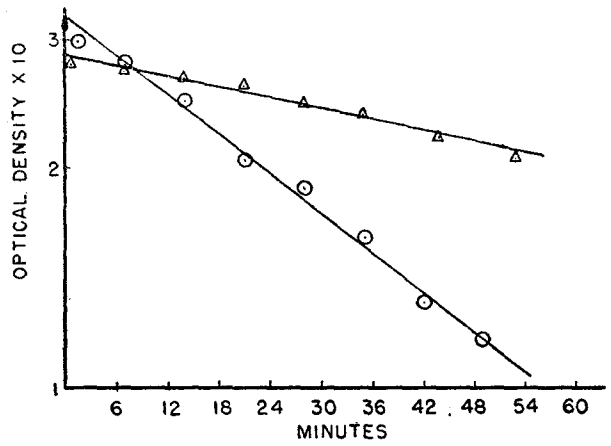

FIG. 6. Kinetics of the over-all reaction between dextran B1355 S4, properdin and C'3 at 37°C., ⊙, and 32°C., △.

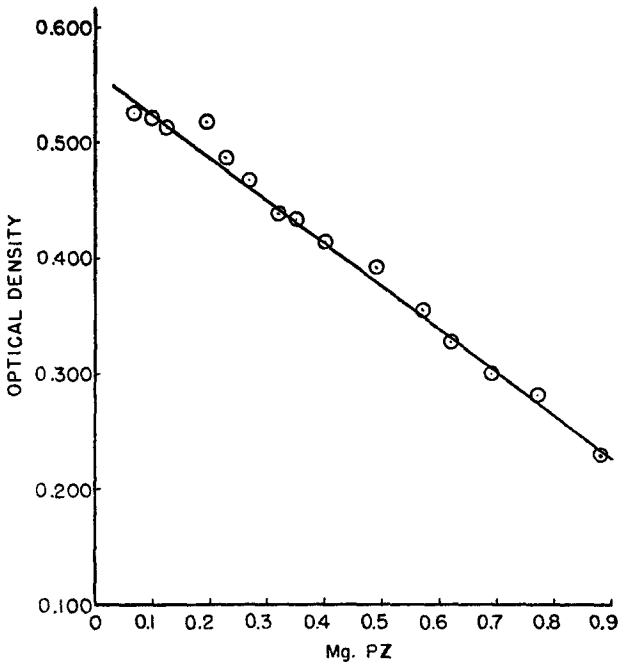

FIG. 7. Effect of varying quantities of PZ on a constant amount of C'_3.

buffer and 0.075 ml. of 0.15 M $Na_3E.D.T.A.$ 1.0 ml. of these mixtures were added to tubes containing 6.0 ml. of buffer and 0.35 ml. of 0.15 M $Na_3E.D.T.A.$ The analyses for C'3 were carried out as in the previous experiment.

From the data, graphed in Fig. 6, an activation energy of 48,000 cal./mole was calculated. The reaction is far more temperature-sensitive than that involving zymosan and may well involve different spatial configurations of the glucose residues.

Stoichiometry of the Reaction Between PZ and C'3.—

20 mg. of zymosan was shaken with 10 ml. of hu C' for 1 hour at 16.3°C., to form the properdin-zymosan complex (PZ) (5). PZ was centrifuged off in the cold, washed once with 5 ml. of cold buffer and resuspended in 10 ml. of cold buffer to give a final concentration of 2 mg./ml. A series of dilutions of PZ in cold buffer was prepared using calibrated pipettes. 1.0 ml. portions of these dilutions were added to 1.0 ml. of RP that had been absorbed with zymosan at both 17°C. and 37°C. (6). The mixtures were incubated at 37°C. for 1 hour. 0.5 ml. of the mixtures were then added to chilled tubes containing 3.0 ml. of cold buffer and 0.2 ml. of 0.15 M $Na_3E.D.T.A.$ 3.5 ml. of these dilutions were mixed with the packed cells from 10.0 ml. of $EAhuC'_A$, and incubated for 1 hour at 32°C.

The optical densities obtained are plotted against the quantity of PZ added in Fig. 7. A direct proportionality between PZ and optical density or C'3 was found.

DISCUSSION

The properdin titration proposed by Pillemer and coworkers (5), permits complications introduced by the unknown serum at both stages of the reaction; the formation of PZ and the reaction of the PZ with C'3. That such complications do occur is evident from the data (Fig. 4) showing that undiluted serum can inhibit the reaction. It is possible, however, to eliminate any interference with the second stage of the reaction, by the unknown, if the procedure of the last experiment is used. The unknown is incubated with RP and zymosan at 15°C. for 75 minutes. The PZ complex is centrifuged in the cold, washed once with cold buffer, and resuspended in a known volume of cold buffer. A measured amount of PZ suspension is added to RP and incubated at 37°C. for 1 hour. The residual C'3 is titrated with $EAhuC'_A$. Since the optical density obtained is directly proportional to the quantity of PZ added, the value obtained with the unknown is simply fitted on the line found with a standard serum pool as in Fig. 7. This method is currently being utilized in a study of the kinetics of the properdin zymosan reaction.

Considerable interest has been aroused by the possibility that properdin levels may be of clinical importance. There is a question, however, as to whether the total properdin content of sera is as important as what may be termed the available properdin. Since unknown cofactors are required for properdin to react *in vitro*, some or all of these factors may be necessary for the *in vivo* re-

action of properdin. If this is true, there may be sera in which the total properdin content is normal, but the amount of properdin available for reaction is limited, owing to a deficiency of some cofactor. This situation is not detectable by Pillemer's assay technique, since the RP added masks the absence of cofactors in the unknown serum. Any investigation attempting to correlate properdin levels with clinical findings should, therefore, measure both total properdin and available properdin. The total properdin content of a serum is measured by forming PZ with RP added to ensure that an optimal concentration of cofactors is present. The available properdin of a serum is measured by forming the PZ complex without any added RP. The quantity of PZ formed will, therefore, depend on the concentration of cofactors present in the serum.

SUMMARY

Quantitative methods have been developed for the estimation of C'3 and properdin. With these methods, the kinetics of the over-all reaction between properdin, zymosan, and C'3 were investigated. The reaction followed first order kinetics, provided zymosan was in excess, and the serum not diluted beyond 1/4. The reaction rate was proportional to the temperature, and an activation energy of 20,500 calories/mole was found.

The distinction between total properdin and available serum properdin is discussed.

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