QUANTITATIVE STUDIES ON THE PROPERDIN-COMPLEMENT SYSTEM

II. Kinetics of the Reaction between Properdin and Zymosan

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Pillemer and coworkers have demonstrated two consecutive steps in the reaction between properdin, zymosan, and the third component of complement (C'3). First, the formation of a complex between properdin and zymosan; second, the reaction of the complex with C'3 (1, 2). In the first paper of this series (3), quantitative methods for the determination of human C'3 based, to a large extent, on ideas developed by Mayer and coworkers in studies of guinea pig C' (4), were applied to a study of the kinetics of the over-all reaction between properdin, zymosan, and C'3. In this paper, kinetic data on the reaction of properdin with zymosan will be presented.

Nomenclature

The following abbreviations will be used:—

Human complement	HuC'
The four recognized components of C'	C'1, C'2, C'3, C'4
EA	Sensitized sheep cells
$EAhuC'_A$	The complex between EA and what are believed to be C'1, C'2 and C'4 of HuC'
Properdin	P
Zymosan	Z
Properdin-zymosan complex	PZ
RP	Serum lacking properdin
R3	Serum lacking properdin and C'3
$Na_3E.D.T.A.$	Trisodium ethylene-diaminetetraacetic acid
$EAg.p.C'_{1,4,2}$	The complex between EA and C'1, C'4 and C'2 of guinea pig C'

Materials and Methods

HuC'.—The pooled serum from healthy human donors was distributed in small vials and kept frozen at −60°C.

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EA.—Sheep cells preserved with Na_3E.D.T.A. and penicillin (5) were standardized and sensitized by the method described in reference 6.

Buffer.—Veronal buffer (7) containing 0.00015 M Ca^{++} and 0.0005 M Mg^{++} was used for all dilutions and washings except where noted.

E.D.T.A. buffer.—Veronal buffer, 0.008 M in Na_3E.D.T.A. was used to bind divalent cations.

Zymosan.—Preparation 5B171[1] was used throughout this work and was activated before use as described in (8).

EAhuC'$_A$.—The EAhuC'$_A$ complex (9), was prepared either by addition of 20.0 ml. of a sublytic dilution of huC', usually huC'/100, or in later work, of R3/20 to a mixture of 40.0 ml. of EA and 40.0 ml. of buffer at 37°C. After 1 to 3 minutes, 2 volumes of chilled saline were added, the mixture was rapidly cooled to 4–6°C. and 15.0 ml. 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. Lysis of EAhuC'$_A$ was carried out at 32°C. (10) for 1 hour.

RP.—Pooled human serum, from which the properdin had been absorbed by treatment with zymosan at 15°C. and then twice at 37°C., was the source of RP. Each batch of RP was then tested for loss of C'3 on incubation with fresh zymosan at 37°C. Only RP showing no loss of C'3 within the limits of error of the experimental method was used.

R3.—Human serum, diluted 1 → 2 was shaken for one hour at 37°C. with 2.5 mg. of zymosan per ml. After centrifugation the R3 was frozen at −60°C. This type of preparation is an R3 only in the sense that it lacks C'3 and can form EAhuC'$_A$. It may not meet the criteria set up for R3 in reference (11).

Kinetic Technique.—All reactions were carried out in baths controlled to ±0.1°C. Mixing was accomplished by using a wrist action shaker. Pipettes cooled in cold buffer were used in all experiments carried out below room temperature.

EXPERIMENTAL

Rate of Inactivation of C'3 by Two Preparations of Zymosan.—Pillemer and coworkers (12) have shown that zymosans vary widely in their activity. Therefore, the activity of 5B171 was compared with that of preparation LE-1,[2] used in the earlier work (3).

5.0 ml. portions of serum were shaken with 10 mg. of LE-1 or 10 mg. of 5B171 at 32°C. 0.5 ml. samples were withdrawn at intervals, added to 1.0 ml. cold buffer, mixed and centrifuged immediately. The supernatants were diluted 1 → 11 using cold E.D.T.A. buffer. 3.5 ml. portions of these dilutions were added to packed EAhuC'$_A$, mixed, stoppered and incubated at 32°C. for 1 hour.

The results, plotted in Fig. 1, show that LE-1 and 5B171 remove C'3 from serum at about the same rate.

Effect of E.D.T.A. on the Reaction of Zymosan with Properdin.—

At 15°C. 5.0 ml. portions of human serum were mixed with Na_3E.D.T.A. and 5B171 as shown in Table I. 0.5 ml. aliquots were removed at intervals and pipetted into 6.0 ml. of ice cold E.D.T.A. buffer. The mixtures were centrifuged and washed once with saline in the

[1] Purchased from Standard Brands, Inc.

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

cold, to remove residual serum and E.D.T.A. The precipitates were broken up by tapping, and analyzed for reactivity towards C'3 as follows: 1.0 ml. of RP/4.5 was added to each tube and the suspension incubated, with shaking, at 37°C. for 1 hour. The tubes were chilled and mixed with 6.0 ml. portions of cold E.D.T.A. buffer. The residual C'3 was determined by adding 4.0 ml. aliquots to packed EAhuC'$_A$. The mixtures were stoppered and incubated

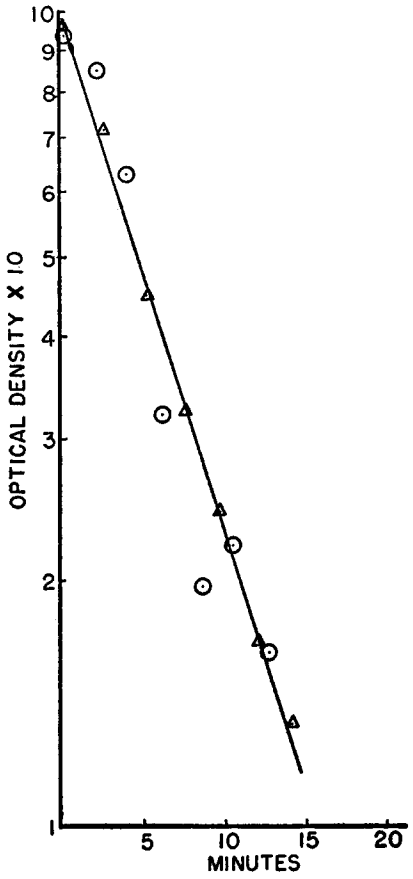

FIG. 1. Inactivation of C'3$_A$ by zymosans LE-1, △, and 5B171, ⊙.

at 32°C. for 1 hour. A blank, consisting of 4.0 ml. of E.D.T.A. buffer plus EAhuC'$_A$ was run concurrently.

While E.D.T.A. stops the formation of PZ, (*cf.* Fig. 2), there may be some dissociation of the complex at 15°C. in the presence of E.D.T.A. Attempts are now being made to isolate properdin from PZ using E.D.T.A.

The Stability of PZ in Cold E.D.T.A. Buffer.—

Two 3.5 ml. aliquots of serum were incubated with 7 mg. portions of 5B171. After 26.4 minutes and 36.0 minutes respectively, at 15°C., 36 ml. of cold E.D.T.A. buffer were added.

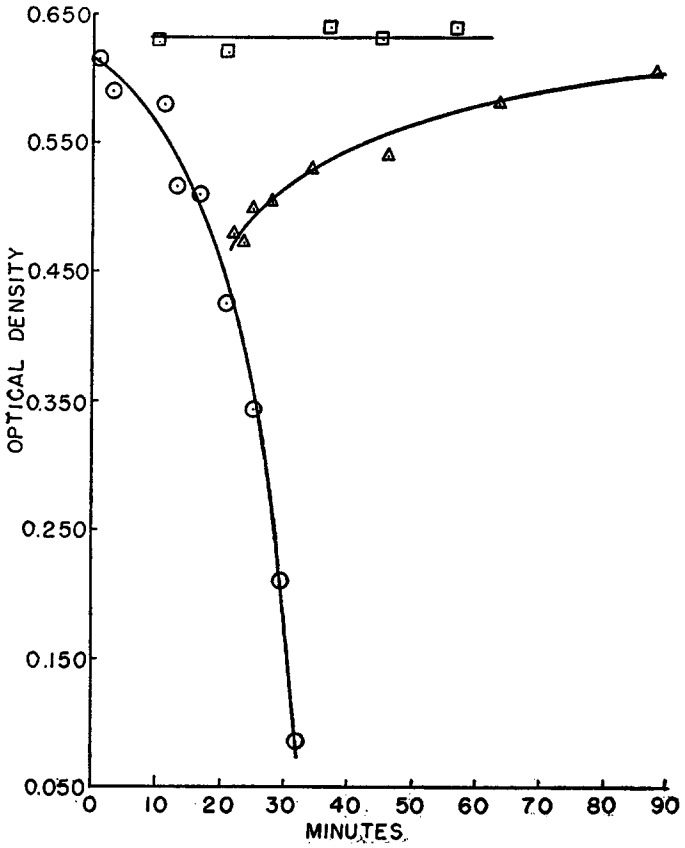

FIG. 2. Effect of E.D.T.A. on the reaction of zymosan with properdin; ⊙, no E.D.T.A.; ⊡, E.D.T.A. added at 12.8 minutes; △, E.D.T.A. added at 36.3 minutes.

TABLE I

Effect of Na_3E.D.T.A. on the Reaction of Zymosan with Properdin

Serum	5B171*	‡t5B171	Na_3E.D.T.A.§	tE.D.T.A.	Sample volume	E.D.T.A. buffer
ml.	*ml.*	*min.*	*ml.*	*min.*	*ml.*	*ml.*
A 5.0	0.5	2.5	—	—	0.5	6.0
B 5.0	0.5	2.8	0.5	12.8	0.5	6.0
C 5.0	0.5	14.3	0.5	36.3	0.5	6.0

* 20 mg./ml.

‡ *t*, time of addition.

§ 0.15 *M* diluted ½ with veronal buffer.

Aliquots measuring 6.0 ml. were pipetted into cold test tubes. At approximately 15 minute intervals, a tube of each series was centrifuged in the cold, washed with cold saline, and tested for activity towards C'3.

The results (*cf.* Table II) show that no significant dissociation of PZ occurs in 75 minutes at 0°C.

TABLE II

Stability of PZ in Cold E.D.T.A. Buffer

T sample	Optical density*	
min.		
0	0.511	0.045
15	0.482	0.092
30	0.530	0.040
45	0.475	0.058
60	0.488	0.065
75	0.480	

* The optical density found with RP unabsorbed by PZ was 0.780.

TABLE III

The Stability of Human C'3 in E.D.T.A. Buffer

Sample	Optical density	
	Fresh sample	24 hr. sample
1	0.270	0.257
2	0.242	0.225
3	0.139	0.126
4	0.073	0.074
5	0.048	0.030

Stability of Human C'3 in E.D.T.A. Buffer.—It became apparent in preliminary experiments on the formation of PZ that considerable economy in time and effort would result from doing the kinetic experiments one day and analyzing for residual C'3, (using EAhuC'$_A$), the next. Accordingly, an experiment was set up to determine the stability of human C'3 in E.D.T.A. buffer.

4.0 ml. aliquots of a series of dilutions of human serum in E.D.T.A. buffer, (from an experiment on the formation of PZ), were added to packed EAhuC'$_A$ and the mixtures incubated at 32°C. for 1 hour. The dilutions were reserved in the cold room for 24 hours for a repeat of the experiment, using fresh EAhuC'$_A$.

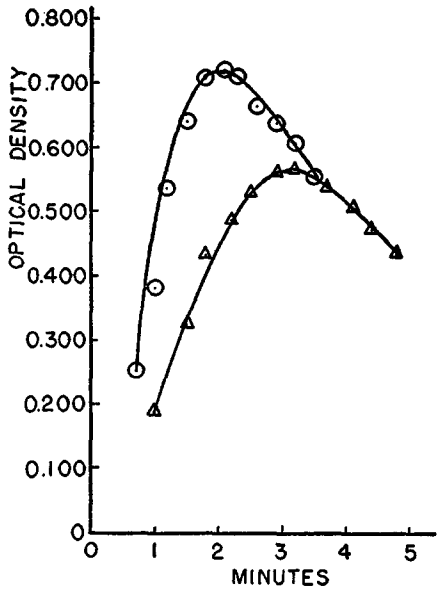

FIG. 3. Lysis of EAhuC'$_A$, formed with R3/20, ⊙; huC'/100, △.

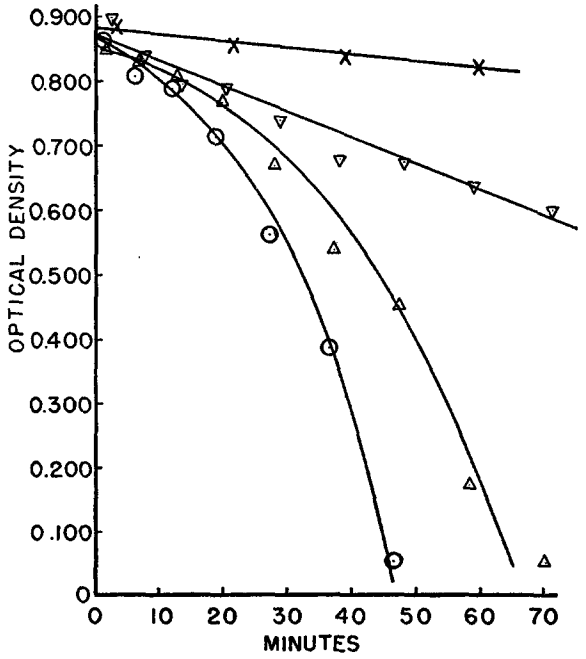

FIG. 4. Effect of varying serum concentration on the reaction of properdin with zymosan; ⊙, undiluted; △, 1 → 2; ▽, 1 → 4; ×, 1 → 8.

The data are presented in Table III, and indicate that human C'3 is stable for 24 hours at 4°C.

Lysis of EAhuC'$_A$ Formed with R3.—In the preparation of RP by absorption with zymosan at both 15°C. and 37°C., ⅓ to ⅔ of the total C'3 is usually lost. This necessitated increasing the sensitivity of the test for C'3 and was

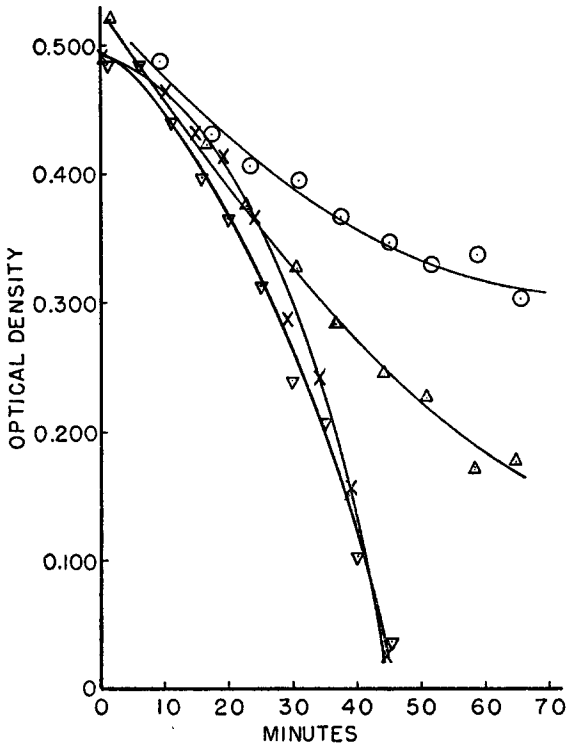

FIG. 5. Effect of varying concentrations of zymosan on the reaction of zymosan with properdin; ×, 1.8 mg./ml.; ▽, 0.91 mg./ml.; △, 0.46 mg./ml.; ⊙, 0.23 mg./ml.

accomplished by substitution of R3 for whole serum in forming EAhuC'$_A$. R3 contains most of the huC'$_A$ in serum (9), and dilutions of R3/10 or R3/20 could be used as routine to prepare EAhuC'$_A$, whereas whole serum usually had to be diluted to 1/80 to avoid large blanks. The lysis obtained by using R3 to form EAhuC'$_A$ is compared with that obtained with huC'/75 in Fig. 3. The experiment followed the technique described in reference (9).

Effect of Varying Serum Concentration on the Reaction of Zymosan with Properdin.—

At 15°C., 0.5 ml. of 5B171 (20 mg./ml.) were added to 4.5 ml. of varying dilutions of human serum. 0.5 ml. aliquots were removed at intervals and pipetted into 6.0 ml. of ice cold E.D.T.A. buffer. The mixtures were analyzed for reactivity with C'3 as usual.

This experiment measures the rate of formation of a complex capable of inactivating C'3. The quantity of C'3 inactivated is directly proportional to the quantity of complex (3). The data, plotted in Fig. 4, showed an increase

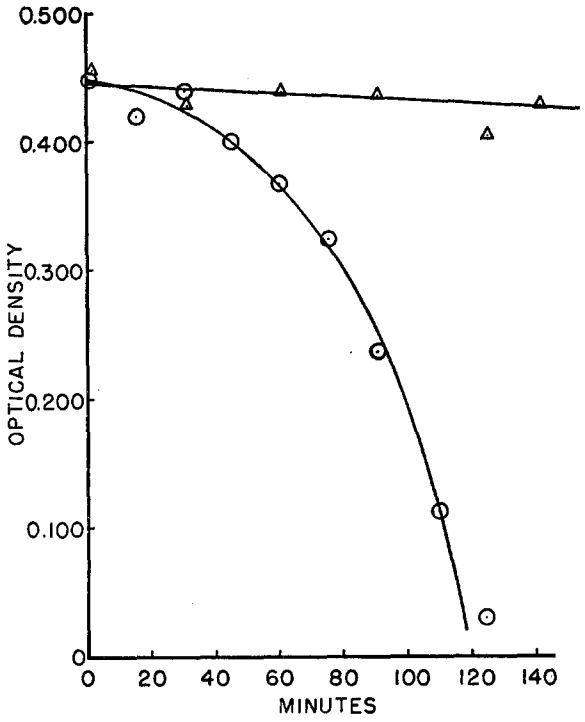

FIG. 6. Effect of temperature on the reaction of properdin with zymosan; △, 5°C.; ⊙, 10°C.

in the rate of formation of the complex with time, an indication that the reaction of properdin with zymosan was complex. The same type of curve was obtained when zymosan LE-1 was substituted for 5B171, and when bovine or pig serum was substituted for human serum.

Effect of Varying Concentrations of Zymosan on the Reaction of Properdin with Zymosan.—

0.5 ml. of varying concentrations of 5B171 were added to 5.0 ml. portions of serum/2 at 15°C. Sampling and testing for the formation of PZ were carried out as described in the previous experiment. The results are shown in Fig. 5.

The kinetics of the reaction are seen to be markedly dependent on the concentration of zymosan. An induction period is noted only with the highest concentration of zymosan (2 mg./ml.).

Effect of Temperature on the Reaction of Properdin with Zymosan.—

5.0 ml. portions of human serum, diluted 1 → 2, were incubated with 0.5 ml. of 5B171 (20 mg./ml.) at 10°C. and 5°C. Sampling and determination of the rate of formation of

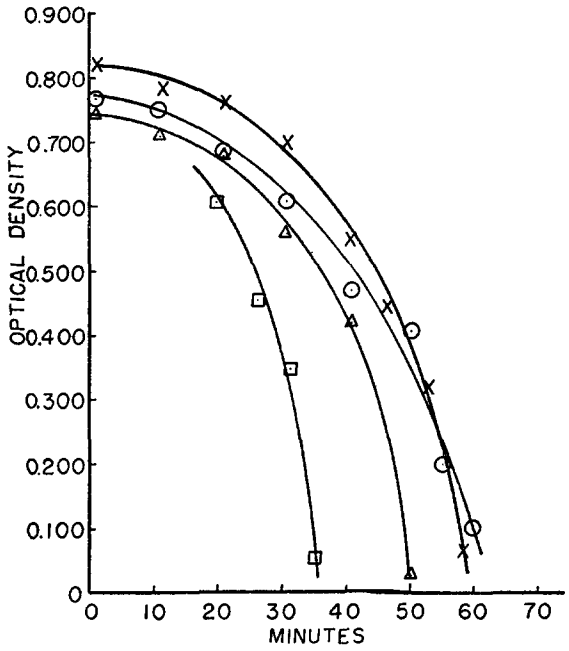

FIG. 7. Effect of pH on the reaction of properdin with zymosan; ×, tris buffer final pH 8.1; △, tris buffer final pH 7.6; ⊙, veronal buffer final pH 7.6; ⊡, acetate buffer final pH 6.2. All buffers contained 5×10^{-4} M Mg^{++}, and 5×10^{-2} M NaCl in a final molarity of 0.15.

complex capable of reacting with C'3 was carried out as usual. The results are shown in Fig. 6.

While active complex was formed at 10°C., none was detected at 5°C. It is, of course, possible that the induction period at 5°C. is longer than the time of the experiment.[3]

Effect of pH on the Reaction of Properdin with Zymosan.—

Mixtures of serum with equal volumes of various buffers were made. The experiment was run at 15°C., using 5.0 ml. aliquots of the diluted sera and 10 mg. of 5B171. The data are graphed in Fig. 7.

[3] In recent experiments, PZ was formed from either human or bovine serum after stirring for 18 hours at 4°C.

The experiments described in this section used relatively large volumes of triply absorbed RP, a reagent which is both expensive and difficult to prepare. Since the sole function of the RP was to provide C'3, a more convenient source of this component was sought. The following experiments were conducted with a pig fraction containing C'1, C'3, and C'4 (5).

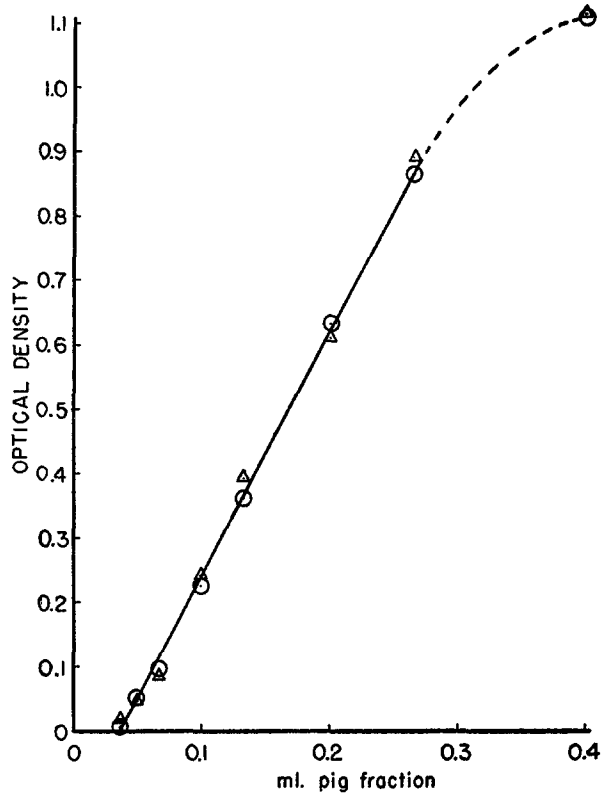

FIG. 8. Effect of varying amounts of 1,3,4 fraction on a constant quantity of EAg.p.$C'_{1,4,2}$.

Stoichiometry of the Reaction between EA g.p. $C'_{1,4,2}$ and the 1,3,4 Fraction.—

The complex between EA and C'1, C'4, and C'2 of guinea pig C' (EAg.p.$C'_{1,4,2}$) was prepared using a sublytic amount of guinea pig C' at 27°C. (13). The reaction was stopped by the addition of 2 volumes of ice cold saline. From this point on, the procedure followed that given in Materials and Methods for $EAhuC'_A$. 4.0 ml. of varying dilutions of two different preparations of the 1,3,4 fraction in E.D.T.A. buffer (to suppress reaction of the C'1 and C'4) were added, and the mixtures incubated at 32°C. for 1 hour.

A direct proportionality between the amount of 1,3,4 added and the resultant optical density was found over a wide range of 1,3,4 concentrations (Fig. 8).

Comparison of Reactivity of 1,3,4 Fraction and RP with PZ.—

Duplicate flasks were set up at 15°C., containing 5.0 ml. of human serum/2 and 10 mg. of 5B171. Aliquots were removed at intervals and assayed for PZ content, using either RP

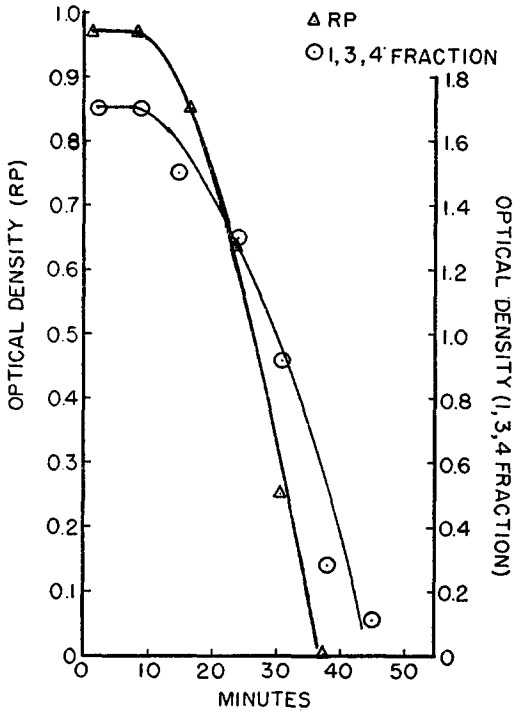

FIG. 9. Rate of formation of PZ using RP or 1,3,4 fraction as an indicator. Optical density of unabsorbed 1,3,4, 1.7, of unabsorbed RP 0.940.

or 1,3,4 fraction. The results are shown in Fig. 9 and indicate that the 1,3,4 fraction makes a satisfactory substitute for RP in titrating PZ.

In this experiment, as well as in all others using the 1,3,4 fraction, no loss of C'3 activity was observed on incubating the 1,3,4 fraction with zymosan.

DISCUSSION

When two substances interact, the rate of reaction generally decreases as the concentration of reacting substances becomes smaller. This is true whether or not catalysis of the reaction is involved. The fact that the rate curves for

the reaction between zymosan and properdin show an induction period, at certain concentrations of zymosan, indicates that the reaction is complex. Experiments currently in progress favor the hypothesis that at least two consecutive reactions must occur prior to the formation of the complex reactive with C'3. Whether this hypothesis is valid or not, the data presented in this paper suggest that considerable caution should be exercised in interpreting properdin titrations.

The substitution of the 1,3,4 fraction for human RP as a source of C'3 should make the technique for measuring PZ accessible to a great many laboratories. The starting material, pig serum, is inexpensive; the fractionation (5) is simple; and the fraction itself is stable for months, in the paste form, at −5°C. When dissolved, the fraction retains its activity at 4°C. for at least 1 week. A further advantage to be gained, using the 1,3,4 fraction, lies in the substitution of EAg.p.$C'_{1,4,2}$ for its human counterpart. These cells are far more stable than $EAhuC'_A$ (14) and it is not necessary to pipette them in a cold room.

In the course of this work it was noted that E.D.T.A. treated g.p.C' lysed both EAg.p.$C'_{1,4,2}$ and $EAhuC'_A$; E.D.T.A. treated 1,3,4 fraction lysed only EAg.p.$C'_{1,4,2}$; and E.D.T.A. treated huC' lysed only $EAhuC'_A$. The reasons for this behavior are somewhat obscure, since the components of C' appear to be, to a considerable extent interchangeable (15, 16) among the three species, and C'3 is presumably the only component active in the E.D.T.A.-treated material.

SUMMARY

The reaction of human, bovine, and pig properdin with excess zymosan exhibits an induction period followed by a rapidly accelerating formation of the properdin-zymosan (PZ) complex. Two methods for determining the quantity of PZ complex are given, one using human serum lacking properdin, the other using a fraction of pig serum containing complement components, C'1, C'3 and C'4.

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