THE PROPERDIN SYSTEM AND IMMUNITY*

III. The Zymosan Assay of Properdin

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Normal human and other mammalian serums contain a protein, properdin (1, 2), which is an important constituent of a natural defense mechanism of blood. Properdin, in conjunction with complement and Mg^{++}, participates in the destruction of certain bacteria and abnormal red cells and in the neutralization and inactivation of certain viruses. Properdin differs from antibody in many respects, particularly in its apparent lack of serological specificity, its requirements for Mg^{++} and complement for its interactions, and in its physical and chemical properties.

A method for the assay of properdin has been briefly described elsewhere (1). This method depends upon the requirement of properdin for the inactivation of the third component of complement (C'3) by zymosan (3). A unit of properdin is defined as the smallest amount of test sample which will reduce the C'3 titer of a properdin-deficient serum (RP) from 120 to 0 units during incubation with zymosan under standard conditions. While there are theoretical and practical objections to the zymosan assay of properdin, it has been found to be more reproducible and reliable than other types of assays now under investigation. The actual test is not difficult to perform, but careful selection and standardization of reagents are necessary.

The present paper describes detailed procedures for the zymosan assay of properdin and for the preparation and standardization of reagents employed in this test.

General Materials and Methods

In this and the following papers of this series, the following symbols (4, 5) will be employed:

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P	Properdin	C'3	Third component of C'
RP	Serum deficient in properdin	C'4	Fourth component of C
Z	Zymosan	R1	Serum deficient in C'1
PZ	Properdin-zymosan complex	R2	Serum deficient in C'2
C'	Complement	R3	Serum deficient in C'3
C'1	First component of C'	R4	Serum deficient in C'4
C'2	Second component of C'		

Human Serum.—Blood from healthy human donors was drawn during the late afternoon and allowed to stand at room temperature for 2 hours. The clot was then rimmed, and the blood stored at 2° overnight. The serum was separated by centrifugation at 2° for 30 minutes at 4000 R.P.M. and recentrifuged under identical conditions to remove residual red cells. The clear serum was then stored at 1°, if used within an 8 hour period or stored at −70° for further use.

pH 7.4 Barbital Buffer (5).—

85.0	gm.	NaCl
5.75	"	5,5-diethylbarbituric acid
3.75	"	sodium 5,5-diethylbarbiturate
5.0	"	M $MgCl_2$
1.5	"	M $CaCl_2$

The ingredients were dissolved in about 1500 ml. of hot distilled water. The solution was cooled, and distilled water then added to a final volume of 2 liters. This concentrated buffer was stored at 1°. *1 part of the buffer was added to 4 parts of distilled water before use.* The diluted buffer was prepared daily and discarded after a 12 hour period.

Sensitized Sheep Cells.—Fresh sheep blood was collected in an equal volume of Alsever's solution (5) and maintained at 1°. The blood was stored for 4 days before use and discarded after 10 days. Sensitized sheep cells were prepared daily, as follows: Cells were washed twice with 10 volumes of 0.15 M NaCl and then once with 10 volumes of barbital buffer. A 2 per cent suspension of washed cells was then made in barbital buffer. 1 ml. of this suspension was hemolyzed by the addition of 24 ml. of distilled water. The hemoglobin concentration of the hemolyzed red cells was determined in a Coleman universal spectrophotometer at 550 mμ with a violet filter using a water blank. The red cell suspension was diluted with barbital buffer to contain 5×10^5 cells/c.mm. by reference to a standard curve relating cell count and optical density at 550 mμ. An equal volume of barbital buffer, containing 4 units of amboceptor, was added quickly and with rapid mixing to the standardized sheep cells. The sensitized cells were used only during a 12 hour period after their preparation.

The Titration of Human Complement (C').—1 unit of C' is the minimum amount of serum necessary to produce complete hemolysis of 1 ml. of sensitized sheep cells in a final volume of 1.5 ml. after 30 minutes at 37°. Normal serums give values ranging from 25 to 75 units/ml. of serum, with a median value of about 40 units/ml. of serum. The latter value corresponds to 0.12 ml. of a 1:5 dilution of human serum. The titration of complement was performed as follows:—

Varying amounts of a 1:5 dilution of human serum ranging from 0.08 to 0.3 ml. were added to the series of 12 × 75 mm. serological test tubes and sufficient barbital buffer was added to bring the volume to 0.5 ml. Then 1 ml. of evenly suspended sensitized sheep cells was added to each tube. The tubes were shaken and incubated in a water bath at 37° for 30 minutes. The least amount of serum showing complete hemolysis was considered to be 1 unit of C'.

Titration of Components of C'.—General methods for the preparations of reagents and for the titration of individual components of C' have been described elsewhere (4–8). In the present work, the sample to be titrated was diluted serially in barbital buffer. To a series of 12 × 75 mm. tubes in an ice bath, 0.2 ml. of the sample dilutions, a constant amount

of reagent (R1, R2, R3, or R4, depending upon the component to be titrated), and 1 ml. of sensitized cells were added. The contents of the tubes were mixed, incubated at 37° for 30 minutes, centrifuged at 2000 R.P.M. for 5 minutes, and compared visually with a 50 per cent hemolytic standard. The titer was expressed as the reciprocal of the sample dilution which gave 50 per cent hemolysis. This value, multiplied by 5, gives the number of units of component per milliliter of sample. The end-point was read to the nearest half-tube; for example, if a dilution of 1:24 gave 60 per cent hemolysis and 1:48 gave 40 per cent hemolysis, the end-point was taken at 1:36 and the titer expressed as 180 units per ml.

The standard precautions were taken before using C′ reagents (5, 6). The reagents employed were not anticomplementary or significantly hemolytic at the levels used in component titrations. Recombination of reagents (R1 and R2, R1 and R3, R1 and R4, etc.) was performed to show that each reagent was hemolytically inactive only because of the absence of the component in question. In most experiments, these criteria were met by using 3 units of R1, 2 units of R2, 2 units of R3, and 3 units of R4 for the titration of C′1, C′2, C′3, and C′4 respectively.

Double distilled water was employed throughout. All glassware was cleaned with sodium dichromate–sulfuric acid solution and washed in distilled water. International PR-I or PR-II refrigerated centrifuges were used for centrifugation. Constant temperature baths, accurate to ±0.5°, were employed for incubation. Hydrogen ion concentration was determined with the Cambridge research model pH meter. Serum and serum reagents were stored at −70° in a Hudson Bay mechanical freezer.

EXPERIMENTAL

This paper is presented in four sections. These deal with the preparation and standardization of (I) zymosan; (II) of purified properdin; (III) of RP and R3; and (IV) with the details of the zymosan assay of properdin. Appropriate methods and materials are presented in each section.

I. ZYMOSAN

Zymosan (3) is a fine, light-gray powder insoluble in water but which disperses readily to give a homogeneous suspension. It is derived from the yeast cell wall. It is composed mainly of carbohydrate as a glucose polymer but contains small amounts of nitrogen, phosphorus, and magnesium. It is possible to remove most of the nitrogen and magnesium-phosphorus complex without destroying the immunological properties of zymosan (3).

In this paper and those that follow, the term *zymosan* is used to designate the above described substances (*a*) that selectively inactivate C′3 when added to human serum and incubated at 37°, (*b*) that will complex with properdin at 17° in the presence of C′ and Mg^{++}, and (*c*) that will form from suitable serums satisfactory R3 and RP which can be used as reagents for the assay of properdin. Not all yeast cell wall preparations fulfill these requirements. This is shown in Table I. It will be seen that zymosan A inactivates C′3, combines with properdin, and makes suitable RP. Zymosan B does not make a suitable R3, but combines with properdin and makes a satisfactory RP. Zymosan C inactivates C′3, combines with properdin, but will not make a

satisfactory RP. Zymosan D will not inactivate C'3 or make a suitable RP but combines with properdin. Zymosan E is entirely inactive. It is of interest that yeast glucan (9) combines with properdin but does not inactivate C'3. Thus, glucan resembles zymosan B. It has also been shown (10) that certain dextrans and levans, mucins, endotoxins, and bacterial cell walls behave like zymosan A, while others show all of the variations exhibited by types B, C, D, and E.

TABLE I

The Effect of Various Zymosans on the Properdin System in Human Serum

Zymosan	Makes satisfactory R3	Makes satisfactory RP	Properdin recovered from PZ complex
A	Yes	Yes	Yes
B	No	"	"
C	Yes	No	"
D	No	"	"
E	"	"	No

It is desirable to have a zymosan preparation that can be employed for all the purposes described above. Such zymosan can generally be prepared by the modified procedure given below:—

Preparation of Zymosan

1. Suspend evenly 1 kg. of yeast in 4 liters of 0.5 M Na_2HPO_4 and boil for 3 hours.
2. Cool to 37° and add 0.5 gm. of trypsin (Wilson 1:250) on the 1st, 3rd, 6th, and 10th day of incubation at 37°. Add 5 ml. of toluene on the 1st day, and 2.5 ml. of toluene on the 3rd, 7th, 11th, and 15th day of incubation. Incubate for 16 days at 37°, with occasional stirring, and adjust pH daily to 7.8–8.0 by the careful addition of NaOH.
3. Collect precipitate by centrifugation. Discard supernatant.
4. Suspend precipitate evenly in 4 liters of tap H_2O at room temperature. Stir thoroughly for 1 hour, centrifuge as in step 3 and discard supernatant.
5. Suspend precipitate evenly with vigorous stirring in 4 liters of boiling tap H_2O. Stir at 95 to 100° for 1 hour. Centrifuge while hot as in step 3. Repeat this step once more.
6. Suspend precipitate evenly in 4 liters of boiling *distilled* H_2O with vigorous stirring. Treat and centrifuge as in step 5. Repeat this step one time. The supernatant should be free of soluble carbohydrate. If not, repeat washing.
7. The precipitate from step 6 is added rapidly with vigorous stirring:—

(*a*) to 8 liters of *absolute ethyl alcohol*; stir for 1 hour; centrifuge.
(*b*) The precipitate is added to 4 liters of *absolute ethyl alcohol* and treated as in (step *a*).
(*c*) The residue is dried *in vacuo*.
(*d*) The dried powder is refluxed for 3 hours with 500 ml. of *absolute ethyl alcohol*.
(*e*) Centrifuge and dry immediately *in vacuo* and store *in vacuo*.

Yield: 1 to 2 per cent.

Care must be taken to avoid bacterial contamination during processing, to allow full tryptic digestion of yeast proteins, and to remove soluble carbohy-

drates by washing. Most of the difficulties in the preparation of zymosan occur during the treatment with ethyl alcohol. It is imperative that *absolute ethyl alcohol* be employed throughout. The use of methanol or of 95 per cent ethyl alcohol may yield zymosans of any of the altered types shown above. Improper dehydration also gives brownish products that are difficult to suspend in water and resemble resins in their appearance.

Before use, zymosan is suspended as evenly as possible in 100 volumes of 0.15 M NaCl. The suspension is placed in a boiling water bath for 1 hour. It is then centrifuged for 30 minutes at 4000 R.P.M., the supernatant discarded and the residue suspended evenly in the diluent of choice to the desired concentration. Such suspensions can be maintained at 1° for at least a month, care being taken to avoid contamination.

The Standardization of Zymosan.—

1. Each lot of zymosan is tested for the minimal amount required for the specific inactivation of C′3. From 0.5 to 2.0 mg. of most zymosan preparations are required to inactivate the C′3 in 1 ml. of human serum. In practice 0.5, 1, 2, and 4 mg. of zymosan are each incubated with 1 ml. of serum for 1 hour at 37° with occasional mixing. The residue is removed by centrifugation and the supernatants tested for C′3 activity. If complete inactivation of C′3 occurs, the sample prepared with the *minimal amount* of zymosan is then tested for C′ and other C′ component activities. At least 75 per cent of the original C′1, C′2, and C′4 should be present for a suitable reagent (R3).

2. While there are marked qualitative and quantitative differences in the ability of zymosans to inactivate C′3, the amounts of most zymosans required for the removal of properdin from serum and for the preparation of suitable RP are remarkably constant. The addition of 2 to 3 mg. of zymosan to each ml. of serum at 17° for 1 hour usually removes over 90 per cent of properdin from human serum, yielding suitable RP and also PZ from which properdin may be eluted.

3. The optimal amount of zymosan required in the assay of properdin is determined as follows: Mixtures of 0.25 ml. of RP and 0.5 ml. of buffer with and without 1 unit of added properdin are incubated at 37° for 1 hour with varying amounts of zymosan. The amount of zymosan employed in the assay of properdin is the maximal amount which will not reduce the C′3 titer of RP more than 25 per cent, but will completely inactivate all the C′3 in the presence of RP and 1 unit of added properdin. Usually, this represents about 4 times the amount of zymosan necessary to inactivate C′3 in untreated serum.

II. PROPERDIN

A general method for the isolation of properdin from human serum has been presented previously (1). A detailed procedure is presented here for the prepa-

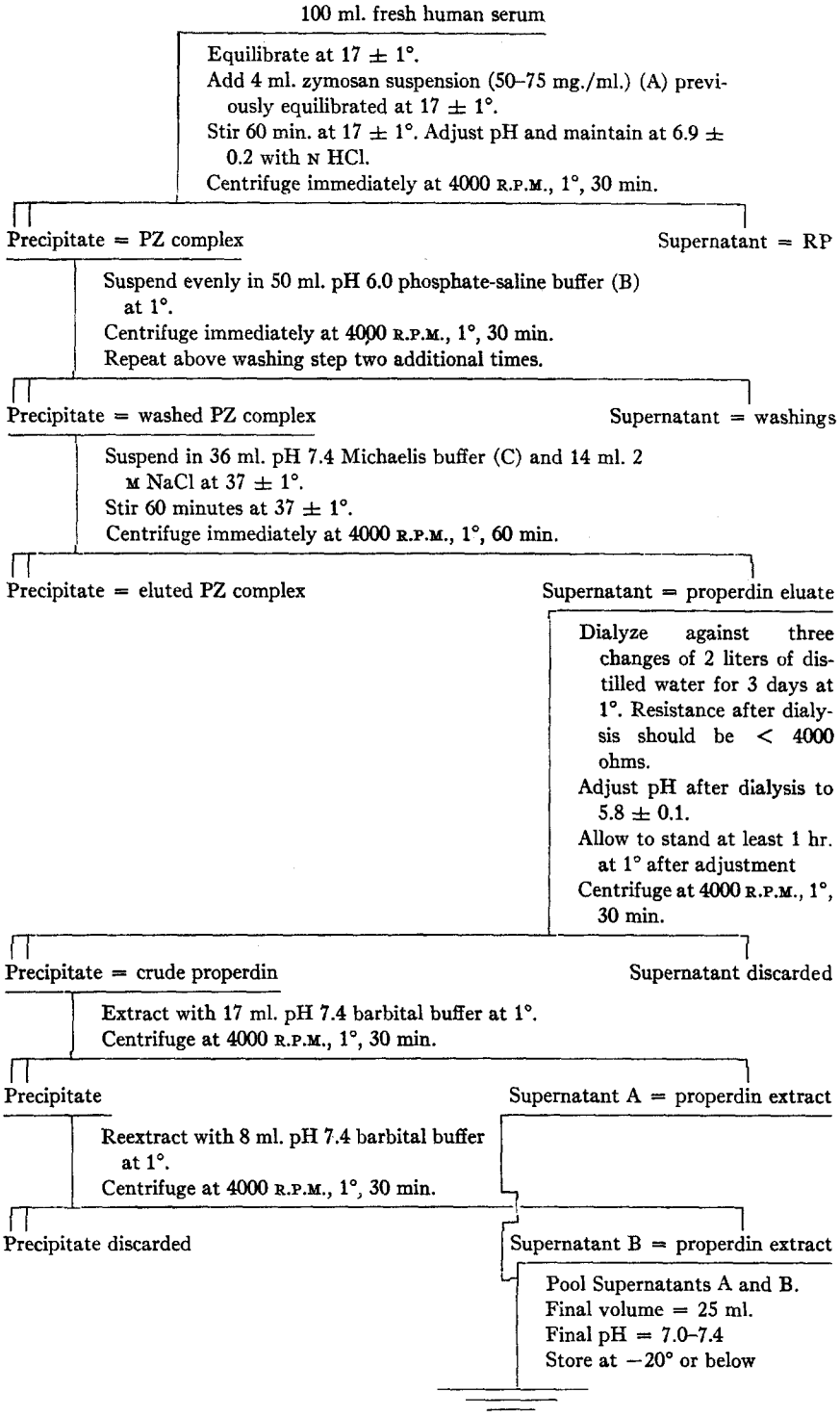

FIG. 1. Flow diagram for the isolation of properdin from human serum.

ration of human properdin suitable for the standardization of the zymosan assay. This material is free of C′ components, but contains traces of plasminogen. The results of further purification studies and the physicochemical characterization of properdin will be described in a subsequent publication.

The purification procedure shown in Fig. 1 has been found to be relatively simple and reproducible. The average yield has been 2 to 4 units of properdin per ml. serum. The purification factor has been in the range of 2000-fold. The titration of purified properdin is given in a later section.

Reagents for the Preparation of Properdin

A. 200 to 300 mg. of zymosan is suspended evenly in 20 ml. of 0.15 M NaCl, placed in a boiling water bath for 1 hour, and then centrifuged at 4000 R.P.M. for 30 minutes. The residue is suspended as uniformly as possible in pH 7.4 barbital buffer to a final volume of 4 ml. (50 to 75 mg./ml.).

B. pH 6.0 phosphate-saline buffer

1768.4 ml. M/10 Na H_2PO_4
231.6 ml. M/10 Na_2HPO_4
Add 1 part of buffer to 19 parts of 0.15 M NaCl

C. pH 7.4 Michaelis buffer

9.714 gm. sodium acetate ($3H_2O$)
14.714 gm. sodium 5,5-diethylbarbiturate
17.00 gm. NaCl
Dissolve in 1960 ml. of H_2O. Adjust pH to 7.4 ± 0.1 with about 43 ml. of N HCl

III. RP AND R3

RP:

The measurement of properdin activity requires an RP *that is deficient only in properdin*. RP must supply C′3 and an excess of all the cofactors necessary not only for the formation of an active PZ and for the inactivation of C′3 by PZ, but also adequate amounts of C′1, C′2, and C′4 for the hemolysis of sensitized sheep cells in the final assay of properdin. While the factors concerned with the properdin system resemble C′ components in many respects, the possibility exists (11, 12) that they differ from C′ components. They may reside in distinct chemical structures or the components of C′ may have dual functions.

There are considerable variations among individual serums in their ability to make suitable RP. While the reasons for these differences are obscure, it is obvious that a serum will produce an unsatisfactory RP if it is deficient in any cofactor, either initially or as a result of treatment with zymosan.

It would, perhaps, be possible to prepare a suitable RP from many more serums by proper choice of conditions for the removal of properdin from each serum. However, this would be a laborious procedure and impractical. Therefore, on the basis of present knowledge, a standard procedure has been followed which allows the preparation of RP from about 20 per cent of the hu-

man serums so far tested. The age, sex, race, blood groups, etc., of the donor bear no relationship to the utility of his serum for the preparation of RP. However, serums extremely high in properdin (greater than 8 units/ml.) are usually unsatisfactory, because they are difficult to free of properdin. Generally, serums low in porperdin but high in C′ make satisfactory RP. It is hoped that work now in progress will not only clarify the nature and the stability of the factors required in RP, but will lead to procedures that will allow most or all serums to be utilized for the preparation of RP.

Meanwhile, sufficient serum for the preparation of RP and R3 may be obtained by a preliminary screening of serums from several donors. Serums obtained from the same donor at different times are remarkably constant.

TABLE II

Variations in the Activity of RP Prepared from Different Serums

Sample	Untreated	Zymosan added	Zymosan and properdin added
	C′3	C′3	C′3
	units/ml.	*units/ml.*	*units/ml.*
RPI	120	90–120	0
RPII	30	30	0
RPIII	120	30	0
RPIV	120	120	60
RPV	0	0	0

All samples are incubated at 37° for 1 hour. The zymosan is removed by centrifugation and the supernatants tested for C′3 activity.

Thus, the same donor, over a period of years, will supply serum that will produce suitable RP or R3.

The Preparation of RP.—Serum is brought to and maintained at 17° in a constant temperature bath. The control of temperature is important. Temperatures below 15° result in an incomplete removal of properdin from serum, while temperatures above 18° result in increased loss of C′3 activity. 2 to 3 mg. of zymosan in 0.08 ml. of barbital buffer are added to each ml. of serum. The mixture is stirred continuously; with small volumes, it is mixed manually every 10 minutes. After 1 hour, the mixture is transferred to chilled centrifuge tubes and centrifuged at 4000 R.P.M. for 30 minutes at 1°. The clear supernatant is brought to 17° and 2 to 3 mg. of zymosan in 0.08 ml. of barbital buffer are again added with stirring to each ml. of treated serum. The mixture is incubated at 17° for 1 hour and centrifuged as above. The clear supernatant (RP) is dispensed into ignition tubes for storage at −70° or maintained at 1° for testing within a 12 hour period.

The Standardization of RP.—A suitable RP should have a C′ titer of at

least 75 per cent of the original serum. It should not be anticomplementary against fresh serum, or any of the serum reagents employed in studies on the properdin system. The addition of zymosan at 37° for 1 hour should not depress the C'3 titer to less than 75 per cent of the untreated RP. The addition of 1 unit of human, cow, or hog purified properdin to the RP under the above conditions should inactivate C'3 completely. RP should measure the properdin in the serums of all species. However, there are occasional variations in the ability of an RP to measure properdin in the serums of different species. This resembles and may be related to the species incompatibilities observed between C' and specific antibody.

Table II shows the results obtained with a suitable RP and with those which are unsatisfactory. RPI is an ideal reagent. RPII lacks sufficient C' and C'3 activities. RPIII still contains more than 0.2 unit of properdin. C'3 in RPIV is not inactivated by properdin, indicating the removal or inactivation of one or more of the accessory factors during its preparation. RPV is anticomplementary or lacks C'3.

R3:

It has been shown previously that there is considerable variation among serums in their C'3 and other C' component activities following (11, 12) zymosan treatment at 37°. A satisfactory R3 must lack C'3 but contain an excess of C'1, C'2, and C'4. The criteria for a suitable R3 are as rigid as those for RP because the assay of properdin is, indeed, based on the *specific inactivation of C'3 by PZ*. Therefore, the reagent employed to measure the presence or absence of C'3 must be deficient only in C'3 but contain an excess of other C' components. Only about 15 per cent of human serums make R3 suitable for use in the assay of properdin. As in the case of the preparation of RP, work is in progress to improve present procedures and to develop other methods for preparing R3.

Preparation of R3.—1 to 2 mg. of zymosan in 0.05 ml. of barbital buffer is added to each ml. of serum at 37° and suspended evenly in the serum. The mixture is incubated at 37° for 60 minutes, with periodic manual mixing. After this time, it is centrifuged at 4000 R.P.M. for 30 minutes at 1°. The clear supernatant is transferred to ignition tubes for storage at −70° or held at 1° for use within a period of 12 hours.

The Standardization of R3.—R3 is tested: (*a*) for lysis against sensitized sheep cells; (*b*) for anticomplementary properties against small amounts of fresh serum; and (*c*) for its ability to measure C'3 in a standard RP in the presence and absence of zymosan and in the presence and absence of properdin. 0.1 ml. of R3 should be non-lytic for 1 ml. of sensitized sheep cells. 0.05 ml. of R3 should, when added to 0.10 ml. of a 1:15 dilution of fresh serum, increase the C' titer of the serum at least 200 per cent. 0.05 ml. of R3 should

measure at least: (*a*) 120 units of C'3 per ml. of RP, (*b*) not less than 90 units per ml. of RP previously treated with zymosan at 37°, and (*c*) no C'3 in RP previously treated with zymosan in the presence of 1 unit of properdin per ml. of RP.

IV. ZYMOSAN ASSAY OF PROPERDIN

Principle of Assay:

The determination of the minimum volume of test sample which, when incubated 1 hour at 37° with constant amounts of RP and zymosan, completely inactivates the C'3 in the mixture.

Definition of Units:

Properdin.—1 unit of properdin is that quantity which, in the presence of an optimal amount of zymosan, completely inactivates 120 ± 30 units of C'3 in 1 ml. of RP during 1 hour at 37°. For example, if 0.1 ml. of sample is the minimum amount which completely inactivates 120 units of C'3 in 1 ml. of RP in the presence of zymosan, the sample contains 1/0.1 = 10 units of properdin per ml.

C'3.—1 unit of C'3 is that quantity which, in the presence of 2 units (usually 0.05 ml.) of R3 and 1 ml. of sensitized sheep cells in a final volume of 1.5 ml., hemolyzes 50 per cent of the cells in 30 minutes at 37°. For example, if 0.2 ml. of a 1:24 dilution of sample gives 50 per cent hemolysis under these conditions, the concentration of C'3 is 24/0.2 = 120 units per ml.

R3.—1 unit of R3 is equal to the smallest volume of the original serum from which the R3 was prepared which, when added to 1 ml. of sensitized sheep cells in a final volume of 1.5 ml., hemolyzes 100 per cent of the cells in 30 minutes at 37°. For example, if 0.12 ml. of a 1:5 dilution of the original serum before conversion into R3 gave 100 per cent hemolysis, then 2 units of the R3 prepared from it is contained in 2 × 0.12/5 = 0.05 ml.

Procedure:

1. Add 0.1 ml. of a standardized suspension of zymosan to each of a series of 13 × 100 mm. tubes in an ice bath.
2. Add 0.25 ml. of RP to each tube.
3. To successive tubes, add decreasing amounts of the test sample; for instance, 0.25 ml. to the first tube, 0.12 ml. to the second, etc.
4. Add barbital buffer to bring the volume in each tube to a total of 0.75 ml.
5. Stopper each tube, shake, and incubate for 1 hour at 37° with periodic shaking every 10 minutes.
6. Centrifuge for 10 minutes at 2000 R.P.M. and pour the supernatants into a series of empty tubes.

The next step determines the concentration of C'3 in these supernatants.

7. With each supernatant from step 6, make a series of 2-fold dilutions from 1:1 to 1:16

in barbital buffer, using 0.2 ml. volumes, in 12 × 75 mm. tubes. Add 2 units (0.05 ml.) of R3 to each tube and make the volume to 0.5 ml. with buffer. Add 1 ml. of sensitized sheep cells, incubate 30 minutes at 37°, and then centrifuge 5 minutes at 1500 R.P.M.

Determine which dilution gives 50 per cent hemolysis by comparing each tube with a 50 per cent hemolysis standard prepared by adding a trace of saponin to a mixture of 0.5 ml. of sensitized sheep cells and 1 ml. barbital buffer. If the 50 per cent hemolysis end-point is intermediate between 2 dilutions, *e.g.*, between 1:8 and 1:16, the end-point dilution is taken midway at 1:12. If 1:12 is the end-point in the C'3 titration, then the concentration of C'3 in the supernatant from step 6 is 12 × 1/0.2 = 60 units per ml. However, C'3 concentrations are expressed in terms of undiluted RP and, since the RP is diluted threefold in the supernatants at step 6, a 1:12 end-point in the C'3 titration is read as 60 × 3 = 180 units of C'3 per ml. of undiluted RP. If the end-point in the C'3 titration is given by the undiluted supernatant from step 6, the C'3 titer is 15 units per ml. of undiluted RP. If there is no trace of hemolysis, the C'3 titer is taken as 0, and it is assumed that all the C'3 in the RP has been inactivated. The end-point in the properdin titration is read as the minimal volume of sample in step 3 which shows complete inactivation of C'3 in step 7. *The number of units of properdin in the test sample is determined by dividing this volume into 0.25.*

Controls:

At step 3, four control tubes are set up in parallel with the test samples and carried through the remainder of the procedure.

Tube A contains 0.25 ml. RP + 0.5 ml. buffer. This is used to determine the concentration of C'3 in the RP after incubation, centrifuging, etc., but in the absence of properdin or zymosan.

Tube B contains 0.25 ml. RP + 0.4 ml. buffer + 0.1 ml. of zymosan. This is used to determine the loss of C'3 in the presence of zymosan but in the absence of properdin.

Tube C contains 0.25 ml. RP + 0.05 ml. (0.25 unit) of purified properdin + 0.35 ml. of buffer + 0.1 ml. zymosan. This sample checks that the addition of 1 unit of properdin (per ml. of RP) results in the complete inactivation of C'3 in the presence of zymosan. The contents in Tube D are identical to those in tube C, except that 0.125 unit of properdin is employed instead of 0.25. This measures the effect of 1/2 unit of P (per ml. of RP).

Typical results with the control tubes at the end of step 7 are:—

Tube A	120 units C'3/ml.
Tube B	90 units C'3/ml.
Tube C	0 units C'3/ml.
Tube D	Trace to 15 units C'3/ml.

For properdin titrations on human or other serums, additional controls consisting of a standard normal serum of the species under test are set up at step 3. These standard normal serums are made up of large numbers of individual normal serums of each animal species and have been carefully titrated against several RP and R3. They are stored in small aliquots at −70°. Stand-

ard normal serums in this laboratory contain the following concentration of properdin:—

Human	6– 8	units/ml.
Mouse	12–15	" "
Rat	25–30	" "
Dog	12–15	" "
Rabbit	8–12	" "

If on any particular occasion the titers are significantly higher than these values, it is possible: (*a*) that the sheep cells are resistant to hemolysis, (*b*) that the R3 lacks C'1, C'2, or C'4, (*c*) that the RP is extremely sensitive to properdin, or (*d*) that R3, RP, or the test serum are anticomplementary.

TABLE III

Sample	Properdin	
	Calculated	Found
	units/ml.	*units/ml.*
1 ml. RP + 1 unit P	1	1
1 " " + 2 " "	2	2
1 " " + 4 " "	4	4
1 " " + 8 " "	8	8
0.25 ml. of serum* + 0.75 ml. of RP‡	2	2
0.50 " " " + 0.50 " " "	4	4
0.75 " " " + 0.25 " " "	6	6

* Serum contained 8 units of properdin per ml.

‡ RP contained less than 0.1 unit of properdin per ml.

Titers lower than the expected values are usually due to: (*a*) fragile sheep cells, (*b*) a lytic or non-specific R3, (*c*) an RP lacking the various factors required for the combination of properdin with zymosan, or (*d*) incompatibilities between the reagents and test sample.

Useful Simplification of the Test:

Since the end-point in the properdin titration is that volume of sample which inactivates all the C'3 in the mixture, it is not necessary to *titrate* C'3, but merely to determine whether it is present or absent. Thus, at step 7, it is sufficient merely to test the undiluted supernatants for C'3. However, a complete C'3 titration is always done on the control samples A and B to check that the RP has a satisfactory C'3 titer, both in the presence and absence of zymosan.

Table III summarizes experiments carried out to determine the reliability of the zymosan assay of properdin. Varying amounts of purified properdin

were added to constant amounts of RP. At the same time, normal human serum was diluted with varying amounts of RP. Properdin titers were determined on the mixtures. It will be noted that the actual properdin titers of the samples are identical with the calculated titers. It is important to conduct such experiments periodically in order to verify the reliability and reproducibility of the zymosan assay.

SUMMARY

A detailed method for the zymosan assay of properdin is described, together with procedures for the preparation and standardization of reagents employed in the test.

The reliability and reproducibility of the assay, as well as its limitations, are discussed.

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