agents or in the presence of large inocula. 5. Rate of primary isolation of orphan virus from stool suspensions was substantially decreased when acid producing tissue culture medium was used.

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Assay of Properdin in Biological Fluids.* (22959)

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A naturally occurring serum protein, properdin, has been described which participates in certain types of non-specific immunity(1). The known constituents of the properdin system are magnesium ions, complement (C'), and properdin (P)(1,2). In the presence of magnesium ions, properdin complexes with zymosan, a complex of insoluble polysaccharides derived from yeast cell wall, and various other polysaccharides(1-4). The third component of complement (C'3) is specifically inactivated by this properdin-zymosan complex. Various assays for properdin based on inactivation of C'3 have been reported(1-3,5). These employ two reagents, (a) a serum depleted in properdin but containing all components of complement (RP) and (b) a serum lacking properdin and C'3 but containing a large excess of all other components of complement (R3).

Since the source of human sera for preparation of satisfactory test reagents is restricted to 10 to 20% of the population, surveys of large numbers of sera are necessary to obtain reagent sera in practical quantities (1,2). The previously described method for removal of properdin from RP preparation has not been demonstrated to be quantitative in our laboratory and complete removal of C'3 from human sera has also been difficult to achieve. In previously described properdin assay there is no provision for the contribution made by C'3 of the test serum, and variable end points result when large particles of zymosan sediment to bottom of the tube. This paper reports a simple reproducible method for preparation of 2 reagent sera and a more sensitive assay for properdin in biological fluids.

Materials. A. *Barbital Buffer.* The composition of stock pH 7.4 buffer was reported elsewhere(2). The diluted buffer was prepared from stock daily and discarded after 12 hours to ensure constancy of pH. B. *Activated Sheep Cells.* Sheep blood in equal volume of Alsever's solution was obtained

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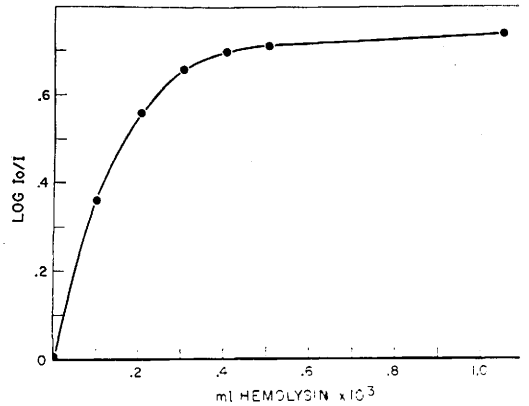

FIG. 1. Hemolysin titration curve.

from commercial supply house. The sheep cells were washed 3 times with 10-15 volumes of barbital buffer. A 4% suspension of washed cells was made in barbital buffer. Hemoglobin concentration of red cells was determined after hemolysis in Spectronic "20" spectrophotometer at 540 mμ. The red cell suspension was then diluted until concentration of cells was exactly 4% by reference to a curve relating number of red cells to hemoglobin. Activated sheep cells were prepared by adding one volume of barbital buffer containing a zero order excess of hemolysin as determined in titration of hemolysin below. C. *Titration of Hemolysin.* Varying amounts of 1:1000 hemolysin were added to a series of photometrically standardized 13 × 100 mm. test tubes containing 0.5 ml of sheep cells. The tubes were incubated at 37°C for one-half hour. 1 ml of a 1:50 dilution of guinea pig complement or RP + R3 (1:50 each) prepared in 0.5% bovine serum albumin in barbital buffer was then added. The tubes were again incubated for one-half hour at 37°C; 0.5 ml of versene was added to stop the reaction. The tubes were centrifuged at 1,000 to 1,500 x g for 10 minutes and read at 540 mμ (Fig. 1). Concentrations of hemolysin beyond 1:2000 do not cause appreciable changes in extent of hemolysis. Each new lot of hemolysin must be standardized. D. *Versene.* 0.1% Versene (N, N′ ethylene diamine tetra-acetic acid) was made in physiological saline and adjusted to pH 7.4. E. *Zymosan.* Commercially obtained zymosan† was ground to a fine, easily dispersible powder with mortar and pestle and suspended uniformly in barbital buffer. Concentration of zymosan routinely used in the test was established by determining the zero order range under conditions of the test (Fig. 2). Each new lot of zymosan must be standardized. F. *Inulin.* Commercially obtained inulin‡ was ground as in E. Vigorous shaking was required to disperse small clumps in some preparations. Concentration as in E (Fig. 2). Each new lot of inulin must be standardized. G. *Serum.* Syringes, needles, and all glassware, see Methods section. Blood was allowed to clot at room temperature for one or two hours or overnight in refrigerator. Clear, unhemolyzed sera, animal and human, were stored at −20°C. No loss of properdin activity took place for at least two months. Serum was

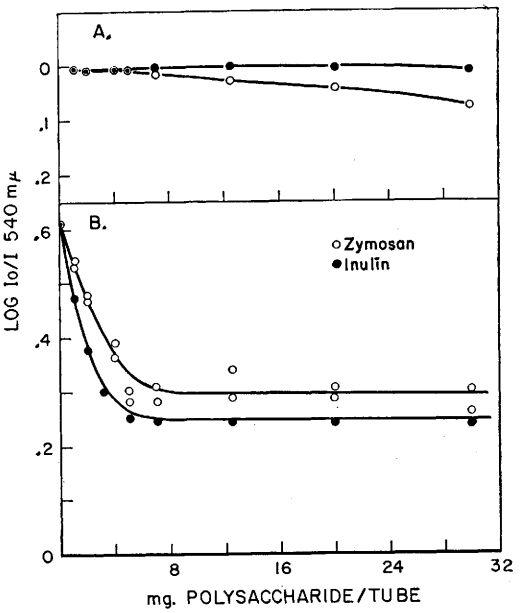

FIG. 2. A. Effect of inulin and zymosan on guinea pig RP in absence of properdin, comparing extent of C′ binding by the 2 polysaccharides. B. Effect of inulin and zymosan on guinea pig RP in presence of properdin contrasting reliability of the calibration curve readings.

† Zymosan Lot #B-61 was obtained from Immunological Specialties, Los Angeles, California. Zymosan Lot #5B171 was obtained from Fleishman Laboratories, Standard Brands, Stamford, Conn.

‡ Inulin was obtained from Matheson Co., East Rutherford, N. J.

thawed and diluted with chilled barbital buffer immediately before assay. Human serum was diluted 1:50; rat was diluted 1:200; mouse was diluted 1:50. H. *Properdin* was obtained from normal human serum by methods reported elsewhere(2). I. *RP* is prepared from commercial guinea pig complement by slowly adding and stirring 4 mg of finely powdered zymosan for each ml of serum. The serum-zymosan suspension is stirred continuously during one hour incubation at 20° to 25°C. The resulting suspension is then centrifuged at 1000-1500 X g for 10 minutes. The precipitate is discarded and the process repeated twice. In the last incubation the temperature is maintained at 37°C for 20 minutes and centrifuged for 15 minutes at 1000-1500 X g. The supernatant is frozen in vials in one or two ml volumes at –20°C. No loss in activity took place with samples continuously frozen for 2 months. J. *Properdin-zymosan complex.* Human serum containing 4 mg of zymosan/ml was incubated with constant stirring for one hour at 20°C and centrifuged at 1000-1500 X g for 10 minutes. The supernatant was discarded. The precipitate was washed 2 times with pH 6 phosphate buffer as reported elsewhere(2). K. *R3* is prepared under the same conditions as RP, except that temperature of incubation is 37°C throughout and during the first incubation instead of zymosan the properdin-zymosan complex is used in excess to ensure complete removal of C'3. Although guinea pig sera are routinely used for R3 preparations, we found that human R3 can be made by this process. All our attempts to deplete human sera of C'3 have been successful. The C'2 component titers of some sera are not, however, sufficiently high to be useful for R3 preparation.

Methods. Since chromic acid washed glassware occasionally contains sufficient chromate ions to inactivate complement, all glassware was washed in trisodium phosphate solution and rinsed with triple distilled water. All incubation at 37°C took place in constant temperature circulating water bath. Each reagent used was individually standardized. In addition to determining the C'3 titer of RP and R3, the C'1, C'2, C'4 titers were also determined for each lot. It has been our experience that activity of RP and R3 does not change measurably for at least 2 months continuous storage at –20°C.

TABLE I. A Typical Properdin Assay Protocol.

Tube	Serum 1:50	Buffered saline	RP + R3 (1:50 each)	Inulin	Code
1	1.0	.5	1.0		SRPR3
2	1.0		1.0	.5	SRPR3I
3	1.0	1.5			S
4		1.0	1.0	.5	RPR3I
5		2.5			
6		2.0		.5	I
7		1.5	1.0		RPR3
8		2.5			
9		2.3	.2		
10		2.1	.4		
11		1.9	.6		
12		1.7	.8		
13		1.5	1.0		
14		1.3	1.2		
15		1.1	1.4		

Properdin assay procedure. 1. Serum to be tested is diluted with chilled barbital buffer immediately prior to test. 2. 1 ml of the diluted test serum is added to tubes as indicated in protocol (Table I). 3. 0.5 ml of inulin or zymosan is added to tubes which are shaken briefly to suspend inulin or zymosan uniformly. 4. Saline is then added to make the volume 1.5 ml in each tube. 5. 1 ml of RP + R3 is added to test and varying amounts of RP + R3 is added for control tube 8 through 15. 6. Incubate at 37°C in circulating water bath for 1½ hours. 7. Centrifuge at 1000-1500 X g for 10 minutes to remove properdin-inulin- (or zymosan) C'3 complex from supernatant. 8. 2 ml of activated sheep cells are added with gentle shaking so that properdin-inulin-C'3 button is not disturbed and tubes incubated at 37°C for one-half hour. 9. 0.5 ml of 0.1% versene solution is added to each tube with gentle mixing. 10. Centrifuge immediately at 1000-1500 X g for 10 minutes. 11. The hemoglobin liberated in the supernatant is read at 540 mμ.

The difference between SRPR3 and SRPR3I represents the decrease in C'3 concentration. This decrease is equivalent to the properdin concentration and therefore

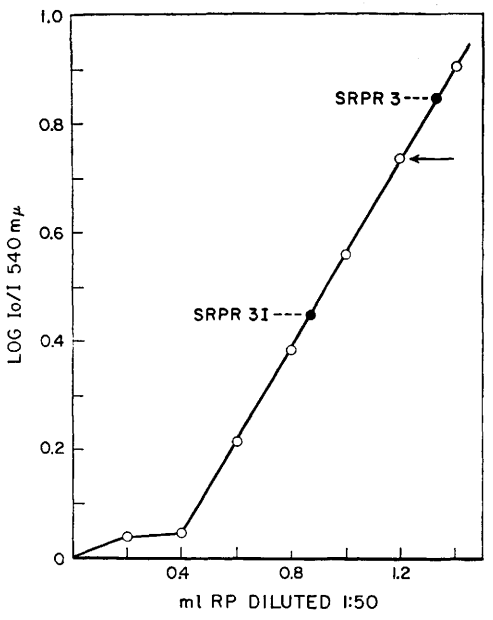

FIG. 3. Relation between extent of hemolysis and varying amounts of RP + R3 and readings of a typical test sera. Arrow indicates level of RP + R3 which corresponds to 50% hemolysis.

properdin concentration may be expressed as a function of optical density difference (Fig. 3).

The optical density of SRPR3 may be taken as 100% C'3. The decrease of C'3 activity (SRPR3I reading) is expressed in percent of SRPR3 optical density reading.

The plot of RP + R3 against optical density (Fig. 3) is a control to ensure that all components except C'3 are in zero order excess. When this is true, a linear plot is obtained between 10% hemolysis and 80% hemolysis. The RP + R3 concentrations selected for the test fall into the range between 10 and 60% hemolysis. If components other than C'3 are limiting, a curve rather than a linear dependence will result.

If C'3 is limiting in RP + R3, a straight line passing through the origin is obtained in a plot of SRPR3-SRPR3I versus ml of serum (or properdin) (Fig. 4). If residual C'3 is still present, the line will not pass through the origin but will intercept the SRPR3-SRPR3I at a point above the origin. If a component other than C'3 is limiting in RP + R3, this plot will pass through a point below the origin. When both these conditions occur together, a curve results.

The properdin content of 1 ml of 1:50 human serum diluted with barbital buffer generally falls into upper region of test range. Sera containing higher or lower properdin levels can be appropriately diluted to fit into the test range. If the dilution is carried out immediately following thawing and just prior to the test, no discernible effect on dilution is obtained. Data obtained from direct pipetting micro-quantities, *i.e.*, 0.02 ml, give the same properdin level as 1 ml of 1:50 dilution of the same serum.

Since in the test SRPR3 contains both complement content of RP + R3 and the complement present in test serum, it was necessary to determine whether the complement content of human test serum and the guinea pig RP + R3 were equivalent in such mixtures. Secondly, it is important to establish what effect, if any, is observed in the C'3 inactivation of mixtures of sera obtained from the two sources. In all cases there was an

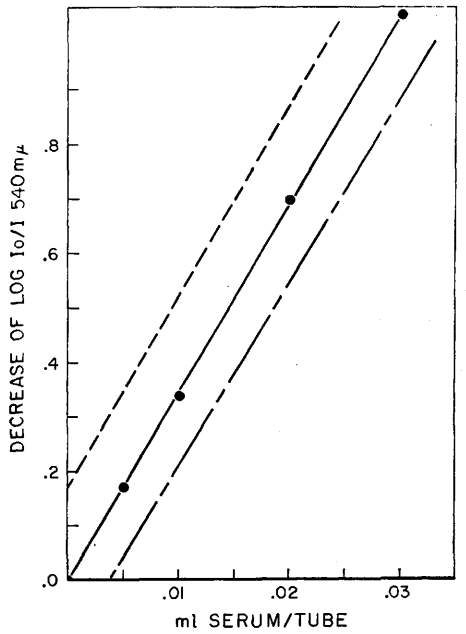

FIG. 4. Relationship between the SRPR3-SRPR3I of a serum containing properdin and the ml test serum. Dash line indicates RP + R3 not completely depleted of properdin; interrupted line indicates RP + R3 containing an excess C'3 (not in limiting concentrations).

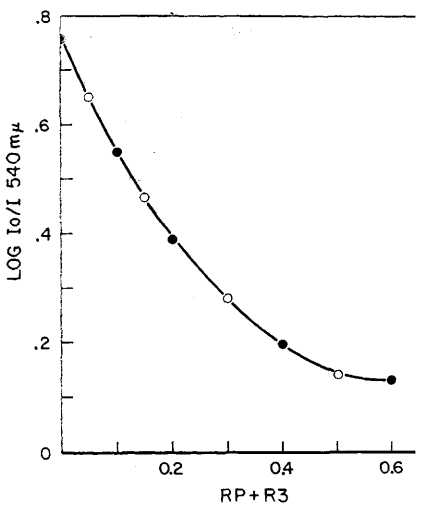

FIG. 5. Rates of inactivation of C'3 by human properdin in ● human RP + R3 and ○ guinea pig RP + R3. Human RP + R3 was diluted (1 : 20) to give the same density as guinea pig RP + R3 (diluted 1 : 50).

additive effect between the 2 complements in the SRPR3. To establish whether there was any difference in C'3 inactivation from RP + R3 preparations obtained from the 2 sources, varying concentrations of mixtures of the 2 preparations were used. This experiment is summarized in Fig. 5.

Discussion. Guinea pig serum was routinely used as the source for RP and R3 reagents utilized in the test. The advantages of using this serum are a) guinea pig serum has the lowest properdin content of any experimental animal(1), and b) the limiting component in guinea pig complement is C'3 (6). With the exception of C'2 the other components are in large excess so that most RP preparations do not actually require addition of R3 reagent. To ensure that C'2 does not become limiting, an equal volume of R3 is always added as supplement to the RP reagent.

Human serum has a much higher properdin level than that of guinea pig, and C'3 is usually not the limiting component(1,6). It is, therefore, difficult to obtain human sera to make satisfactory RP and R3 reagents as described in the literature. To obtain a suitable human RP, it is necessary to use sera which originally contain higher than average levels of C'2 and lower than average levels of properdin. We found that satisfactory R3 reagents can be made with human sera from at least 50% of random human sera tested utilizing incubation with an excess of PZ complex.

Tube (S), which is the test serum alone, reflects the overall C' titer of this serum. The difference between SRPR3 and RPR3 reflects the C'3 titer of the test serum. Occasionally the contribution of C'3 made by the test serum is high relative to the properdin content; therefore, without above controls on complement derived from the test serum, error would arise.

Zymosan varies from lot to lot in activity of properdin binding as well as in inactivation of C'3. There is extremely wide variation between duplicate tubes when the coarse zymosan preparation described in the literature is used. Finely powdered zymosan gives more consistent readings.

Inulin binds properdin and inactivates C'3 in a manner similar to zymosan. Since this polysaccharide is chemically descript and can be uniformly suspended in barbital buffer, inulin can be used in higher concentrations than zymosan. It does not absorb out other components of complement as is frequently the case with zymosan in high concentrations (Fig. 2). Inulin is used in this test as the C'3 inactivating polysaccharide in preference to zymosan.

Spectrophotometric determination of hemolysis is a sensitive and highly reproducible means of quantitating properdin and complement activity since an infinite number of concentrations can be extrapolated on the linear plot relating C'3 activity to properdin activity as well as C' activity of the test serum. A single tube establishes a finite concentration of C'3 in the absence of inulin and another tube establishes its concentration in the presence of inulin. Two tubes (run in triplicate) give the properdin activity of a single serum.

The properdin content of serum that shows anticomplementary activity can be established by a series of dilutions of this serum in the presence and absence of known amount of

purified properdin to determine recovery value of the added properdin. This recovery value can then be used to calculate the true properdin content of such serum.

Summary. 1. A sensitive, reproducible assay for properdin is described using spectrophotometric determinations of C'3 content. 2. RP and R3 reagent sera for the properdin assay may be readily prepared from guinea pig serum. 3. RP and R3 from both human and guinea pig sources may be used interchangeably in the reported properdin assay. 4. Inulin was found to be more satisfactory than zymosan as a properdin binding and C'3 inactivating agent.

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Isolation of Anticoagulant Fractions from Crude Fucoidin. (22960)

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Studies on methylpentose-containing polysaccharide material of plant origin have been in progress in this laboratory for more than two years(1,2). During this work, it occurred to us in view of the known anticoagulant activity of some other sulfated polysaccharides(3), that fucoidin, a polymer of L-fucose monosulfate, present in large amounts in the brown marine algae *Phaeophyceae,* might possess anticoagulant activity. This was found to be the case. The present paper describes the preparation of fucoidin fractions and gives data on some of their chemical properties and anticoagulant activities.

Material and methods. Source and preparation: Air dried *Fucus vesiculosus* was obtained from Woods Hole Marine Laboratories, Mass., between April and July, 1956. The preparation of crude material was based on older procedures(4). Purification procedures were guided by determinations of the anticoagulant activity employing the protamine titration test. Crude fucoidin was prepared as follows. About 1 kg dried *Fucus vesiculosus* was ground for 28 hrs. in a ball mill. To the powder obtained, 4 l of tap water were added and the mixture heated with occasional shaking in boiling water bath for 18 hrs. Toluene was added and the material left at room temperature overnight. The supernatant fluid amounted to about 500 ml to which 1 l of water was added. The fluid was then freed of insoluble matter by filtering through muslin. One hundred grams of lead acetate in 300 ml water were added and the precipitate removed by filtration. Barium hydroxide was added to the supernate until it became pink to phenolphthalein. The resulting precipitate was allowed to settle, the supernate siphoned off, discarded, and 300 ml of 4N H_2SO_4 were added for every 700 ml of precipitate containing fluid. The mixture was then shaken mechanically 4 hrs. This was followed by dialysis for 3 days against running tap water. The nondialyzable material was concentrated *in vacuo* to 500 ml, freed of insoluble material by spinning at 12000 g for 45 min. and the supernate poured into 10 vol. of 100% ethanol containing 0.3% sodium acetate. The precipitate was washed with

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