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A Quantitative Agglutination Method for the Study of the Specific Agglutination of Micro-organisms

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SUMMARY: A quantitative method for the study of the specific agglutination of micro-organisms is described. The degree of agglutination is estimated from the sedimentation curves of suspensions to which varying amounts of serum are added, which are compared with that of a control suspension without serum. The time of centrifugation required for sedimentation of 50 % of the organisms in the serum-suspension mixtures related to the corresponding time for the control suspension gives the relative 50 % sedimentation times which, within a certain region, show a linear relation with the amounts of serum used. The activity of sera is expressed by their agglutinin content and by their agglutinating capacity, given by the slope of the time-serum regression line.

Rickettsias of the typhus group and their corresponding sera are clearly distinguished by the method described. *Proteus* OX19 agglutinating sera of different origins (pregnant women, murine typhus and *Proteus*-immune rabbit), with the same agglutinating titre by the dilution method, are distinguished according to their origin by the quantitative method. The application of the method to the study of other groups of micro-organisms is suggested.

The study of the specific agglutination of bacteria, rickettsias and viruses by immune sera is usually made by the serial dilution method, and the results are expressed as the highest dilution of a serum which causes a standard degree of agglutination. To obtain greater uniformity and reproducibility of the results of such tests, Hedén (1946) described a method for the determination of the 50 % end-point based on the method of Reed & Muench (1938) for the evaluation of end-points in animal tests. This method has the advantage of taking into consideration a number of dilutions for the evaluation of the titre but it suffers from the influence of the personal bias which may be considerable when agglutination tests are read by different workers. The method described by Hirst & Pickles (1942) for the study of virus haemagglutination, if applied to the study of the agglutination of micro-organisms, as suggested by Kabat & Mayer (1948), would probably yield valuable results. In this test, the degree of agglutination in individual tubes is estimated by measuring the density of the layer of unsedimented cells after a fixed period of time, and the course of the haemagglutination is translated by a sigmoid curve. In our experience (unpublished data) a similar type of curve was observed for bacterial and rickettsial agglutination. A great advance in the study of the agglutination reaction was made with the introduction of the chemical quantitative method of Heidelberger & Kabat (1934*a, b*) in which the amount of antibody combined with agglutinated bacteria is measured by weight. This method, making possible the study of the course of bacterial agglutination as a function of

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antigen and antibody, is a valuable tool for research, but its practical applications are limited by the technical difficulties involved.

In the present paper we describe a method in which the degree of agglutination is estimated by the sedimentation curves of suspensions to which various amounts of serum are added; these are compared with the sedimentation curve of a control suspension without serum. The sedimentation curves are built by reading the light extinction of suspensions in a photoelectric colorimeter after successive periods of centrifugation. The term sedimentation is used in a wide sense, as the variations of the light extinction depend both on the sedimentation proper and on the aggregation of the organisms. In such tests, a linear relationship is observed, within a certain range, between the amount of antibody present and the relative time necessary for 50 % of the bacteria or rickettsias to be deposited.

MATERIAL AND METHODS

Antigens

Rickettsial suspensions were prepared from infected chick embryo yolk-sacs, purified by ether treatment and differential centrifugation. Murine (Wilmington strain) and classical (Breinl strain) typhus suspensions were prepared by method 2 of Topping & Shepard (1946). The suspension of Q fever rickettsias was prepared by method 1 of the same authors. The suspensions were washed shortly before use by two centrifugations in an angle-head centrifuge at 4000 r.p.m. for 1 hr. and re-suspended in 0.2 % formalized 0.005 M phosphate-buffered (pH 7.3) saline (Kent, Bukantz & Rein, 1946), the diluent used throughout. The density of the suspensions was adjusted so as to give, when diluted 1/10, a light extinction reading of 50.

Bacterial suspension. Ethanol-treated suspension of *Proteus* OX 19 was prepared according to the method of Bridges (1944). The density was adjusted so as to give, when diluted 1/10, a light extinction reading of 100.

Sera

Murine typhus serum. Pooled sera from guinea-pigs bled about 30 days after infection with the Wilmington strain.

Classical typhus serum. Obtained from a guinea-pig infected with a yolk-sac suspension of the Breinl strain and bled 30 days later.

Q fever serum. Guinea-pig immune serum kindly supplied by the late Dr R. R. Parker, from the Rocky Mountain Laboratory, Hamilton, Montana.

Proteus antisera. Obtained from human cases of murine typhus, from pregnant women, and from a rabbit immunized with an ethanolic suspension of *Proteus* OX 19.

Measurement of opacity

The density of the suspensions was determined by the light extinction observed in a Klett-Summerson photoelectric colorimeter with a 54 filter. A special holder was adapted to take standardized 13 × 100 mm. tubes. The

window was slightly reduced to allow the use of 1 ml. volumes. Within the region in which we worked, a linear relationship was obtained between the concentration of the suspension and the light extinction, as seen in Table 1 in which the light extinctions of various concentrations of a *Proteus* OX 19 suspension are given. The value of 100 % was chosen arbitrarily to give a reading of approximately 100.

Table 1. *Opacity measurements of Proteus OX 19 suspensions*

Suspension (ml.)	Saline (ml.)	Suspension (%) (x)	Light extinction (y)
0.1	0.9	10	9
0.2	0.8	20	19.5
0.3	0.7	30	30.5
0.4	0.6	40	41
0.5	0.5	50	52
0.6	0.4	60	60
0.7	0.3	70	70
0.8	0.2	80	81
0.9	0.1	90	92
1.0	—	100	99

The parameters of the line $y = bx + a$ gave values of 0.154 and 0.99 respectively for a and b , allowing the direct reading of the percentage of bacteria in suspension from the light extinction.

For economy, less concentrated rickettsial suspensions were used and the 100 % density was standardized to give a light extinction reading of 50. The same linear relationship was observed as in bacterial suspensions, and the percentages of rickettsias were given by the light extinction readings multiplied by 2.

Agglutination methods

Serial dilution tests

Serial twofold dilutions of the serum were made in saline and an equal volume of the suspension was added. *Proteus* OX 19 agglutination was done in 12 × 75 mm. tubes with a total volume of 1 ml. and the rickettsial agglutinations in 10 × 75 mm. tubes with a total volume of 0.2 ml. The tests were incubated in a water-bath at 37° for 2 hr. and left overnight at 4°. The titres represent the highest final dilution of serum causing definite agglutination. The 50 % end-points were calculated according to the method described by Hedén (1946). The density of all the suspensions was adjusted to give a light extinction reading of about 85.

Quantitative agglutination test

0.1 ml. volumes of the suspension were distributed with a 0.2 ml. pipette at the bottom of a series of standardized 13 × 100 mm. tubes. Increasing volumes (in arithmetical progression) of an appropriate dilution of serum were added and the volumes in all tubes were made up to 1 ml. with buffered saline. The control tube received 0.1 ml. of the suspension and 0.9 ml. of buffered

saline. The tubes were shaken, left to stand for a few minutes, and the light extinctions were read with the photoelectric colorimeter to check the 100 % density. The tubes were then incubated in a water-bath at 37° for 2 hr. and were then centrifuged in a horizontal centrifuge at a speed previously determined to yield 50 % sedimentation within 10 and 20 min. (about 2200 r.p.m. for rickettsial suspensions and 1000 r.p.m. for bacterial suspensions). After each 5 min. period the tubes were carefully removed from the centrifuge and the light extinctions determined with the photoelectric colorimeter. Centrifugation was continued until over 50 % of the organisms in the control tube were deposited; usually three or four periods of 5 min. were sufficient (Table 2). The tubes were then centrifuged at high speed for all the organisms to be deposited, and the light extinctions of the supernatants were read; these values were deducted from the previous readings obtained with the corresponding tubes to eliminate error introduced by haemolysis or opalescence of the sera. The percentage of organisms deposited after each period of centrifugation was calculated and plotted against time. The 50 % sedimentation times (T_{50}) were read from the curves (Fig. 1). The T_{50} of the control was adopted as the unit time (U) and the relative 50 % sedimentation times (T') were given by the relation T_{50}/U , which may vary from very nearly 0 (complete agglutination) to 1 (absence of agglutination). The relative 50 % sedimentation times plotted against the amounts of serum (S) gave points approximately on a straight line (Figs. 2-5), and the reaction may be expressed by the equation $T = bS + a$. The agglutination unit was defined as the amount of serum giving a relative 50 % sedimentation time of 0.5, that is, an amount of serum with which the 50 % sedimentation time is half that of the control. This unit was obtained from the line $T = bS + a$; the parameters a and b were calculated by the method described by de Almeida (1951).

Table 2. *Quantitative agglutination test with Proteus OX 19*

Suspension (ml.)	Serum 1/200 (ml.)	Saline (ml.)	Serum concentration (ml.) (S)	Sedimentation			T_{50} *	T_{50}/U † (T)
				5 min.	10 min.	15 min.		
0.1	0.4	0.5	0.0020	27	43	56	12.5	0.86
0.1	0.5	0.4	0.0025	31	48	61	10.5	0.72
0.1	0.6	0.3	0.0030	38	56	70	8.5	0.58
0.1	0.7	0.2	0.0035	41	61	72	6.5	0.44
0.1	—	0.9	—	18	38	52	14.5	—

* Time in minutes for 50 % sedimentation; read from the curves.

† $U = T_{50}$ for the control tube.

RESULTS

Agglutination of rickettsias

Q fever immune guinea-pig serum, with a titre of 1/1280 by the dilution method, showed by the quantitative method an agglutination unit of 0.00075 (1333 units/ml.). The relative sedimentation times plotted against the amounts of serum gave points approximately on a straight line with a regression coefficient of -628.

Murine and classical typhus sera were tested against both the homologous and the heterologous suspensions; the results are shown in Table 3 and in Figs. 3 and 4.

Fig. 1. Sedimentation curves of *Proteus* OX19 suspension in the presence of varying amounts of agglutinating serum. (From Table 2.)

Fig. 2. Agglutination of Q fever rickettsias of homologous serum.

Fig. 3. Agglutination of murine typhus rickettsias by murine and classical typhus sera.

Fig. 4. Agglutination of classical typhus rickettsias by classical and murine typhus sera.

Table 3. *Agglutination of murine and classical rickettsias by homologous and heterologous sera*

Serum	Antigen	Titre by dilution method	Quantitative method		
			Agglutination unit	Units/ml.	Regression coefficient
Murine	Murine	1/80	0.012	83.3	-29.4
Classical	Murine	1/40	0.0625	16.0	-8.0
Murine	Classical	1/20	0.05	20.0	-5.2
Classical	Classical	1/80	0.0085	117.6	-12.5

It was possible to differentiate the two systems by both the quantitative and the serial dilution methods. The former method allowed a clear differentiation by two characteristics: the agglutination unit, which was always lower for the homologous than for the heterologous systems; and the regression coefficient, with higher values for the homologous than for the heterologous systems.

In the serial dilution method, both sera gave titres of 1/80 with the homologous antigens and lower titres with the heterologous antigens; the differences however, were of the order of one or two dilutions, and, in view of the difficulty

Fig. 5. Agglutination of *Proteus* OX19 by sera of different origins. □, rabbit *Proteus* antiserum; ▲, murine typhus serum; △, pregnancy serum A; ●, pregnancy serum B.

in reading the finer degrees of agglutination, the end-point was not always easy to define. We found the estimation of the 50 % end-point (Hedén, 1946) to be impractical in rickettsial agglutination tests because of the difficulty in distinguishing five or even four different grades of agglutination.

Agglutination of Proteus OX 19 (Weil-Felix test)

In Table 4 we give the results of the serial dilution and of the quantitative agglutination methods obtained with four sera, all of which gave a titre of 1/320 by the first method.

Table 4. *Agglutination of Proteus OX 19 by sera of different origins*

Sera	Dilution method		Quantitative method		
	Titre	50 % end-point	Agglutination unit	Units/ml.	Regression coefficient
Rabbit <i>Proteus</i> antiserum*	1/320	1/201	0.006	166	-107
Murine typhus	1/320	1/226	0.016	62	-44.4
Pregnancy A	1/320	1/201	0.021	47	-33
Pregnancy B	1/320	1/127	0.027	37	-32

* 1/32 dilution of a rabbit *Proteus* antiserum with an original agglutination titre of 1/10,240.

The quantitative method clearly distinguished these sera both by the agglutination unit and by the regression coefficient, the latter providing a particularly clear distinction (Fig. 5). A certain correspondence was observed between the values obtained for the 50 % end-points calculated according to Hedén (1946) and those obtained by the quantitative method, except in the case of the rabbit immune serum in which the 50 % end-point was low in relation to the agglutination unit and to the slope.

DISCUSSION

The linear relationship between the degree of agglutination, judged by the relative 50 % sedimentation time, and the amounts of serum, provides valuable information for the study of the agglutination reaction. The activity of sera studied by this method may be expressed by two characteristics: the agglutinin content, represented by the agglutination unit; and the agglutinating capacity, represented by the slope (b) of the time (T)-serum (S) regression line $T = bS + a$.

In this respect, our results are analogous to those obtained with the quantitative complement-fixation test, in which the complement-fixing capacity of certain systems is better expressed by the slope of the complement-antigen and complement-serum regression lines (Rice, 1946; Wolfe & Kornfeld, 1949).

From our results with Proteus OX 19 agglutination, it is interesting to note that in the case of sera of the same origin (pregnancy) the time-serum regression lines show a striking parallelism (Fig. 5), whereas a comparison of the sera of different origins reveals an increasing slope corresponding to increasing specificity of the reaction. These results are in agreement with the view that the Weil-Felix reaction is due to the presence, in the sera of typhus patients, of an antibody reacting with one of the antigenic groupings of Proteus OX 19 (Weil & Felix, 1921; White, 1933; Castaneda, 1935). They also agree with the observation that the agglutination of Proteus OX 19 by sera from pregnant women is due to a factor distinct from that in sera from typhus patients, as shown by indirect evidence by Travassos, de Gois, Pereira & Bruno-Lobo (1951).

From the results obtained, it may be expected that the application of the present method to the study of other groups of micro-organisms will be of value in providing evidence of inter-group antigenic differences. The method may, with the help of analytical centrifuges, also prove to be of use in the study of the antigenic structure of the smaller viruses. Its use as a routine serological test requires further investigation.

In the case of a few sera tested against rickettsial suspensions, a time-serum linear relationship was not observed. The same sera showed in serial dilution tests a wide range of dilutions with partial agglutination. The significance of these results was not investigated.

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