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QUANTITATIVE STUDIES OF COMPLEMENT FIXATION

I — A simplified and accurate procedure based on 50 per cent hemolytic end point

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SUMMARY

A quantitative method for estimating complement, (C')-fixing titers is described. In this method, fixed amounts of antigen (maximally reactive dose) and complement (e.g. 2 $C'H_{50}$) are incubated with amounts of antiserum adjusted to leave residual amounts of non-fixed C' in the range of 0.6 to 1.5 $C'H_{50}$. Titers are calculated by dividing D, the reciprocal of serum dilution, by f', the conversion factor estimated from h'_s, the slope of the line expressing the regression of the logarithm of serum dilution to the logit of the percentage of hemolysis.

INTRODUCTION

The introduction of accurate spectrophotometric techniques [6, 9] for estimation of the 50 per cent hemolytic unit of C' ($C'H_{50}$) was followed by improvement of older C'-fixation procedures and by development of a refined method [10] that permitted quantitation at a level of precision approaching that of the quantitative precipitin method.

As reviewed by OSLER [12], this refined method found many important applications in theoretical studies such as those concerning the parallelism in C'-fixation and specific precipitation, the relationship between C'-fixing potency and antibody nitrogen content, and the estimation and characterization of antigens. The method is, however, too elaborate for serodiagnostic purposes, since it involves the use of a large excess of complement (50, 100 or even 200 $C'H_{50}$) and therefore requires a large number of titrations to determine the number of units of C' fixed in each reaction mixture.

In contrast to the method described in [10], traditional quantitative methods like those developed by KRISTENSEN [7], MALTANER et al. [8, 17], and STEIN & VAN NGU [14] are based on conditions adjusted to provide amounts of residual, unfixed C' in the range of 0.6 to 1.5 $C'H_{50}$.

Intended for needs of the clinical laboratory these traditional methods lack the precision required in replicated tests such as those needed in statistically controlled assays of the kind reported in 1955 [4]. This requisite of precision, combined with simplicity of procedure, is obtained by the method described in the present paper.

Although the essential elements of the procedure have been previously described, it was considered advantageous to devote a separate paper to its complete description in order to save the reader the time required to consult scattered publications.

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MATERIALS

Buffered diluent — Isotonic veronal-bicarbonate buffer, pH 7.3-7.4, containing 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$, as well as 0.1% gelatin, was used as diluent for the various reagents and reaction mixtures. The incorporation of gelatin in the diluent serves to minimize errors arising from adsorption of antigen, antibody, or C' at water-glass and water-air interfaces. Also, gelatin is said to diminish spontaneous lysis of red cells and low level erratic hemolysis attributable to residual traces of cleaning agents left in the glassware [13, 14]. Since the presence of gelatin increases the likelihood of bacterial contamination on prolonged storage even in the cold, sterile gelatin was added to amounts of buffer sufficient for only 1 or 2 days-work.

The buffered diluent was prepared in the following manner: Eighty-five grams of sodium chloride and 3.75 grams of sodium 5,5-diethyl barbiturate were dissolved in about 1400 ml of distilled water. 5.75 grams of 5,5-diethyl barbituric acid were dissolved in about 500 ml of hot distilled water. The two solutions were mixed, and 5 ml of a stock solution containing 1 M $MgCl_2$ and 0.3 M $CaCl_2$ were added. The volume was then made up to exactly 2000 ml. This solution, the stock buffer diluent, is 5 times isotonic. To prepare the isotonic buffer, 50 ml of a sterile solution of Difco gelatin 1% were added to 100 ml of the hypertonic buffer in a 500 ml volumetric flask, and made up to volume with distilled water. It is important that buffer be prepared accurately because lysis of sheep erythrocytes by antibody and C' is highly affected by slight changes in electrolyte concentration [11].

Glassware — In the primary standardization of the hemolytic system the use of calibrated pipettes and volumetric glass vessels is deemed highly advisable. Widemouth tubes of neutral glass, 20 x 100 mm, and 20 ml capacity, were used for the reactions to facilitate mixing of the contents. The glassware should be cleaned preferably with sulfuric acid-sodium dichromate mixture. If detergents are used for cleaning, rinsing must be done with utmost care and thoroughness; otherwise, residual traces of the cleaning agent may remain on the glassware surface and produce erratic hemolysis.

Hemolytic antibody — Rabbit antisheep erythrocyte serum preserved at 0-2°C with an equal volume of glycerol was used as the source of hemolytic antibody.

Guinea pig complement — Healthy, non-pregnant guinea pigs of about 500 grams were bled from the heart (10-15 ml) after a period of fasting of at least 6 hours. The sera were carefully separated from the clot to minimize hemolysis, and a pool of at least 20 sera was prepared. The C' pool was distributed in approximately 1.2 or 2.2 ml portions into acid-cleaned Pyrex test tubes (13 x 100 mm). The tubes were closed with clean rubber stoppers and immediately stored in an electric deep-freeze at -18°C. Individual tubes were thawed by dipping into a water bath at 37°C for a few minutes with occasional mixing of the contents. A measured volume was suitably diluted with cold veronal buffer, and the non-utilized portion was discarded since repeated thawing may deteriorate complement.

Sheep blood — Sheep blood was drawn aseptically into an equal volume of sterile, modified Alsever's solution (24.6 grams glucose, 9.6 grams sodium citrate, and 5.04 grams sodium chloride in 1200 ml distilled water, adjusted to pH 6.1 with citric acid and sterilized by filtration). The citrated blood was preserved at 2°-5°C. As shown by CROFT [5], under these conditions of storage, the susceptibility of sheep red cells to specific hemolysis remains uniform for at least 2 months, following an initial stabilization period of about 1 week.

Sera — The C'-fixing immune sera were obtained from a clot of freshly drawn blood, centrifuged with care to minimize hemolysis, and inactivated at 56°C for 30 minutes.

Antigen — Soluble antigens like bovine serum albumin (BSA) were stored frozen at -18°C in stock solution of suitable concentration. Particulate antigens, e.g. cardiolipin antigen, were subjected to special manipulations.

METHODS

A) *Standardization of the hemolytic system*

Standardization of red cell suspension — A portion of citrated sheep blood sufficient for the day's work was centrifuged, and the cells were washed three times with about 20 volumes of cold veronal buffer. The sediment of washed, packed cells was then suspended in 30 volumes of cold buffer (preliminary 3% suspension), and the suspension was filtered through a small wad of absorbent cotton.

Standardization was accomplished by diluting 1 ml of the suspension with 9 ml of distilled water or 0.1% sodium carbonate. The optical density of the clear lysate was then read in a Coleman Junior spectrophotometer, model 6, with a 13 mm square cuvette at a wavelenght of 550 mμ. The standardized concentration was given by $O.D._{550} = 0.560$ and corresponded to approximately 700 million cells per ml in the standardized suspension.

Since the preliminary "3% suspension" usually does not yield a lysate giving the desired standardization value, it was necessary to adjust the suspension by dilution or concentration (centrifugation and resuspension in a smaller volume) according to the formula: $\frac{O.D. \times 100}{0.560} - 100 = V$, where O.D. is the optical density of the lysate and V is the amount of diluent to be added or subtracted from 100 ml of the preliminary suspension.

If a lysate gave a value $O.D._{550} = 0.588$ instead of the standard value 0.560, correction was made by adding 5 ml of buffered diluent to 100 ml of the non-standardized suspension. since $\frac{0.588 \times 100}{0.560} - 100 = 5$.

Standardization of hemolytic antibody — The standardization of hemolytic antibody was carried out as follows: 2.0 ml of different dilutions of antiserum were added to 2.0 ml of a standardized cell suspension with constant swirling of the contents. Tubes were incubated at 37°C for 15 minutes and kept in an ice bath thereafter. To 1.0 ml of the sensitized cells were added 2.0 ml of guinea pig C' diluted to approximately 0.5 $C'H_{50}$/ml, and 2.0 ml of cold veronal buffer. The test was incubated at 37°C for 60 minutes, with mixing every 5 minutes in the first 30 minutes, and every 10 minutes thereafter, to prevent settling of the cells. After incubation, tubes were centrifuged at 1,500 rpm for 10 minutes, and supernates were analysed to determine the percentage of hemolysis.

The dilution chosen for preparing sensitized cells must be in a range where the degree of hemolysis becomes independent of antibody concentration. This is necessary in order to avoid increase in C' titers resulting from hemolysin in some human or animal sera used in the fixation test. For sensitizing cells, it is customary to use an amount of hemolytic antibody corresponding to 2-4 times the dose beyond which further increments of amboceptor do not appreciably affect the degree of lysis. There is no inconvenience in using such an excess of hemolysin provided one avoids the range in which agglutination of red cells is observed.

Sensitized cells — A measured volume of the adequate dilution of amboceptor was slowly poured into an equal volume of standardized cell suspension with constant swirling of the contents. The suspension was kept in a water bath at 37°C for 15 minutes with occasional mixing and then kept in the refrigerator until used. Sensitized cells were prepared daily as required.

Standardization of complement — Different volumes of a dilution of guinea pig C' 1/500 (1.5 — 2.0 — 2.5, and 3.0 ml) were added to a series of tubes containing 1.0 ml of sensitized cells and an amount of buffer sufficient to bring the final volume in each tube to 5 ml. The tubes were incubated and read as for amboceptor standardization.

The 50 per cent unit of C' was calculated on the basis of the von Krogh equation *,

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TABLE I

Conversion factors for h = 0.2

y	f	y	f	y	f	y	f
10.0	0.644	30.0	0.844	50.0	1.000	70.0	1.185
10.5	0.651	30.5	0.848	50.5	1.004	70.5	1.190
11.0	0.658	31.0	0.852	51.0	1.008	71.0	1.196
11.5	0.664	31.5	0.856	51.5	1.012	71.5	1.202
12.0	0.671	32.0	0.860	52.0	1.016	72.0	1.208
12.5	0.678	32.5	0.864	52.5	1.020	72.5	1.214
13.0	0.684	33.0	0.868	53.0	1.024	73.0	1.220
13.5	0.691	33.5	0.872	53.5	1.028	73.5	1.226
14.0	0.696	34.0	0.876	54.0	1.033	74.0	1.233
14.5	0.701	34.5	0.880	54.5	1.037	74.5	1.239
15.0	0.707	35.0	0.883	55.0	1.041	75.0	1.246
15.5	0.712	35.5	0.887	55.5	1.045	75.5	1.252
16.0	0.718	36.0	0.892	56.0	1.049	76.0	1.259
16.5	0.723	36.5	0.895	56.5	1.054	76.5	1.266
17.0	0.728	37.0	0.899	57.0	1.058	77.0	1.273
17.5	0.733	37.5	0.903	57.5	1.062	77.5	1.281
18.0	0.738	38.0	0.907	58.0	1.067	78.0	1.288
18.5	0.742	38.5	0.911	58.5	1.071	78.5	1.295
19.0	0.748	39.0	0.914	59.0	1.076	79.0	1.303
19.5	0.753	39.5	0.918	59.5	1.080	79.5	1.312
20.0	0.758	40.0	0.922	60.0	1.084	80.0	1.319
20.5	0.763	40.5	0.926	60.5	1.089	80.5	1.328
21.0	0.767	41.0	0.930	61.0	1.094	81.0	1.336
21.5	0.772	41.5	0.934	61.5	1.098	81.5	1.345
22.0	0.776	42.0	0.937	62.0	1.103	82.0	1.354
22.5	0.781	42.5	0.941	62.5	1.108	82.5	1.364
23.0	0.785	43.0	0.945	63.0	1.112	83.0	1.373
23.5	0.790	43.5	0.947	63.5	1.117	83.5	1.384
24.0	0.794	44.0	0.953	64.0	1.122	84.0	1.394
24.5	0.798	44.5	0.957	64.5	1.127	84.5	1.404
25.0	0.803	45.0	0.960	65.0	1.132	85.0	1.415
25.5	0.807	45.5	0.964	65.5	1.137	85.5	1.426
26.0	0.811	46.0	0.969	66.0	1.142	86.0	1.438
26.5	0.815	46.5	0.972	66.5	1.147	86.5	1.450
27.0	0.820	47.0	0.976	67.0	1.152	87.0	1.463
27.5	0.824	47.5	0.980	67.5	1.157	87.5	1.476
28.0	0.828	48.0	0.984	68.0	1.162	88.0	1.490
28.5	0.832	48.5	0.988	68.5	1.168	88.5	1.504
29.0	0.836	49.0	0.992	69.0	1.174	89.0	1.519
29.5	0.840	49.5	0.996	69.5	1.179	89.5	1.535
						90.0	1.552

f = conversion factor; y = percentage of lysis.

which describes the relationship between x, the amount of C' and y, the percentage of lysis: $\log x = \log k + h. \log \frac{y}{1-y}$ (1)

$$x = k. (y/1-y)^h \quad (2)$$

where k = x for y = 0.5 corresponds to $C'H_{50}$, the amount of C' required for 50% hemolysis; and h, the slope of the straight line represented by equation (1), is a parametric constant which expresses the shape characteristics of the sigmoid curve represented by equation (2). The value of h is usually in the range 0.18 to 0.22 so that the values of $(y/1-y)^{0.2}$, given in Table I, may be used as "conversion factors" (f) to estimate k ($C'H_{50}$) or D_k, the titer of C' expressed in $C'H_{50}$ per ml **:

$$k = x/f$$

$$D_k = D \times f.$$

For example, 2.5 ml of guinea pig serum diluted 1/500 gives 52.3 per cent of lysis under the conditions of the test described above. In this particular case, for D = 500/2.5, f = 1.096 (see Table I for y = 52.3), the titer of C' may be evaluated as $200 \times 1.096 = 219.2$.

B) *Estimation of the maximally reactive dose of antigen*

Determination of the maximally reactive dose of antigen is an essential prerequisite in the performance of quantitative C'-fixation. This may be done in a two-dimensional bloc titration according to the following protocol: Mix 0.2 ml of serial dilutions of serum with 0.4 ml of a fixed dilution of C' and 0.2 ml of serial dilutions of antigen. After a fixation period of 20 hours at 0-2°C, add 0.2 ml of sensitized cells. Incubate at 37°C for 60 minutes, centrifuge, and read approximate degrees of lysis with the aid of color standards prepared as follows: To 0.5 ml of standardized cells, add 4.5 ml of distilled water. The hemoglobin solution thus obtained will serve as the 100% reference standard. Prepare a series of dilutions from this 100% standard by mixing 0.1, 0.2, 0.3 ml, etc. of the deep-colored hemoglobin solution with amounts of distilled water sufficient to make up the volume to 1 ml in order to obtain the standards equivalent to 10, 20, 30%, etc.

If a line is drawn through the tubes showing 50 per cent lysis, an "isofixation curve" is obtained [1] that describes the relations between antigen, antibody, and the particular dose of C' used in the test. The vertical branch of this curve is asymptotically parallel to the antibody axis and corresponds to conditions proper for the titration of antigen, because the amount of C' fixed will depend only on the amount of antigen if antibody is in great excess. In the region of excess antigen, however, two main possibilities occur: 1) Isofixation curve, type I, shows an horizontal branch asymptotically parallel to the antigen axis; 2) In isofixation curve, type II, the horizontal branch bends towards higher antibody concentrations.

As pointed out by ALMEIDA [1], for different doses of C' the horizontal branch of type I curves, or its ascending segment in the case of type II isofixation curves, are parallel to each other, and any dose of antigen situated in the region of parallelism of the isofixation curves may be used for comparative titration of antisera. In type I curves, the *doses of parallel reactivity* are also maximally reactive. This is not obviously the case with isofixation curves of type II, where the maximally reactive dose of antigen should be read at the inflexion point of the horizontal branch.

The latter situation is characteristic of systems in which an excess of antigen exerts inhibitory effect on C' fixation, as in the example given in Table II. It is seen in this example that beyond 0.04 μg BSA N, fixation of C' required higher amounts of antibody at the three tested C' levels. A solution of 0.25 μg/ml of BSA, i.e., 0.04 μg

* According to von Krogh [16] y is related to x by equation: $y = \frac{x^n}{x^n + k^n}$ or $x = k\left(\frac{y}{1-y}\right)^{1/n}$, whose graphic expression is a sigmoid line. For the sake of simplicity and following the notation suggested by Thompson et al. [15], h has been used instead of von Krogh's 1/n.

** If the value of h is not encompassed within the usual range 0.2 ± 10%, the value of k is better estimated from the plot of log x versus log (y/1−y) as intercept of the line for y/1 − y = 1. The use of three cycle logarithmic paper is convenient for this purpose.

BSA N/ml was, therefore, used to titrate rabbit anti-BSA in tests with 2.5 or 10 $C'H_{50}$.

C) *Estimation of C'-fixing potency*

For the estimation of C'-fixing potency of an antiserum, mixtures were set up with the following composition:

Serum (serial dilution) ..... 1 ml
Antigen (maximally reactive dilution) 1 ml
Complement (a fixed dilution) ..... 2 ml

After 20 hours of incubation at 0-2°C, 1 ml of sensitized cells was admixed. Spectrophotometric readings of supernates were made after incubation for 1 hour at 37°C.

O.D. values were converted into percentages of hemolysis, y, by reference to the O.D. value of a tube showing complete lysis, e.g., the buffer control (buffer + C'). Controls for anti-or pro-complementary effects of serum and antigen dilutions were also included in the test. In tests conducted with 2 $C'H_{50}$, the mixtures corresponding to the controls were diluted with 4 ml of saline before 2 ml of sensitized cells were added. For instance, if the supernates of serum or antigen controls, diluted 1/2 as specified above, gave O.D. readings corresponding to 61.5 per cent hemolysis, an identical value being obtained in the buffer control, it was concluded that neither serum nor antigen exerted any anti- or pro-complementary effect.

Table III reproduces the protocol of the titration of a rabbit serum anti-BSA according to the method described.

The degrees of lysis in the test conducted in a final volume of 5 ml, as outlined before, should be determined spectrophotometrically. In less precise studies, however, the final volume may be reduced to 1 or even 0.5 ml. In such a case, supernates are read by comparison with visual color standards, as recommended for the titration of antigen.

The titer (T) is expressed by the dilution of serum corresponding to 50 per cent hemo-

TABLE II

Estimation of maximally reactive dose of antigen in tests with different amounts of complement

AbN/ml *	BSA N/ml, μg							
μg	0.004	0.012	0.04	0.12	0.36	1.1	3.3	10
			A) 2 $C'H_{50}$					
5	0	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0	0
1.25	20	0	0	0	0	0	30	30
0.625	60	10	0	0	10	40	70	70
0.312	80	80	80	60	60	80	80	80
0.156	80	80	80	80	80	80	80	80
0.078	80	80	80	80	80	80	80	80
			B) 5 $C'H_{50}$					
3.3	20	0	0	0	0	0	0	0
2.2	50	0	0	0	0	0	10	20
1.5	90	0	0	0	0	0	20	80
1.0	90	10	0	0	0	10	80	80
0.66	90	70	10	10	20	80	80	80
0.44	90	90	40	80	90	80	80	80
0.3	90	90	90	90	90	80	80	80
			C) 10 $C'H_{50}$					
3.3	100	10	0	0	0			
2.2	100	20	0	0	0			
1.5	100	50	0	0	0			
1.0	100	80	10	10	10			
0.66	100	100	50	30	50			
0.44	100	100	100	100	100			
0.3	100	100	100	100	100			

* Rabbit serum anti-BSA no. 21, 0.707 mg AbN/ml

lysis, i.e., by the dilution that fixes the amount of C' added less 1 $C'H_{50}$.

Two methods may be used in the estimation of titer:

A) *Method of graphic interpolation*

In this method T is graphically estimated from the plot log D versus log (y/1−y) by using a 2x3 cycles log paper, or from plotting log D against the probit of y, by using log probability paper *. The intercept on the ordinate axis for 50 per cent hemolysis (y/1−y = 1) gives the value of T (Fig. 1).

Calculation of T by this method is advantageous because it does not require assumption of a constant value of h'_s, the slope of residual, unfixed C'. It should be emphasized that only sera yielding parallel lines in the above mentioned plot, in tests performed with identical reagents, are comparable in terms of T.

B) *Method based on a constant value of* h'_s

An alternative method for calculating T involves the use of conversion factors derived from h'_s. The titer is simply calculated by dividing the serum dilution by its corresponding conversion factor: T = D/f'.

This method is based on the assumption of constant value for h'_s and has two obvious disadvantages:

a) Use of conversion factors based on a slope which may not correspond to the particular conditions on the day of the test;

b) Use of the same conversion factor for sera exhibiting significantly different slopes. This last possibility probably accounts for the "atypical reactions" observed with the technique of WADSWORTH et al. in quantitative complement fixation studies with sera of syphilis and Chagas' disease [2, 18].

DISCUSSION

The quantitative procedure outlined in this paper combines ease of performance of methods based on direct measurement of non-fixed, residual C' with the technical accuracy of the quantitative method introduced in [10].

The new procedure particularly resembles

TABLE III

Protocol of the titration of rabbit serum anti-BSA no. 21 with maximally reactive dose of BSA and 5 $C'H_{50}$

Serum dil., D	O.D. of supernates	% lysis, y	y/1−y	Conversion factor, f'(1)	Titer (D/f')
58	0.105	0.184	0.225	—	—
63.9	0.160	0.281	0.391	0.893	71.5
70.2	0.245	0.430	0.754	0.967	72.6
77.3	0.370	0.650	1.857	1.078	71.7
"Complete"	0.570	—	—	—	—

(1) Calculated for h'_s = 0.12

* no. 3128 designed by Hazen, Wipple & Fuller. Codex Book Co., Inc., Norwood, Mass.

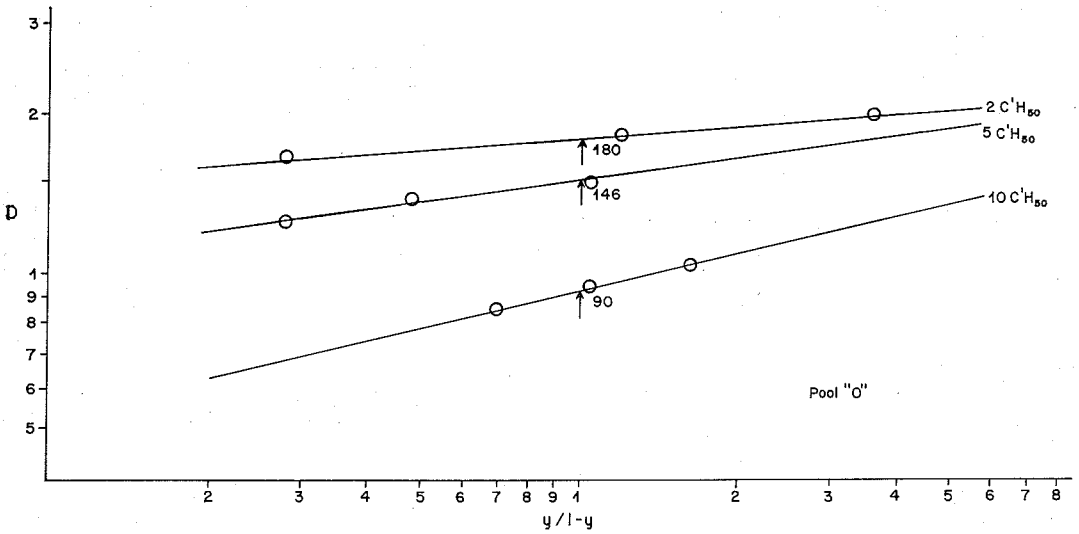

Fig. 1 — Graphical determination of the C'-fixing potency of the same antiserum (Pool "0") at three different levels of complement

TABLE IV

Experimental results from single titration of rabbit anti-BSA with a maximally reactive dose of antigen at three C' levels

Serum *	Values of h's in tests with			Titers obtained in tests with		
	2 $C'H_{50}$	5 $C'H_{50}$	10 $C'H_{50}$	2 $C'H_{50}$	5 $C'H_{50}$	10 $C'H_{50}$
21	0.09	0.12	0.17	95	72	39
22	0.07	0.15	0.26	122	98	59
25	0.09	0.11	0.19	102	79	49
M	0.09	0.10	0.18	102	81	50
O	0.07	0.14	0.22	180	146	90

* Following groups of sera were tested on the same day, with same reagents: 21 — 22, and 25 — M — 0. Sera were previously diluted to the same level of precipitating antibody (40 μg AbN/ml)

previously described methods [7, 8] in which fixation mixtures are set up with serial dilutions of serum, a maximally reactive dose of antigen, and a fixed dose of C'.

As shown in the second contribution of this series, there is a linear relationship between the logarithm of serum dilution (D) and the percentage of lysis (y), according to the following equation:

$$\log D = \log T + h'_s . \log (y/1-y),$$

where log T, the zero intercept, and h'_s, the

slope, are parametric constants for the conditions of the test.

From the equation above,

$$D = T. (y/1 - y)^{h'_s}$$

and, by substituting f' for $(y/1-y)^{h'_s}$,

$$T = \frac{D}{f'}.$$

This expression of titer represents a submultiple of $D.K'_{sa}$, as used in MALTANER and GHESH's procedure, under the provision that K'_{sa} be calculated on the basis of the same conversion factor, f':

$$K'_{sa} = \frac{n}{f'}$$

n being the amount of C' added in the test.

The numerator of the "index ratio titer", $D.K'_{sa} = D. \frac{n}{f'} = n. \frac{D}{f'}$, is, then, obviously a multiple of the expression of titer, T, as used in this study.

According to the method described by the Albany investigators, K'_{sa} is calculated with the aid of conversion factors based on the slope of the plot log x versus logit y, in experiments in which varying amounts of C', x, are tested in the presence of serum and antigen in the range of partial lysis. This requires previous adjustment of serum and antigen dilutions, and, besides unnecessary technical complications, introduces an important source of error, since the value of the slope under conditions described above may be significantly different from the slope h'_s obtained by the plot log D versus log $(y/1 - y)$.

This slope is fundamentally distinct from ALMEIDA's h'_x [3], since h'_x usually varies as a decreasing function of C' added, whereas h'_s increases as a direct function of C', e.g., from 0.07 — 0.09 in tests with 2 $C'H_{50}$ to 0.17 — 0.26 in tests with 10 $C'H_{50}$ (cf. Table IV).

Table IV also illustrates the wide variation of T observed with five different sera previously adjusted to the same level (40 μg/ml AbN) of precipitating antibody. From the sera tested, only two (25 and M) showed identical titers. Serum O, examined on the same day with 25 and M, exhibited a titer 1.8 times higher at the three tested C' levels. Values obtained with serum 22 were 1.3 — 1.5 higher than those given by serum 21 tested on the same day. This may be explained either by the variable content in non-precipitating, C'-fixing antibody in the above sera, or by qualitative differences of the antibodies, as suggested by the work of WALLACE et al. [19].

As shown in the second contribution of this series, the variations of the value h'_s in tests with different batches of C' and red cells do not affect the values of relative potency of antisera compared under identical experimental conditions with a reference standard. This provides additional basis to the recommendation put forward thirty nine years ago by KRISTENSEN [7] in his pioneer contribution towards the establishment of a simplified C'-fixation assay based upon a *parallel line assay*.

RESUMO

Estudos quantitativos sôbre fixação de complemento. I — Método simplificado e preciso baseado na leitura do ponto correspondente a 50% de hemólise

Descreve-se um método para determinar a potência fixadora de complemento, no qual quantidades fixas de complemento (e.g. 2 $C'H_{50}$) e de antígeno (dose de reatividade máxima) são incubadas com quantidades variáveis de anti-sôro ajustadas de maneira a deixar, na mistura fixadora, uma quantidade residual de complemento compreendida entre 0.6 e 1.5 $C'H_{50}$.

O título do sôro é calculado simplesmente dividindo D, a recíproca da diluição do anti-sôro, por f', o fator de conversão correspondente à percentagem de lise, y, calculado com base em h'_s, inclinação da reta log D versus log $(y/1 - y)$.

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