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A Method for Serial Determinations of Serum Complement

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Summary. A method is described for making serial estimations of serum complement. Titrations were made in a standardized haemolytic system and the 50 per cent haemolysis end point obtained by relating the probit percentage haemolysis to the reciprocal of the corresponding serum dilution. Possible variable factors such as changes in *p*H and red cell concentration were studied and it was found that the most important source of error arose from the use of different batches of sheep red cells. This was overcome by running a lyophilized standard reference serum in parallel with all titrations and expressing the unknown sera in terms of this standard. Observations were then made on a group of selected subjects to form the basis for a study of changes in patients suffering from renal disease.

INTRODUCTION

IT HAS been known for a long time that serum complement is lowered during acute nephritis (Gunn, 1914–15; Kellett and Thomson, 1939; Reader, 1948). Recently Lange *et al.* (1951) have reported low levels in the nephrotic syndrome in children and have suggested that complement determinations may be of diagnostic and prognostic significance in this condition.

To assess the value of complement determinations in renal diseases it is necessary to have a method which is acceptably reproducible for serial estimations over prolonged periods of time. This has not always been the case in previously reported studies. The purpose of this paper is to emphasise the difficulties which arise in such a study and to describe a method which has been found suitable for serial complement estimations in individual patients over periods of years if necessary. Experiments have been designed to test the reproducibility and limits of error of the method, which has then been applied to the study of a group of healthy normal people and patients suffering from certain non-renal diseases. A control group of complement values has been compiled from these results. In a subsequent paper values obtained in patients suffering from various renal diseases at different stages are compared with this control group.

MATERIALS AND METHODS

Collection of Serum

Venous blood was allowed to clot at room temperature or defibrinated using a bent glass rod. The serum was used within four hours, but occasionally after storage at 4° C. overnight.

Buffered Saline

All reagents were diluted and sheep red cells suspended in a veronal buffered saline at *p*H 7·4 prepared according to the method of Mayer, Eaton and Heidelberger, (1946).

Standard Complement

A standard reference preparation of complement was used throughout these studies. Initially guinea pig serum preserved by Richardson's method (1941) was used, but it was later found more convenient to use a lyophilized human serum stored at 4° C. in sealed glass ampoules. 0·5 ml. volume of fresh serum was lyophilized in ampoules and a 1 in 10 dilution made in buffered saline when required.

Haemolytic System

Formolized sheep red cells and glycerinated anti-sheep cell serum ('Wellcome' brand Wassermann reagents) were obtained from Burroughs Wellcome and Co. Batches of sheep red cells were obtained each week and as far as possible treated identically. They were washed three times in buffered saline and suspended to give a concentration just greater than 6 per cent. Haematocrit determinations were made on this suspension (centrifuged at 3000 r.p.m. for 30 minutes in Wintrobe tubes) and sufficient buffered saline added to adjust the concentration accurately to 6 per cent, in the manner suggested by Price and Wilkinson (1947). Haemolytic antibody was diluted in buffered saline to give a solution containing 6 minimal haemolytic doses. When not in use these reagents were stored at 4° C. When required, the cells were sensitized by incubating with an equal volume of haemolytic antibody at 37° C. for 30 minutes. The sensitized cells were used immediately.

Procedure

Suitable serial dilutions of the serum were made in buffered saline to give a final volume of 0·5 ml. in each case. For normal sera it was found to be convenient to make an initial dilution of 1 in 10 in saline and from this further dilutions up to 1 in 60 in six tubes. To each tube was added 1 ml. of saline followed by 0·5 ml. of the sensitized cells. Two control tubes were included. In one (representing zero haemolysis) the serum dilution was replaced by 0·5 ml. of saline, in the other (representing 100 per cent haemolysis) 1·5 ml. of distilled water was added to 0·5 ml. of the sensitized cells. A similar series of tubes was put up for the standard complement.

The tubes were incubated in a water bath at 37° C. for 60 minutes and then stored at 4° C. for an hour. If more convenient this time could be extended overnight without significantly altering the result. The tubes were then centrifuged at 3000 r.p.m. for 5 minutes and a 1 in 10 dilution of each supernatant made in 0·85 per cent sodium chloride solution.

Measurements of haemoglobin concentration in these diluted supernatants were made in a Unicam Spectrophotometer as previously described (Walton, Ellis and Taylor, 1957). An absorption reading of a suitably diluted serum was deducted if the original serum was excessively lipaemic or icteric. The amount of haemolysis produced in each tube was then expressed as a percentage of that occurring in the second control tube.

RESULTS

When the degree of haemolysis, expressed as percentage values, was related to the corresponding serum dilution, the well-recognized sigmoid relationship was obtained (Fig. 1A). On transforming the per cent haemolysis into probit units and plotting these results against the reciprocal of the corresponding serum dilution, a linear relationship

was obtained over the range 20 to 80 per cent haemolysis (Fig 1B). From this line an accurate estimate of the dilution of serum associated with 50 per cent haemolysis could be made.

For convenience in comparing results an arbitrary system of unitage was adopted and a serum was said to contain 1 unit of activity per ml. when a 1 in 25 dilution gave rise to 50 per cent haemolysis. The values for the reciprocal of the serum dilution could thus be transformed into unit values and the serum complement activity in units per ml. of original serum read off directly at the 5 probit units (50 per cent haemolysis) level.

All values obtained were referred to that of a standard complement titrated in parallel. The initial value for this standard serum was obtained by calculating the mean of sixteen estimations using sixteen different batches of sheep red cells.

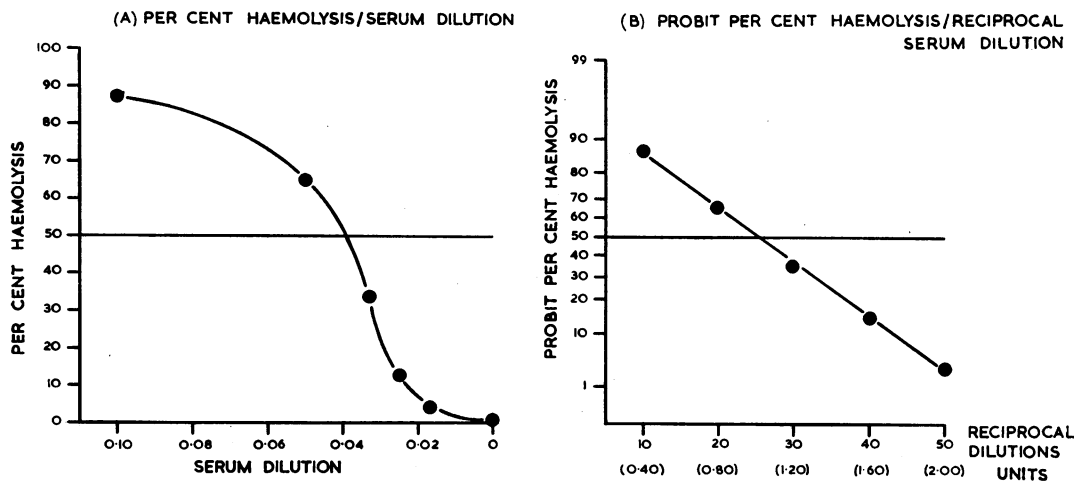

FIG. 1. Illustrating alternative methods of expressing the relation between the degree of haemolysis and the dilution of serum (i.e. concentration of complement) in a standardized haemolytic system. (*a*) Sigmoid relation between percentage haemolysis and serum dilution; (*b*) Transformation of (*a*) to linear relation by plotting percentage haemolysis (probit scale) against reciprocal of serum dilution. The figures bracketed also express the latter scale in arbitrary units.

REPRODUCIBILITY AND SOURCES OF ERROR

Stability of Serum Complement

Seifter, Pillemer and Ecker (1944) studied the stability of human serum complement under varying conditions of storage and stated that the complement activity of undiluted serum was stable for up to one week when kept at 1° C. We have found that complement values are unchanged for periods varying from 3 to 5 days when sera are stored at 4° C. As all estimations were made well within these limits, no error was anticipated from this source.

Hydrogen Ion Concentration of Medium

The effect of variation in *p*H on complement activity was investigated. In one experiment red cells from one batch were washed in saline and then the usual 6 per cent suspensions made in isotonic saline at a range of *p*H from 4·2 to 7·5. These suspensions were used to determine the complement value for a single serum. The cells

were sensitized by adding an equal volume of haemolytic antibody diluted in saline at a corresponding *p*H in each instance. The results obtained are shown in Table 1 and are in agreement with those of Seifter, Pillemer and Ecker (1944) who found that complement activity was inhibited by extremes of *p*H, but was relatively unaffected over the range 6·1 to 8·4. Mayer *et al.* (1946) likewise found no significant variation in complement activity over the range of *p*H 6·9 to 7·6. In our experience there has been a marked variation in the *p*H of physiological saline as usually prepared. To eliminate possible errors from this source all titrations have been made at *p*H 7·4 using a buffered saline throughout.

Concentration of Sheep Cell Suspensions

In this experiment red cell suspensions were made at varying concentrations from a single batch of cells and used to determine the complement value for a single serum. The titration in each case was carried out at *p*H 7·4. The results obtained are shown in Table 1B.

TABLE 1

EFFECT OF VARIATION IN (A) *p*H AND (B) RED CELL CONCENTRATION, ON SERUM COMPLEMENT

(A)

p*H of reaction*	*Complement units per ml.*
4·2	Nil
4·7	Nil
5·5	2·90
6·1	4·60
7·0	4·92
7·5	4·92

(B)

Concentration red cells %	*Complement units per ml.*
4·0	2·34
5·0	2·14
6·0	2·00
7·0	1·75
8·0	1·77

In (A) a single guinea pig serum was titrated at varying *p*H with a fixed concentration of sheep red cells, and in (B) a single human serum was titrated at constant *p*H (7·4) using red cell suspensions at varying concentrations.

With low red cell concentrations there was an apparent increase in complement activity. To eliminate this source of error red cell suspensions were standardized by reference to haematocrit determinations. Attention has recently been drawn to this problem by Plescia, Amiraian and Heidelberger (1957).

Concentration of Haemolytic Antibody

It is well known that there is a direct relationship between the concentration of haemolytic antibody used in a haemolytic system and the apparent complement activity (Kent, 1946). This has been amplified recently by Walton, Ellis and Taylor (1957). Haemolytic antibody was therefore used at a constant concentration of six minimal haemolytic doses throughout.

Sensitization of Sheep Red Cells

Complement titrations were made on a single serum using red cells from one batch sensitized by incubating with haemolytic antibody for varying periods of time. There was surprisingly little difference for periods from 5 minutes up to 60 minutes. For convenience all titrations were made after a 30 minute sensitization period.

Variation in Sheep Red Cell Batches

It was thought that the most likely source of error in an uncontrolled system would arise from variations in the batches of sheep cells obtained each week. Figs. 2 and 3 show the values obtained for stable preserved specimens of guinea pig and human sera using different batches of red cells obtained at weekly intervals.

The variation was of surprising magnitude and made it imperative that a standard reference complement be run in parallel with each batch of titrations if comparable results were to be obtained. It appeared that this variation was due to some intrinsic difference

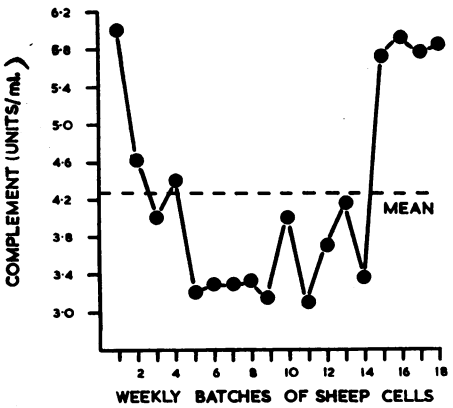

FIG. 2. Apparent variation of complement activity in a single sample of preserved guinea pig serum tested with different batches of sheep red cells.

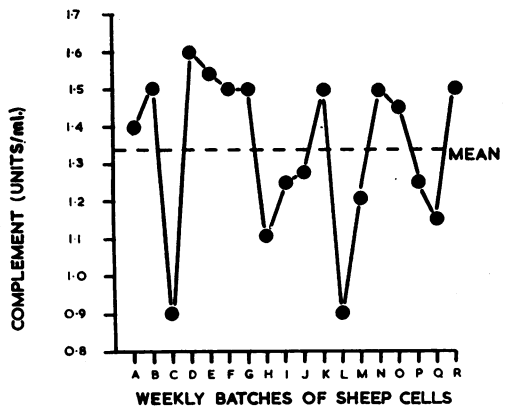

FIG. 3. Apparent variation of complement activity in a single sample of lyophilized human serum tested with different batches of sheep red cells.

in the cells. Experiments designed to investigate the effect of variation in *p*H, ionic strength, temperature, concentration and osmotic fragility of the cells failed to reveal any other satisfactory explanation.

REPRODUCIBILITY OF THE METHOD

The previous experiments had suggested that the main source of variability of results from week to week was due to an apparently inherent variation in susceptibility to immune lysis between different batches of sheep red cells. An experiment was undertaken to see whether allowance could be made for this variation between batches of cells by the introduction of a standard reference source of complement. Having established the titre for 50 per cent haemolysis of this standard complement against one arbitrarily chosen batch of red cells, the aim was to see whether the titres obtained with subsequent batches of red cells would allow the derivation of a simple correction factor, and whether the application of this factor would allow valid serial comparisons of the complementary activity of sera in spite of differences in susceptibility to haemolysis between batches of the cells used.

This necessitated a preliminary investigation of the uniformity and stability of complement activity in the individual ampoules of lyophilized human serum adopted as the reference source.

Uniformity between Ampoules of Lyophilized Serum

Fresh serum was lyophilized in 0·5 ml. volumes as previously described. The complement activity in each of three ampoules was estimated in triplicate using the same reagents in parallel so that variations in the values obtained would be attributable only to differences between the ampoules. The results obtained are shown in Table 2.

TABLE 2

VALUES OBTAINED FOR TRIPLICATE RUNS ON EACH OF THREE AMPOULES OF LYOPHILIZED SERUM

Runs	*Complement activity (units per ml. serum)**		
	Ampoule 'A'	*Ampoule 'B'*	*Ampoule 'C'*
1	2·44	2·38	2·44
2	2·40	2·46	2·43
3	2·43	2·38	2·47

* Each result derived from the common regression of an experiment in triplicate.

Analysis of Variance: (i) between runs V.R. $= \frac{n_1}{n_2} = \frac{2}{4} = 0{\cdot}034$; $P < 0{\cdot}20$

(ii) between ampoules V.R. $= \frac{n_1}{n_2} = \frac{2}{4} = 0{\cdot}81$; $P < 0{\cdot}20$

From an analysis of variance into the components due to differences between individual runs and individual ampoules, it was concluded that there was no significant difference in the complement activity between different ampoules of the standard complement.

Adjustment of Difference between Red Cell Batches

The standard complement was now used to investigate (*a*) the effect of ageing of red cell suspensions by making complement titrations in triplicate on a single batch of red cells (i) within 4 hours of preparation of the cell suspension, and (ii) when the cell suspension was 24 hours old. (*b*) The variation between different red cell batches received over a period of three weeks. For the test serum, blood was drawn each week from the same healthy individual and the usual titrations set up in triplicate. The results obtained are shown in Table 3.

TABLE 3

VALUES OF COMPLEMENT ACTIVITY FOR A SINGLE FRESH SERUM OBTAINED USING DIFFERENT BATCHES OF SHEEP CELLS

Serum	*Complement values* (Units per ml. serum)*					
	W1		*W2*		*W3*	
	D1	*D2*	*D1*	*D2*	*D1*	*D2*
Standard	2·60	2·67	2·30	2·37	1·72	1·73
Unknown (uncorrected)	1·83	1·85	1·64	1·67	1·25	1·20
Unknown (corrected)	1·83	1·80	1·85	1·83	1·89	1·80

* Each result derived from the common regression of an experiment in triplicate.

The values are given uncorrected and after correction by reference to the standard. W1, W2 and W3 are weekly batches of sheep red cells; D1, and D2, 4-hour- and 24-hour-old cell suspensions respectively.

No significant difference in slopes or intercepts was found between replicates and the values obtained for their common regression, in each titration. The figures given were therefore derived from the intercept of the common regression line with the 5 probit unit (50 per cent) level on the Y-axis.

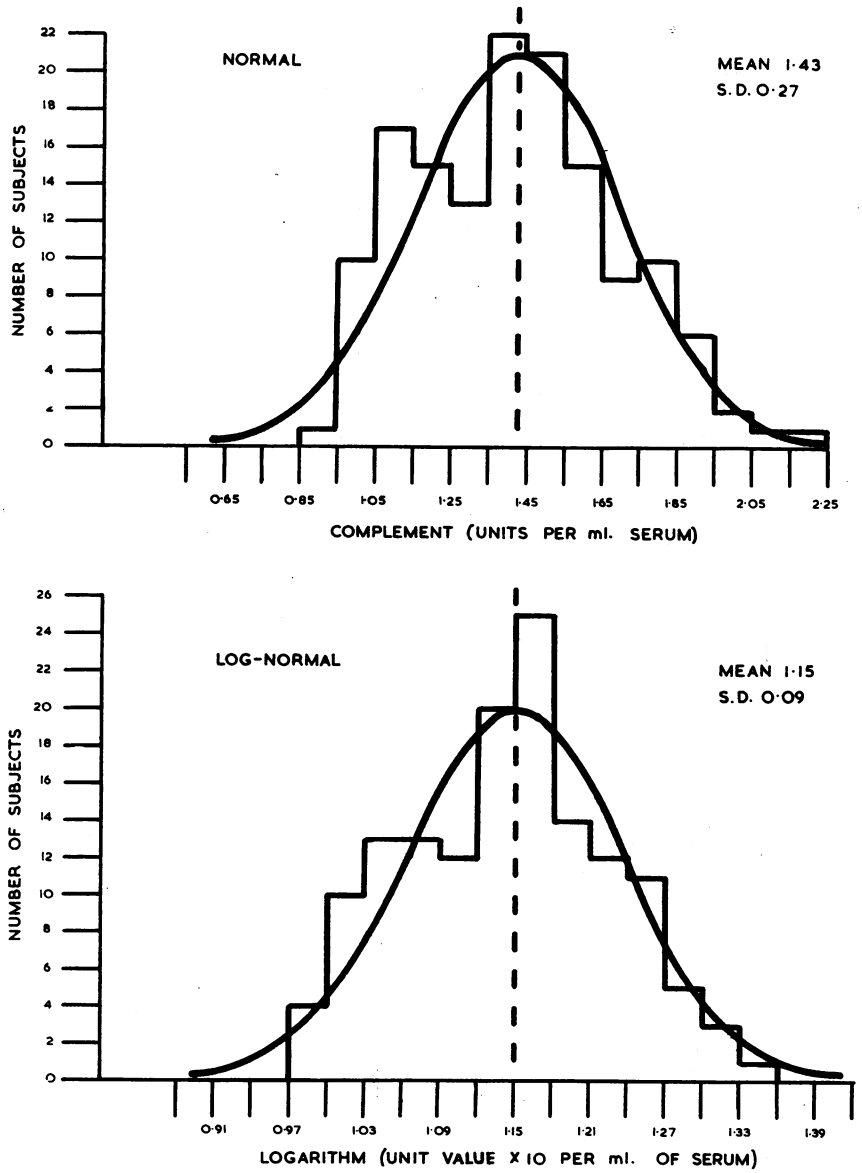

FIG. 4. Range of serum complement values (in arbitrary units) in 143 subjects. (*a*) Distribution of arithmetic values; (*b*) Distribution of logarithmic values. In each case the calculated normal distribution curve is superimposed on the histogram.

A small variation was encountered from day to day using the same batch of cells. But when estimations were made at weekly intervals with different batches of cells the error

was considerable (mean 1·57, coefficient of variation 16·5 per cent). However, it will be seen that the values for the unknown serum obtained by reference to the standard over the 3 week period were acceptably uniform (mean 1·83, coefficient of variation 1·8 per cent). The variation between different batches of formolized red cells over long periods found here is greater than that encountered when studying, over a more limited period, the effect of using different batches of sheep cells for the measurement of the anticomplementary activity of a single sample of heparin (Walton, Ellis and Taylor, 1957). In each case, however, the final result was adequately corrected by reference to a standard preparation.

APPLICATION OF THE METHOD TO THE STUDY OF SERUM COMPLEMENT IN A CONTROL GROUP

Sera were examined from healthy normal adults and children and from patients suffering from a variety of selected diseases. These included peptic ulcer, various malignant diseases, hypertension and ulcerative colitis. For each group of disorders there was no significant variation in either the range or the mean value when these were compared with those for the healthy normals. The results obtained for the pathological sera were therefore added to those for the healthy sera for inclusion in a control group. Patients suffering from non-renal diseases known to be associated with low serum complement levels, such as portal cirrhosis and infective hepatitis, were excluded. The results obtained are shown in Fig. 4.

It will be seen that the histogram of the distribution of these values expressed in terms of units per ml. of serum is somewhat asymmetrical with regard to the superimposed normal curve calculated for the data (Fig. 4). This asymmetry is diminished by expressing the complement values in terms of logarithm-units (Fig. 4). It appears therefore that the distribution is probably a log-normal one rather than normal.

The results were further analysed into groups related to the age and sex of the individuals, but no significant differences were found between these groups.

DISCUSSION

Several studies of serum complement have previously been made in patients suffering from renal disorders, but the methods used have not always been satisfactory. In earlier work the titration end point was frequently taken at 100 per cent haemolysis using a series of tubes containing doubling dilutions of the serum. Reader (1948) utilized such a method when he studied the changes occurring in acute nephritis and did not include an internal standard. This method was not capable of yielding sufficiently accurate or reproducible results for long-term studies. It has been pointed out by Kabat and Mayer (1948) that in the haemolytic system, where a sigmoid relationship exists between the degree of haemolysis and concentration of complement, the most sensitive index of activity is an end point at the 50 per cent level where the reaction is most sensitive to minimal changes in the amount of complement available.

Kellett (1954) improved his original (1939) method when he adopted the 50 per cent haemolysis level as an index of complement activity. However, the use of the sigmoid relationship between percentage haemolysis values and serum dilutions and the absence of a reference standard complement made the interpretation of variations in patients over periods of months difficult or impossible.

Lange (1951) used the method suggested by Wadsworth (1939) and used guinea pig serum as the reference standard complement. Fifty per cent haemolysis end points were estimated by comparison with a series of colour standards arranged to represent increments of 5 per cent haemolysis. If no one tube corresponded to 50 per cent haemolysis, the tubes closest above and below were estimated and the actual 50 per cent level obtained by interpolation. Clearly, this method is less accurate than the spectrophotometric one used in the present study. The usual objection raised to the use of spectrophotometric methods is that false values may be obtained with lipaemic or icteric sera. A correction for this possible error was introduced in the present method as detailed above.

Wedgwood and Janeway (1953) used a method similar to that described by Kabat and Mayer (1948) and estimated the 50 per cent haemolysis titre of sera by means of the von Krogh formula using log-log graph paper in the manner suggested by Mayer, Eaton and Heidelberger (1946).

We believe that the method now described, based on a design relating the probit per cent haemolysis to the reciprocal of the corresponding serum dilution, is a simpler means of obtaining an accurate estimate of that concentration of serum associated with 50 per cent haemolysis. The reasons for the adoption of this design have been considered elsewhere (Waksman, 1949; Walton, Ellis and Taylor, 1957).

The experiments outlined serve to illustrate the many difficulties which arise with respect to certain variables such as *p*H and red cell concentration. Previous workers have discussed these and other factors such as the concentration of magnesium and calcium ions required (Mayer *et al.*, 1946), but in our opinion insufficient emphasis has been placed upon the extreme variability due to different batches of formolized red cells. We have not investigated whether similar variation occurs with untreated cells. The procedure adopted here of referring all estimations to a standard preparation of complement run in parallel with each batch of titrations gives acceptably reproducible results even when observations have to be made over periods of months in individual patients.

It is difficult to compare our range of normal controls with those of other writers because complement activity is so dependent upon the relative concentration of the individual reagents and the total volume used in the titrations. The results agree fairly closely with those given by Lange (1955), however, so that his system of unitage appears to be approximately interchangeable with the present one.

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