126. A SIMPLE COLORIMETRIC METHOD FOR THE ESTIMATION OF HAEMOLYSIS AND ITS APPLICATION TO THE STUDY OF STREPTOLYSIN

By DENIS HERBERT¹

From the Biochemical Laboratory, Cambridge

(Received 1 November 1941)

THE method to be described arose as the result of an investigation² of a haemolysin produced by group A haemolytic streptococci ('streptolysin O'). In the course of this work, many measurements of the haemolytic activities of different streptolysin O preparations had to be made, and the need was felt of a method for measuring haemolysis more accurate than those in common use.

The method of 'serial dilutions' is usually adopted for the measurement of haemolytic activity. Falling amounts of haemolysin solution are incubated in a series of tubes with constant amounts of a red blood cell suspension; the 'end-point' is then taken as the last tube in which complete haemolysis occurs, as judged by eye. Widely adopted as this method is, it is extremely inaccurate. If, as is usual, the serial dilutions are so spaced that each tube contains half as much haemolysin as the preceding one, then the possible error is anything up to 100%. This error cannot be greatly reduced by a closer spacing of the dilutions, for this makes it more difficult to determine the end-point by visual inspection. In addition to this there are other sources of error in the method as it is usually practised, such as inadequate control of pH and other factors to be discussed below.

Other more refined methods have occasionally been used for measurement of haemolytic activity; these have mostly been too time-consuming or have needed too complicated apparatus to come into routine use among pathologists.

Eagle [1929], studying the haemolysis of sensitized red cells by complement, observed the time taken for complete haemolysis of a certain amount of red cells, which was a function of the amount of complement present. The author is inclined to doubt the accuracy of this method unless some form of optical apparatus is used to assist in determining the exact moment of complete haemolysis, which is rather difficult to determine by the naked eye alone.

A more refined application of the same principle has been designed by Ponder [1932] and applied to the study of haemolysis by saponins etc. In this method the red cells are mixed with haemolysin in one cell of a Stufenphotometer immersed in a constant temperature bath and with an automatic stirrer to prevent the red cells settling. The amount of light passing through the suspension is determined from moment to moment; this allows the time taken for complete haemolysis and for any degree of partial haemolysis to be measured with high accuracy. This is probably the most sensitive arrangement for the measurement of haemolysis yet designed, but it has the disadvantage of needing rather complicated apparatus.

¹ Fellow of King's College, Cambridge.

² Reported in the following paper by Herbert & Todd [1941].

Vles *et al.* [1931] incubated red cells with amounts of haemolysin sufficient to cause only partial haemolysis in a given time; after centrifuging unlysed red cells the degree of haemolysis was measured by determining the amount of haemoglobin in the supernatant. This was done by measuring the extinction coefficient of the absorption band at $542\text{ m}\mu$ in a visual spectrophotometer. This method is accurate but again needs complicated apparatus and in addition is very time-consuming.

It may be pointed out that the method of Vles *et al.* [1931] and the customary method of 'serial dilutions' both measure the amount of haemolysis occurring in a given time, while the method of Eagle measures the time taken to effect a certain amount of haemolysis; the apparatus of Ponder may be used in either way.

The method to be described is based on the first principle, and is similar to that of Vles *et al.* in that the extent of partial haemolysis in a given time is measured by determination of the amount of haemoglobin set free from the haemolysed red cells; this is done however by a colorimetric method to be described below, which is extremely rapid and simple and does not need complicated apparatus.

EXPERIMENTAL

Briefly, the method consists in measuring out into a series of tubes falling amounts of the haemolysin solution whose activity is to be measured, and incubating them all with a standard amount of a red cell suspension; after incubation the tubes are centrifuged and the amounts of haemoglobin in the supernatant measured. Other important features of the method are as follows.

(1) *Control of pH.* The medium in which haemolytic tests are usually carried out is 'normal' saline; in other words it is a substantially unbuffered medium. This is liable to lead to error since the haemolytic activities of most haemolysins vary with the *pH*, and the *pH* at which they exert their maximum activity is not necessarily at neutrality. The haemolytic activity of streptolysin O, for example, is maximum at *pH* 6.5 and falls off quite sharply on either side of this *pH* [Herbert & Todd, 1941].

For accurate work, therefore, haemolysis tests should be carried out in isotonic buffer solutions. For measurements on streptolysin O a phosphate buffer of *pH* 6.5 (68 ml. $0.158\text{ M Na}_2\text{HPO}_4$ + 32 ml. $0.158\text{ M NaH}_2\text{PO}_4$) was used; this buffer, which is itself isotonic, was mixed with an equal volume of 0.9% NaCl. This isotonic phosphate-NaCl mixture was used for washing and suspending the red cells, and also for diluting haemolysin solutions. For other haemolysins, of course, buffers of different *pH* and hence of different molarity might have to be used. As far as I am aware, the literature contains no references to isotonic phosphate buffers; for the purposes of this work, the data of Grollmann [1931] on the vapour pressures of *M*/15 phosphate buffer mixtures were used to calculate the molarities of buffers isotonic with 0.9% NaCl.

(2) *The use of red cell suspensions of an exactly standardized strength.* Red cell suspensions were standardized by colorimetric estimation of their haemoglobin content, and the standard suspensions used always contained exactly 7.5 mg. haemoglobin per ml. This figure was chosen because it corresponds very closely (for rabbit's red cells) with the $2\frac{1}{2}\%$ by volume suspension commonly used by pathologists. Standardization by measuring the haemoglobin content, however, is much more accurate than by measuring the volume of the red cells.

The standard conditions used throughout this work for measuring haemolytic activity were as follows.

Varying amounts of the haemolysin solution, made up to 1 ml. with isotonic saline-phosphate pH 6.5, were mixed with 1 ml. of a standard red cell suspension and incubated for 30 min. at 38°. The tubes were then immediately centrifuged and the amounts of haemoglobin in the supernatant fluids determined colorimetrically. When adding the red cell suspension to the haemolysin it is important (in the case of streptolysin O at least) that mixing should be immediate and complete; the red cell suspension is therefore rapidly blown into the haemolysin solution from a 1 ml. 'blow-out' pipette.

The amount of haemolysin that under these conditions liberates 3.75 mg. haemoglobin (corresponding to haemolysis of 50 % of the standard dose of red cells) is called one haemolytic unit (H.U.). Thus if 0.001 ml. of a certain haemolysin solution caused exactly 50 % haemolysis under the standard conditions, then the solution would contain 1000 H.U./ml.

The haemoglobin estimations were carried out in a Leitz colorimeter. The principle of the method is that if a suitable green filter is used in the eyepiece of the colorimeter, a haemoglobin solution may be matched against a neutral grey filter of known density, which serves as a permanent standard and obviates the necessity for continually preparing fresh standard haemoglobin solutions. The no. 7 green filter in the eyepiece of the Leitz colorimeter was used, together with Ilford neutral grey filters of densities 1.0 and 1.5. With the above green filter haemoglobin solutions could be matched most perfectly against the neutral grey filters, and the matching was found to be very sharp and much easier than matching two haemoglobin solutions in an ordinary colorimeter without a filter in the eyepiece.

Micro (1 ml.) cups were used in the colorimeter, and coupled plungers, which move together so that the two liquid columns are always the same height. The left-hand cup contains water, and the right-hand cup the solution whose haemoglobin content is to be measured; a neutral grey filter is interposed between the left-hand cup and the eyepiece. This arrangement is not strictly necessary, but is useful especially if the haemolysin solution contains some coloured substance other than haemoglobin; in such a case the coloured solution can be added to the left-hand cup instead of water, when its effect is automatically compensated for, since the two liquid columns are always the same height.

The Ilford neutral grey filters have densities guaranteed by the makers to be accurate to within 1 %; however, they cannot be used directly to measure the strength of haemoglobin solutions since the light transmitted by the eyepiece filter is not perfectly monochromatic, but stretches over a comparatively wide region of the spectrum. The neutral grey filters must therefore first be calibrated in terms of haemoglobin. This was done by matching them in the colorimeter in the manner described against a solution of haemoglobin of known strength, determined by measuring the extinction coefficient of the 575 m μ band in a Hilger visual spectrophotometer. This has only to be done once; the neutral grey filters then constitute permanent standards, and the strength of unknown haemoglobin solutions matched against them can be read off directly in terms of mg. haemoglobin per ml.

A rheostat is incorporated in the Leitz colorimeter by which the intensity of the light may be varied; by the use of this and of a series of neutral grey filters of different densities, a wide range of haemoglobin concentrations can be measured.

It will be apparent that the method described above is a form of absolute colorimetry, and that any other type of absolute colorimeter such as a Stufenphotometer or a photoelectric colorimeter could be substituted for the arrangement described. Alternatively a simple colorimeter, preferably with a green

filter in the eyepiece, could be used, and the unknown haemoglobin matched against a standard haemoglobin solution of known strength, as in ordinary colorimetry. The disadvantage of this method is the constant need to prepare fresh haemoglobin solutions.

The above method was also used to prepare the standard red cell suspensions. 1 ml. of a concentrated (*ca.* 20 %) suspension of red cells, previously washed three times in isotonic buffer, was laked in 25 ml. of distilled water, and after centrifuging down stromata the haemoglobin content was measured as above. The concentrated suspension was then diluted until it contained exactly 7.5 mg. haemoglobin per ml. Rabbit red cells were used throughout. The red cells were kept in the refrigerator and were not used after they were 3 days old.

Some examples will now be given of the application of the method to the measurement of the haemolytic activity of streptolysin O preparations. It should be mentioned that this haemolysin is unusual in that it is only active in the presence of reducing agents such as sodium hydrosulphite, cysteine, thiolacetic acid etc., and is inactivated by oxidation. This phenomenon is described in detail by Herbert & Todd [1941]; since the present paper is chiefly concerned with the method of estimation of haemolysis, it will suffice to say here that all the experiments to be described were carried out on fully activated streptolysin.

Fig. 1 shows an experiment in which varying amounts of a streptolysin O solution, activated with thiolacetic acid, were incubated with red cells, and the degree of haemolysis measured in each case. The upper dotted line, at 7.5 mg. haemoglobin, indicates complete haemolysis; the lower, at 3.75 mg. haemoglobin indicates 50 % haemolysis. It will be seen from the curve how the amount of streptolysin which liberates exactly 3.75 mg. haemoglobin in 30 min. at 38° can be found, and hence the number of H.U./ml.

Fig. 2 shows similar experiments with four different haemolysin solutions of different strengths. In Fig. 3 the results of Fig. 2 are replotted, the scales of the abscissae being adjusted so that the mid-points of all four curves coincide; it will be seen that when this is done all the curves coincide completely throughout their length.

Many hundreds of such determinations have been carried out in the course of this work, and in all cases, curves of the same characteristic sigmoid shape have been obtained.

The nature of the reaction underlying the peculiar shape of this curve can be seen from Fig. 4, in which the experimental data of Fig. 1 are plotted in a different way. Instead of the degree of haemolysis ('H') being plotted against the concentration of streptolysin ('L') as in Fig. 1, in Fig. 4 dH/dL is plotted against L. The curve then appears as what it really is—a frequency diagram of the red cell population, expressing the resistance of different red cells to haemolysis by different concentrations of streptolysin. It is to be presumed that for each individual red cell there is a critical concentration of streptolysin below which it is not haemolysed and above which it is completely haemolysed in a given time. This critical concentration will of course be different for different red cells, varying with their age, size, thickness of cell membrane etc.

Haemolysis of a single red cell, it must be presumed, is an 'all-or-none' process; degrees of partial haemolysis are only possible with a heterogeneous collection of many red cells. On this view, if the action of streptolysin O could be tested on red cell suspensions in which all the red cells were exactly alike, the resulting curves instead of being sigmoid in shape like the curves of Figs. 1-3, would be a series of vertical straight lines.

Fig. 1. Concentration-haemolysis curve for streptolysin O. In this and all succeeding curves, experimental conditions were as follows: the amount of streptolysin solution shown was incubated for 30 min. at 38° with 1 ml. of a red cell suspension containing 7.5 mg. haemoglobin per ml., and the amounts of haemoglobin liberated by haemolysis measured as described in the text.

Fig. 2. Concentration-haemolysis curves for streptolysin O. The four curves represent four different streptolysin preparations.

Fig. 3 For explanation see text. ○●●● represent the four curves of Fig. 2 and Fig. 1.

A study of Figs. 1-3 reveals another point of more immediate practical importance. The slope of these curves is greatest in the region of about 50 % haemolysis, while in the region of almost complete haemolysis the curve flattens out and becomes nearly asymptotic.

In this region of the curve, large variations in the amount of haemolysin added produce but small changes in the degree of haemolysis; e.g. a 50 % increase in the amount of haemolysin added only increases the degree of haemolysis from 96 to 100 %; in the middle region of the curve, however, a 6 % increase in the amount of haemolysin added will increase the degree of haemolysis from 48 to 52 %.

Fig. 4. For explanation see text.

This clearly shows how inaccurate it is to measure the activity of haemolysins by taking complete haemolysis as an end-point; it is far more accurate to take 50 % haemolysis as an end-point, even if the degree of haemolysis is estimated by visual inspection only.

In this work, the activity of an unknown streptolysin solution was determined by setting up (usually) 5 tubes containing falling amounts of haemolysin at 25 % intervals, and after incubating with red cells etc. as described above, constructing a curve similar to Fig. 1; the amount of haemolysin necessary to cause exactly 50 % haemolysis of the standard dose of cells can then be determined by interpolation. Alternatively this can be done by reference to the standard curve of Fig. 3.

Theoretically the activity of an unknown streptolysin solution could be determined by putting up a single tube and referring to the standard curve; in practice it was considered more accurate to put up a series of tubes and work by interpolation.

The above method has been in routine use for a year and has proved extremely satisfactory. It is about ten times more accurate than the method of serial dilutions commonly employed, the mean error being about $\pm 5\%$.

The method contains a possible source of error which is worthy of mention, namely, that haemolytic activity is being measured by a biological reagent, red blood cells, whose natural variation might be a disturbing factor. Prigge [1937], discussing the haemolysins of *Cl. welchii*, lays great stress on this point, and states that the same haemolysin solution may have apparently different activities

if measured with different red cell suspensions, or with the same red cell suspension at different times. He therefore recommends that whenever an unknown haemolysin is to be tested, a parallel experiment should be put up with a 'standard' haemolysin preparation, and the activity of the unknown expressed in terms of the standard. Theoretically this is a sound principle; in practice I have not found it necessary to take this precaution, since I have never observed such variation in different batches of red cells as Prigge apparently found.

Table 1 shows some measurements made on the same streptolysin solution with the same batch of red cells on the same day. In all cases the agreement

Table 1. *Haemolytic activities of streptolysin O preparations tested repeatedly on the same red cells*

Streptolysin batch no.	Haemolytic activity found (H.U./ml.)
(3)	278, 278, 280, 283; mean 279.8
(6)	1210, 1200, 1190; mean 1202
(9)	1520, 1510; mean 1515
(10)	800, 800, 790; mean 796.7

Table 2. *Haemolytic activities of various streptolysin O solutions tested upon different occasions*

Date tested	Haemolytic activity found (H.U./ml.)
I. Purified streptolysin preparation 8	
12. xii. 40	1870
13. xii. 40	1850
13. xii. 40	1840
15. xii. 40	1760
15. xii. 40	1940
15. xii. 40	1745
II. Purified streptolysin preparation 9. Stored in refrigerator at 2°	
13. ii. 41	890
14. ii. 41	910
19. ii. 41	877
22. ii. 41	870
23. ii. 41	890
1. iv. 41	666
1. iv. 41	625
2. iv. 41	588
2. iv. 41	575
3. iv. 41	564
6. iv. 41	567
16. iv. 41	524
III. Purified streptolysin preparation 13. Kept frozen solid at -10°	
10. vi. 41	877
11. vi. 41	862
3. ix. 41	862
4. ix. 41	877
20. x. 41	852

between duplicates is very good. Table 2 shows experiments in which streptolysin solutions have been repeatedly tested on different occasions with different batches of red cells. Here again the agreement is good. In the measurements on preparation 9 in Table 2 it is interesting to compare the series made in February, 1941, which all give about the same value, with those made two months later; the solution had stood in the refrigerator at 2° in the interval and its activity had decreased, but the second series of measurements, though all lower than

the first, are in good agreement with each other. Preparation 13, in the same Table, had been kept frozen solid at -10° , and in this case a series of measurements made over an interval of four months (and moreover, in this case not only with different samples of blood, but with blood from different animals) all give values in close agreement.

It is difficult to reconcile these results with those of Prigge; one can only conclude either that there is much more variability in the red cells of sheep, which he used, than in those of rabbits, which I used, or else that some other source of error was present in his technique.

Although I have not found it necessary to allow for biological variation by running each measurement of an unknown haemolysin solution in parallel with a measurement on a standard, it is obvious that if necessary this could easily be done, and the principle of the method is not affected thereby. In this case unknown and standard solutions would both be measured in the manner above described, on the same red cells at the same time. If the amounts of unknown and standard haemolysin solutions required to cause haemolysis of exactly 50 % of the standard dose of red cells were x ml. and y ml. respectively, and if the standard had S units per ml., then the unknown would have $S.y/x$ units per ml.

It is hoped that the method of determining haemolytic activity described in this paper will be found useful by pathologists. It has been used routinely in this laboratory over the last twelve months and has been applied to the study of streptolysin O, streptolysin S, and the haemolysins of *Cl. welchii* and *Cl. tetani*. It should be applicable to the study of all bacterial haemolysins, and also of haemolysis by complement and by saponin etc.

SUMMARY

A simple and accurate method for the measurement of haemolysis is described, together with some results obtained by the application of the method to the study of streptolysin O.

The author is indebted to the Medical Research Council for a research grant, and to Drs E. W. Todd and W. E. van Heyningen for much helpful discussion and advice.

REFERENCES

- Eagle (1929). *J. gen. Physiol.* 12, 821.
- Grollmann (1931). *Biochem. J.* 25, 166.
- Herbert & Todd (1941). *Biochem. J.* 35, 1124.
- Ponder (1932). *Proc. roy. Soc. B*, 101, 193.
- Prigge (1937). *Z. Immunforsch.* 91, 457.
- Vles, Prager & Bernstein (1931). *C.R. Acad. Sci. (Paris)*, 192, 1506.