THE PRECISE MEASUREMENT OF HEMOLYSIN.

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The content in hemolysin of a solution is commonly measured as the amount which is just sufficient to produce complete hemolysis of an arbitrary quantity of red blood cells, usually 0.5 cc. of a 5 per cent suspension of washed erythrocytes. The method employed for such measurement gives results which are neither exactly comparable in determinations made at different times, nor highly precise. The susceptibility of erythrocytes to hemolysis is influenced by many factors, and the amount of the minimal hemolyzing quantity must vary accordingly for every specimen of test cells. Lack of precision in the measurement results from the fact that relative differences in hemolysin content between adjacent tubes in a titration series must be great, in order to distinguish the end-point, so that the value determined differs often by a large amount from a possible true value.

The method of titration of hemolysin described in this paper was developed for a study of the association of hemolysin with different fractions of immune serum and plasma protein. It measures hemolysin content as the ratio of the hemolytic activity of a given solution to that of standard immune serum. This standard has been usually a portion of the whole serum from which isolated protein fractions have been derived.

This choice of a standard immune serum instead of a given quantity of erythrocytes, as the unit of measurement of hemolysin content, has the advantage that applies to the use of a diphtheria antitoxin for the standardization of toxin and of new antitoxin: the antibody is the most stable biological element of the immune system.

The necessity for a large increment of hemolytic substance in successive tubes in a titration to determine the minimal hemolyzing quantity has long been recognized, and is not peculiar to the immune
hemolytic system. With most, if not all hemolytic agents the increment of substance necessary to produce the final 10 or 15 per cent of complete hemolysis of a given quantity of cells is not in proportion to that which brings about the preceding fractional amount of hemolysis, but greatly exceeds its proportion. Madsen \(^1\) made this observation first for tetanolysin, and to measure the lyric value of this hemotoxin determined the amount required to produce hemolysis equivalent to that of one-third or one-sixth of the total quantity of red cells used as reagent. Schur \(^2\) used a similar method for the estimation of staphylolysin, and plotted the amounts of lysis against the corresponding amounts of hemoglobin liberated. The S-shaped curve so obtained is given also by serum, saponin, and NaOH, according to Handovsky \(^3\), although Mioni \(^4\) had previously reported that the amount of serum hemolysis, with an excess of alexin, is proportional to the amount of sensitizer. Brooks \(^5\) found that the amount of hemolysis is not proportional to the amount of alexin in the presence of a constant amount of sensitizer, but is represented by an S-curve which is similar to those of Handovsky \(^3\), and has devised a method for the titration of complement which makes it possible to compare the amounts of alexin which produce like results in constant time.

The method of titration of hemolysin content described here utilizes the same principle; it compares the amounts of unknown and of standard hemolysin which produce a definite fractional amount of hemolysis of a given specimen of erythrocytes when acting in conjunction with a given specimen of alexin.

The standard immune serum is freshly diluted for each titration and brought to a concentration such that 1.0 cc. will produce almost complete hemolysis of 0.5 cc. of a freshly prepared 5 per cent suspension of sheep erythrocytes. A series of tubes is prepared containing 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 cc. of the standard immune serum dilution. A preliminary titration of the unknown serum or fraction is carried out, if its approximate value is not known, and in a second

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\(^1\)Madsen, Th., Z. Hyg. u. Infectionskrankh., 1899, xxxii, 214.

\(^2\)Schur, H., Beitr. chem. Physiol. u. Path., 1903, iii, 89.

\(^3\)Handovsky, H., Arch. exp. Path. u. Pharmakol., 1912, lxix, 412.


series of three to five tubes are placed graded amounts of the unknown such that approximately 50 per cent hemolysis of the test cell quantity will be brought about by one of the intermediate tubes in the series. The fluid in all tubes of both series is brought to the same volume, 0.5 cc. of the erythrocyte suspension added with a quantitative or standardized pipette, and alexin representing two units added. For reading in the ordinary Duboscq colorimeter the final volume should be at least 4 cc. Both series of tubes are placed simultaneously into the water bath at 37°C., kept agitated, and withdrawn and placed in cold water as soon as hemolysis is complete or almost complete in the highest concentration of the standard series. After cooling the tubes are centrifuged and the percentage amount of hemolysis in each tube of standard and unknown series is determined in the colorimeter, using the “highest” tube of the standard series as the 100 per cent standard. This is permissible since complete hemolysis of one “unit” of cells is not the end-point chosen in the comparative measurement.

A graph is prepared in which the fractional amount of hemolysis in each tube of the standard series is plotted as ordinate against the corresponding amount of diluted immune serum as abscissa. The curve is S-shaped; it varies slightly in form with each specimen of erythrocytes or alexin. From this curve is obtained the value of the abscissa of the standard corresponding to the fractional amount of hemolysis produced in each tube of the unknown series, or to the 50 per cent ordinate determined by interpolation on a curve drawn through the experimental points of the unknown.

Simple calculation then gives the ratio of the hemolysin concentration of the unknown to that of the standard. The concentration of any solution in units which produce a given fractional amount of hemolysis under the conditions of each experiment may be represented by $M/V$ where $M$ is the dilution, or volume in cc. in which is contained 1 cc. of the serum, or protein fraction referred to the original serum volume, and $V$ is the volume of diluted solution which is required for the given amount of hemolysis. Then the ratio of unknown concentration to concentration of standard is given as follows:

$$\frac{M_2}{M_{sd.}} \times \frac{V_{sd.}}{V_2} = \frac{C_2}{C_{sd.}}$$

for each fractional amount of hemolysis.
The ratios obtained from varying amounts of hemolysis have been found to agree within 4 per cent if the ordinates chosen lie between 15 and 85 per cent on the scale of ordinates so that the probable error or divergence from the mean is not greater than 2 per cent for any single reading. Consequently a single tube of dilution of unknown may be used for measurement of hemolysin concentration, if its value falls within the limits mentioned.

It is important that both alexin and erythrocytes be fresh and that the cells be washed in only two changes of isotonic suspending medium. More thorough washing of the cells leads to higher values of hemolysis in the first part of the curve, with little effect on the hemolysis of the final portion of the curve, so that greater error is introduced in determining the abscissa of the standard which corresponds to the ordinate of the unknown.

Comparison at different times, with different specimens of erythrocytes and alexin, between the same unknown solution and the same standard has given values that agree within 2 per cent.

SUMMARY.

A method is described for the measurement of hemolysin concentration, which makes possible exact comparison of results obtained at different times and with different specimens of erythrocytes and alexin; and gives precise values with an error not greater than 2 per cent.