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A MICRO BLOOD VOLUME METHOD USING A BLUE DYE AND PHOTOCELL

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The method described is designed to eliminate as far as possible the factors causing inaccuracy in blood volume determinations while retaining maximum simplicity. The advantages of the method are:

(1) that the errors caused by hæmoglobin and serum pigment are automatically eliminated almost completely;

(2) that the amount of blood required for the samples is very small, making it suitable for small animals;

(3) that it uses a blue dye peculiarly suitable for plasma volume determination;

(4) that the measurement of colour density is made by means of a simple photocell colorimeter, thus eliminating the ordinary visual colorimeter which may be liable to serious error [Gregersen, 1938].[1]

In the measurement of the circulating blood volume the validity of any dye injection method depends upon:

(1) the injection of a suitable dye,

(2) complete mixing of the dye with the blood,

(3) accurate measurement of the dilution of the dye in the plasma,

(4) accurate measurement of the ratio of blood cells to plasma or hæmocrit value.

The criteria of a suitable dye are that it must (1) be non-toxic, (2) not be absorbed by the cellular elements of the blood, (3) have a slow rate of

[1] During the preparation of this paper Gibson & Evelyn [1938] published a photocell method for blood volume determination, derived from the spectrophotometric procedure of Gibson & Evans [1937] which has many points in common with that described here. We put our experience on record for two reasons: first, that this method provides an independent confirmation of the underlying principles common to both; second, that the differences in the two methods are not negligible. This method has important advantages in the colour filter used, the lack of need for a separate correction for hæmolysis, the volume of serum required, and the knowledge of an overall control covering all the sources of error.

disappearance from the blood stream, and (4) have physical characteristics suitable for accurate measurement.

In order to obtain a sample of blood completely mixed with the dye two quantities must be known; the time required for the injected dye to become homogeneously distributed and the rate of disappearance of the dye.

Accurate colorimetric measurement of the dilution of dye in a sample of plasma is made difficult by the presence of (1) plasma colour, (2) hæmoglobin, (3) lipæmia, (4) residual dye, and (5) preparation of the standard for colour comparison [Gregersen *et al.* 1935; Gregersen, 1938].

Accuracy of the hæmatocrit measurement depends upon complete packing of the cells and use of an anticoagulant which allows correct estimation of the normal proportion of cells to plasma.

Fat particles in the plasma will cause an error in any dye injection method for measuring plasma volume.

The results reported in this paper were obtained using sheep blood but should be equally applicable to other bloods, provided the same controls are observed.

Choice and suitability of dye

Dye. The dye used is T 1824 or Evans Blue, the use of which was suggested by H. M. Evans in 1920, extensively studied for plasma volume work by Gregersen and co-workers, and introduced into clinical work by Gibson & Evans [1937].

Toxicity. The dye is non-toxic in dosage up to 20 mg./kg. [Gibson & Gregersen, 1935]. It has been given to several hundred human subjects in dosage of 0·2 mg./kg. without untoward effect [Gibson & Evans, 1937; Thompson *et al.* 1938], and to sheep in dosage of 0·5–1·0 mg./kg. without effect on weight curve [Barcroft & Kennedy, 1938].

Disappearance rate. The average disappearance rate in both dog and man is 5–8 p.c. per hr. [Gregersen *et al.* 1935]. Comparison of a large series of vital dyes used for plasma-volume determination has shown that as a group the red vital dyes disappear from the blood stream 65 p.c. more rapidly during the first hour than T 1824 [Dawson *et al.* 1920].

Stability of dye. Aqueous filtered solutions of T 1824 are remarkably stable and retain a constant colour value for several months [Gibson & Evans, 1937]. This allows use of the same stock solution over a long period. Most of the red vital dyes fade within a few hours.

Standardizing dye solution. It is essential that both calibrating sample (or standard) and test sample be made up in solvents in which the dye has the same light absorption for a given dilution [Gregersen & Gibson, 1937]. This condition, which has not been fulfilled in many previous dye methods, can be satisfied by the choice of suitable concentrations of salt and plasma. Solutions of T 1824 made up in 0·9 p.c. NaCl containing 20 p.c. plasma has been found to give the same light absorption as an equal concentration of dye in whole plasma.

T 1824	In 0·9 p.c. NaCl containing 20 p.c. plasma	In whole plasma
Stock solution A	35·6	35·4
Stock solution B	36·4	36·4

Light absorption of the dye is independent of individual variations in the sheep plasma with which it is diluted as shown in Table I.

TABLE I. Light absorption of T 1824; stock dye solution B diluted 250 times in 0·9 p.c. NaCl containing 20 p.c. plasma

Date	Sheep no.	Colour reading
7 March 1938	309	36·4
9 March 1938	337	36·5
11 March 1938	285	36·4
17 March 1938	262	36·6
23 March 1938	329	36·5
23 March 1938	289	35·5
28 March 1938	298	36·4
29 March 1938	354	36·6
30 March 1938	325	36·7

Greatest variation is 0·3 unit which is equivalent to 1 p.c. error in plasma volume. The reproducibility of the calibration readings eliminates the necessity of frequent recalibration of the stock dye solution.

Effect of blood cells and clotting on dye. Absorption of the dye by the cellular elements of the blood is negligible, and coagulation of blood does not effect the concentration of the dye in the plasma [Gregersen & Schiro, 1938]. This allows the use of clotted blood samples.

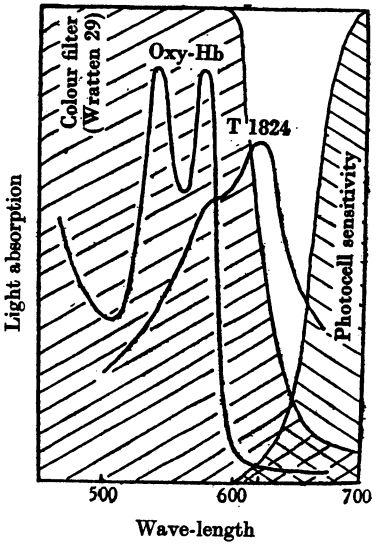

Fig. 1. Absorption spectra of dye T 1824 and of oxyhæmoglobin. The shaded area on the left represents light absorption of Wratten Filter No. 29; that on the right the decrease in sensitivity of the photocell.

Colour filter. As seen in Fig. 1, the combination of the colour filter (Wratten 29 F) and the photocell sensitivity results in the transmission of effective light only from about 610 to 690 mμ. wave-length. Since both

oxyhæmoglobin and serum pigment absorb very little light in this region their presence causes a negligible error in the colour readings. Thus, the effective light absorption is due almost entirely to the dye which has its absorption peak at 620 mμ. It has been found empirically that the dye follows Beer's law under the conditions of the experiment; that is, the negative logarithm of the transmitted light is proportional to the concentration of the dye.[1] This is shown in Fig. 2.

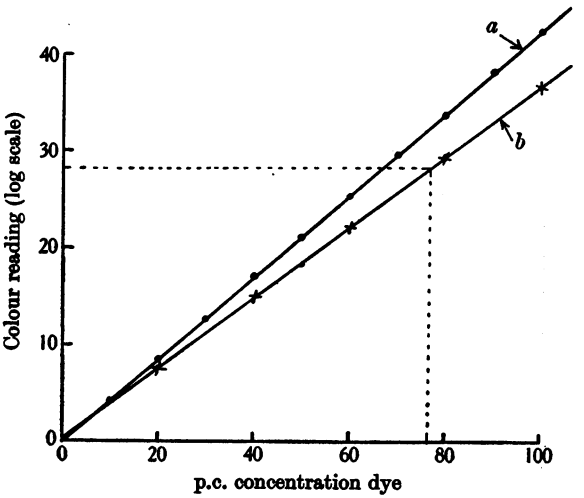

Fig. 2. The validity of Beer's law for T 1824 under the experimental conditions employed, and the equivalence of 20 p.c. plasma and whole plasma as dye solvents. Curve *a*: dye made up in 0·9 p.c. saline; "100 p.c." dye concentration = 40 mg./litre. Curve *b*: dye made up in two different strengths of plasma. × = 20 p.c. plasma in 0·9 p.c. saline. ● = whole plasma.

Mixing time. Large errors may be obtained if the dyed sample is withdrawn before the injected dye is thoroughly mixed with the circulating blood. Since the length of time required for complete mixing varies in different species of animals and even in the same animal with changes in the circulation, it is important to determine this interval accurately. Fig. 3 shows the obvious fallacy of obtaining the correct dilution before mixing is complete.

Disappearance rate curve and extrapolation. Immediately after the injection of any dye it begins to disappear from the circulating blood.

[1] Beer's law theoretically holds only for monochromatic light or over a wave-length region where the absorption coefficient is sensibly constant. The combination of sharp cut off of photocell sensitivity on one side and of light transmission by the red filter on the other, approximates this condition, as shown by the curves (Fig. 2).

This takes place during the process of mixing and continues thereafter at a uniform rate. The error caused by the small amount of dye which has disappeared during the interval up to the time of withdrawal of samples may be corrected by extrapolation back along the line represented by the disappearance rate curve to zero time or time of dye injection (Fig. 3). This theoretical value represents the dilution of dye if it were completely mixed at the moment of injection [Sunderman & Austin, 1936; Gibson & Evans, 1937]. The error caused by the disappearance of

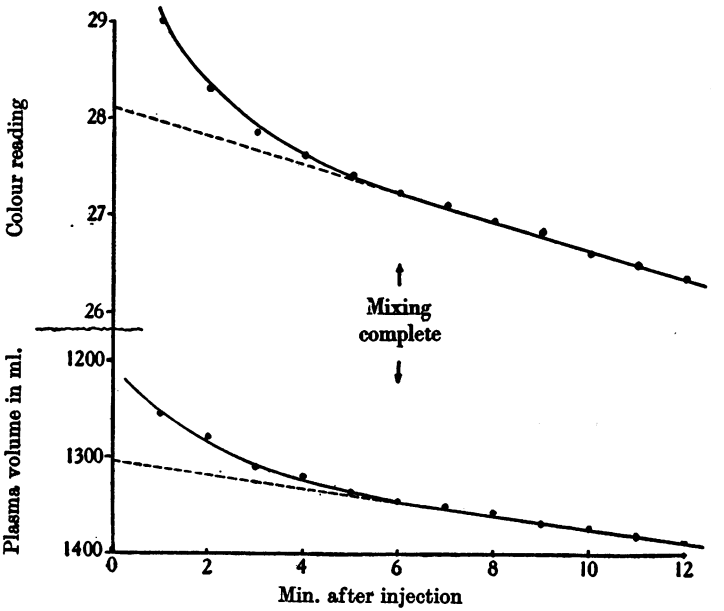

Fig. 3. Mixing curve and disappearance slope curve of T 1824 in a sheep, and extrapolation from the latter to time of injection (dose of dye = 0·8 mg./kg.).

T 1824 during the first 6 min. after injection in sheep is about 2–3 p.c. of the plasma volume. The more rapid the disappearance rate of the dye used the greater the error. Thus, the error is less with T 1824 than with the red vital dyes.

Hæmatocrit. Although it is theoretically possible to separate completely the cells and plasma by centrifuging, it is doubtful whether this ever occurs. The degree of accuracy to which complete separation is approached determines the degree of accuracy to which the corpuscular volume is measured in any plasma-dye method. This point has not been adequately stressed. In our experience centrifuging sheep blood for 30 min. at 3000 r.p.m. in a centrifuge of 15 cm. radius (one of the

commonest sizes generally employed) fails by 10–12 p.c. to pack completely. It is not possible to pack sheep blood completely with 2760 g. centrifugal force (4000 r.p.m. with 15 cm. radius) within 4 hr., and it requires 18,780 g. (12,000 r.p.m. with 11·5 cm. radius) of centrifugal force for 25 min. to obtain complete packing (Fig. 4). In experiments comparing blood of different species of animals, variations in both centrifugal force and time were found in order to obtain maximum cellular packing [Ponder & Saslow, 1930]. It is necessary to test the validity of any centrifuging method employed.

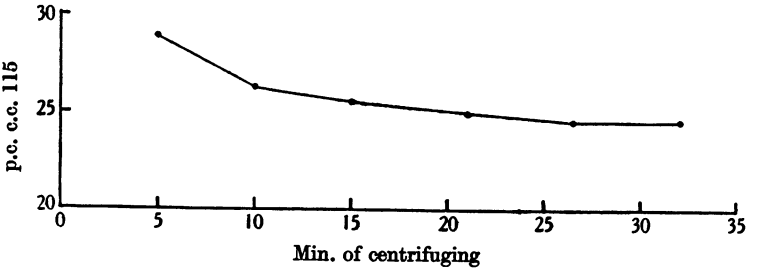

Fig. 4. Sheep blood (adult, heparinized). Time required to pack corpuscles. Centrifuge: 12,000 r.p.m.; 11·5 cm. radius; acceleration 18,780 g.

Description of colorimeter. A very simple type of photocell colorimeter has been used.[1] The light from an incandescent lamp passes through a colour filter, through the trough containing the test solution, and on to the rectifier type photocell, which is connected directly to a pointer galvanometer (Fig. 5). A good deal of latitude in the choice of all the components of the colorimeter except the colour filter would probably cause little error, so long as light intensities on the photocell are kept low. The components actually used are as follows:

Lamp: 6 V., 24 W. car head-lamp bulb supplied by a 6 V. car accumulator. With a suitable lens or reflector much smaller lamps can be used (e.g. a 2 V., 0·6 W. pocket torch lamp).

Colour filter: Wratten No. 29 F. It is important that this filter or a very similar one be used.

Trough and trough holder: Simple, easily made troughs, with a thickness of 1 mm. and a fluid capacity of 0·1 c.c., are made out of 1 mm. bakelite sheet, with microscope cover slips fastened on as sides with wax. The trough is U-shaped, 7 mm. wide and about 15 mm. deep. Troughs of the same thickness must be used for a given series of readings. Each trough must be standardized.

Photocell: Electrocell, Berlin.

Galvanometer: Pointer type, about 10 divisions per microampere. Two scales are provided, a linear one giving readings proportional to the percentage of light transmitted, and a logarithmic one giving readings proportional to the concentration of pigment where Beer's law holds. It is the logarithmic or "concentration" scale which has been used throughout for this work. Where the logarithmic scale is valid, "blank" corrections made with undyed plasma can be subtracted directly from the observed unknown dyed

[1] More particulars of the instrument than are given here may be obtained from the makers, Unicam Instruments, Ltd., Cambridge.

"concentration" reading without any further calculation (see protocol). This gives the dye concentration alone.

A determination is made as follows:

(1) Lamp off; check galvanometer zero.

(2) Lamp on; no trough in place; adjust light intensity till full scale deflexion is obtained =0 on concentration scale.

(3) (*a*) Insert trough containing test solution and take reading on concentration scale.
(*b*) Take reading on dye-free plasma.

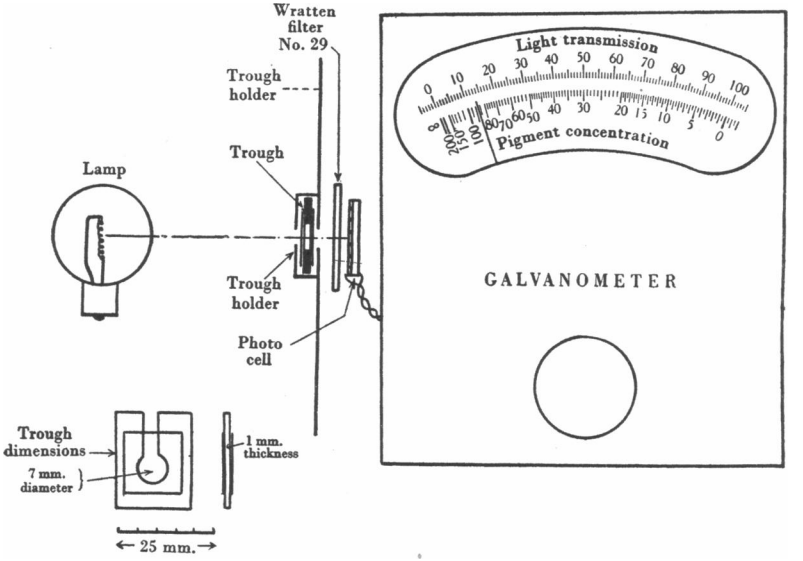

Fig. 5. Diagram of colorimeter.

(4) Subtract 3*b* from 3*a* to obtain reading for dye alone.

(5) If necessary, check reading with known dilution of stock dye.

Sample calculation of blood volume. Data from Fig. 3, using Fig. 2 as calibration curve:

	6 min. sample	9 min. sample	12 min. sample
(1) Observed reading of unknown plasma sample:	31·2	30·8	30·3
(2) Observed reading of dye-free plasma	4·0	4·0	4·0
(3) Corrected reading for dye alone	27·2	26·8	26·3

(4) Calculated extrapolated reading for 0 min. on disappearance curve = 28·1.

(5) Concentration of dye from calibration curve (Fig. 2*b*):

Reading 28·1 = 76·5 p.c. of 1/250 = 1/327 dilution or dilution of dye in plasma at 0 time.

(7) Since 4 c.c. stock dye solution were injected, 4 × 327 = 1308 c.c. = plasma volume.

(8) Hæmatocrit value of dyed sample (Fig. 4) = 24·5 p.c. cells.

(9) Thus, 1308 × 24·5/75·5 = 425 c.c. = corpuscular volume.

(10) Total blood volume = 1308 plasma
425 cells
1733 total

Repeated determinations. For successive determinations on the same animal it is necessary to take account of any dye which remains in the plasma from a previous injection. A blank sample is withdrawn and used in the same way as the dye-free blank in an initial determination.

Errors and controls

(i) *Colour determination.* The usual accuracy may be judged from the calibration values given in Table I. The greatest variation is 0·3 unit, which is equivalent to about 1 p.c. error in plasma volume. The galvanometer needle can be read with accuracy to 0·2 unit over the range used. This is equivalent to about 0·6 p.c. error.

(ii) *Effect of hæmolysis.*

Whole serum no hæmolysis	Same serum slight hæmolysis	Same serum "badly hæmolysed"
33·0	32·95	32·8
19·1	19·15	—
36·0	—	35·8

Slight hæmolysis causes essentially no error; "badly hæmolysed" samples cause an error of about 1 p.c.

(iii) *Overall control.* By adding dye directly to a known volume of blood which is then treated exactly as a withdrawn sample, a "blood-volume" determination can be calculated which includes all the hazards of the method (i.e. dye standardization, colour determination, hæmatocrit reading), except the possibility of specific changes in the dye due to its presence in the living body and errors dependent upon its mixing and disappearance. This is a rigorous test and we strongly recommend it being applied to any dye method as a critical test. The results in the second part of the following table were so obtained:

(i) *Controls testing validity of optical system*

Dye in distilled water		Dye in sheep plasma	
Measured volume c.c.	Calculated volume c.c.	Measured volume c.c.	Calculated volume c.c.
10·0	9·99	100·0	100·3
200·0	201·4	100·0	100·0
—	—	100·0	100·3
—	—	100·0	99·7
—	—	100·0	100·0
—	—	100·0	100·0
—	—	100·0	100·3
—	—	100·0	99·7
—	—	100·0	99·4

(ii) *Controls testing validity of optical system and accuracy of hæmatocrit*

Dye in whole rabbit blood		Dye in whole sheep blood	
Measured volume c.c.	Calculated volume c.c.	Measured volume c.c.	Calculated volume c.c.
50·1	51·2	100·0	100·1
100·0	100·4	100·0	98·9
—	—	100·0	100·0
	—	100·0	102·3

Summary

A method for plasma-volume determination is described, using a simple photo-electric cell colorimeter, a red light filter and a blue dye. The method minimizes the error caused by hæmoglobin and serum pigment and requires small blood samples for measurement, a complete determination being obtainable on 5 c.c. or less. It has several advantages over methods using a visual colorimeter and a red dye. Its accuracy is about 3 p.c.

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