CLINICAL STUDIES OF THE BLOOD VOLUME. IV. ADAPTATION OF THE METHOD TO THE PHOTOELECTRIC MICROCOLORIMETER

By JOHN G. GIBSON, 2d, and KENNETH A. EVELYN [1]

(From the Department of Medicine, Harvard University, the Medical Clinic of the Peter Bent Brigham Hospital, Boston, Massachusetts, and the McGill University Clinic, Royal Victoria Hospital, Montreal, Canada)

(Received for publication October 18, 1937)

In a previous communication (1) a method was described for determining the plasma volume by injecting intravenously an azo dye "Evans Blue" and measuring the dye concentration of undiluted serum samples with the spectrophotometer. The cost of the spectrophotometer and the specialized training required for its successful operation limit the applicability of the method to the study of clinical problems. Studies with this method by one of us (2, 3) have indicated the desirability of employing a simpler and less expensive type of photometer.

In this communication we will describe the adaptation of the plasma volume method of Gibson and Evans (1) to the photoelectric microcolorimeter of Evelyn and Cipriani (4, 5). This adaptation has been accomplished without the introduction of any essential change in the technique, and with no sacrifice of accuracy, or restriction of the range of applicability of the method. In addition, the simplified technique described has the advantage of greater objectivity and rapidity of the photometric readings, as compared with the spectrophotometric method.

In estimating the dye concentration of blood serum samples with the spectrophotometer, the absorption measurements are made in terms of optical density at the wavelength (620 millimicrons) of maximum absorption of the blue dye. In the photoelectric photometer, measurements of light transmission are made in a narrow spectral region isolated by a color filter which transmits light in the vicinity of 620 millimicrons.

In order that the same mathematical formulae used in the original technique may be employed, the transmission values obtained with the microcolorimeter are converted (by simply taking the negative logarithm) into quantities called L values which are analogous to optical densities as measured on the spectrophotometer. Since an L value is merely the average optical density of the solution over the narrow band of wavelengths transmitted by the filter, it is possible, by making the filter sufficiently selective, to obtain the same linear relation between concentration and L value as exists between concentration and optical density. One may, therefore, employ standard spectrophotometric formulae by merely replacing optical densities by the corresponding L values.

Two color filters are used. The filter referred to below as number 620 has a maximum transmission of 620 millimicrons, and is used for measuring dye concentration in serum samples, just as readings are made with the spectrophotometer at 620 millimicrons. The other filter, number 540M, has a maximum transmission at 540 millimicrons and is used for correction of dye values for hemolysis in serum samples (with the spectrophotometer the measurements on which this correction is based are made at 574 millimicrons). Both filters are mounted in the same holder and may be interchanged readily. The transmission curves of the two filters, of "Evans Blue" and of oxyhemoglobin are shown in Figure 1.[2]

APPARATUS

The instrument used was the photoelectric microcolorimeter described by Evelyn and Cipriani (5) with the plunger type absorption cells described by Evelyn and Gibson (6). All measurements were made with a 10.0 mm. depth of serum accurately enclosed between the bottom of the

[1] This work was done during the tenure of a grant from the Banting Research Foundation.

[2] These measurements were obtained with the recording spectrophotometer of the Color Measurements Laboratory of the Massachusetts Institute of Technology, through the courtesy of Professor A. C. Hardy.

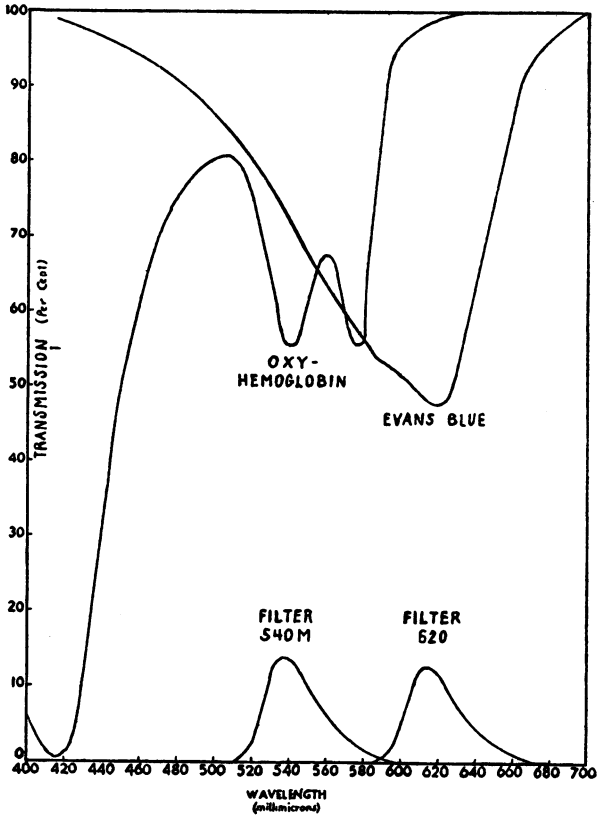

FIG. 1. TRANSMISSION SPECTRA OF EVANS BLUE IN SERUM, OXYHEMOGLOBIN AND OF FILTERS 620 AND 540M.

absorption cell and the lower surface of the plunger.

Determination of plasma volume

The technique of injecting dye, taking the blood samples and preparing the serum samples for colorimetric reading, is exactly as described before (1). The same absorption cell can be used for readings on all the dyed samples, provided it is well drained with a fine bulb pipette between samples; but a separate cell should be used for the dye-free sample. The same plunger must be used for an entire series of dyed samples and should be rinsed with water after reading each sample. Care must be taken that no bubbles form beneath the plunger when it is lowered into the serum. A little over 1 cc. of serum is pipetted into the cell, this being enough to allow the lower 2 or 3 mm. of the plunger to dip into the serum.

Single volume determinations

The cell containing the dye-free serum is placed in the apparatus, filter 620 is inserted, and the galvanometer is adjusted to read 100. As soon as the reading has become absolutely steady, the center setting aperture [3] is racked into place, and the exact value of the center setting is noted. The dyed samples are then read in turn, the center setting being kept constant throughout by readjustment of the rheostats if necessary. When the readings for the entire series of dyed samples have been made, the dye-free sample is again inserted, and should check at the initial reading of 100.

The galvanometer readings obtained are plotted against time (exactly as in the case of the optical density values in the original method), and the extrapolated galvanometer reading G is used in the calculation which is given in detail below.

Since the amount of light transmitted by the dye-free blank is always set at 100, the light transmission of the dye in the samples is given by

$$T = \frac{G}{100},$$

and since the concentration of the dye is proportional to the negative logarithm of the light transmission, the concentration of the dye in mgm. per cc. of serum is

$$C = \frac{-\log T}{K} = \frac{-\log \frac{G}{100}}{K} = \frac{2 - \log G}{K}.$$

The quantity $2 - \log G$ is always written L, hence

$$C = \frac{L}{K},$$

where K is a constant whose value must be determined for each new lot of dye by making a number of measurements of L for various known values of C.

The plasma volume in cc. is given by the formula

$$PV = \frac{M}{C} = \frac{MK}{L}, \qquad (1)$$

[3] The center setting aperture is used to allow the operator to make sure that the galvanometer setting for the dye-free sample remains constant throughout the series of readings without the necessity of reinserting the dye-free sample between each sample reading.

where M [4] is the number of mgm. of dye injected.

The calculation of the plasma volume thus is made from the extrapolated G value by substituting the corresponding L value in formula 1.

$$\text{Total blood volume} = \frac{\text{Plasma volume}}{100 - \text{hematocrit}},$$

$$\text{Red cell volume} = \text{Total blood volume} - \text{plasma volume.}$$

Repeated volume determinations

When a second dye injection is given shortly after an initial volume determination has been made, the so-called dye-free sample contains a certain amount of residual dye from the previous injection. The effect of this residual dye is compensated for automatically by reading all the new dyed samples against a new center setting obtained by first setting the galvanometer to 100, with the dyed dye-free sample, exactly as though it contained no dye. If several dye injections have been made in a short interval so that the amount of residual dye is very great, it may be necessary to adjust the initial setting to 50 instead of 100, and to multiply all the galvanometer readings obtained from this setting by 2. The G values so obtained are used to determine the extrapolated L value in the manner described above.

An alternative method, which may sometimes prove useful when the original dye-free sample (which contains no dye) still is available, is as follows. All the dyed samples including the dyed dye-free are read after setting to 100 with the original dye-free sample. The L value is obtained from the extrapolated G values in the usual way and is corrected for residual dye by subtracting from it the L value of the dyed dye-free sample.

Indirect determinations

The procedure in this type of experiment is exactly as described for the original method except that L values are used instead of optical densities. The disappearance slope is drawn from the L values of samples taken during the control period, and changes in plasma volume during the experimental period are calculated from the deviation from the disappearance slope of the L values of successive serum samples. The same formulae are employed for the calculation as in the original method:

$$\text{P.V.}_{1,\,2,\,\text{etc.}} = \frac{\text{P.V.} \times L_{\text{Sp. 1, 2, etc.}}}{L_{\text{S. 1, 2, etc.}}}, \quad \text{where}$$

P.V. = the initial plasma volume in cc.,

$\text{P.V.}_{1,\,2,\,\text{etc.}}$ = the plasma volume to be calculated,

$L_{\text{S. 1, 2, etc.}}$ = L value of serum sample from which $\text{P.V.}_{1,\,2,\,\text{etc.}}$ is calculated,

$L_{\text{Sp. 1, 2, etc.}}$ = L value on disappearance slope corresponding to $L_{\text{S. 1, 2, etc.}}$.

Standardization of dye solutions

The standardization of each dye solution consists of the determination of the corresponding K values for a 10.0 mm. depth of serum with filters 620 and 540M. Our practice has been to prepare a series of standards in concentrations of 1.0, 0.5, 0.333, 0.25 and 0.2 mgm. per cent for the 0.1 and 0.15 per cent solutions, and in concentrations of 3.0, 1.5, 1.0, 0.75, and 0.6 mgm. per cent for the 0.3 per cent solutions. The average of the values for the individual standards so obtained is taken as the K value for the solution. K values for filters 620 and 540M for 4 solutions of the original lot of T–1824, and for 2 solutions of Evans Blue (EK number 3873) are shown in Table I.

Correction for hemolysis of samples

The principles of correction for hemolysis in the photometric technique are the same as those employed with the spectrophotometer. As shown in Figure 1, the absorption value of hemoglobin with filter 620 is small compared with its absorption value with filter 540M, while on the contrary, Evans Blue absorbs more strongly with filter 620 than with filter 540M. We have determined the ratio $R = \frac{L_{540M}}{L_{620}}$ for hemoglobin to be 20; for the original lot of T–1824 to be 0.7; and for Evans Blue (EK number 3873) to be 0.6 (see Table I).

These ratios are used in the formula as given in the original paper (1).

After the L values at filter 620 have been obtained for the entire series of samples, filter 540M

[4] This value is corrected for the calibration value of the dye delivery syringes.

TABLE I

Standard K values for Evans Blue solutions in serum

$\left(K = \frac{L}{C} \text{ for 10 mm. depth of serum}\right)$

Date	Original lot of T-1824					
	Dye solution	Concentration	$K_{620} = \frac{L_{620}}{C}$	Average K_{620} for solution	$\frac{K_{540M}}{C} = \frac{L_{540M}}{C}$	$\frac{R_d}{K_{620}} = \frac{K_{540M}}{K_{620}}$
1937		*mgm. per cent*				
February 5...	S	100	0.767			
April 20.....	S	100	0.765	0.766	0.535	0.700
March 2.....	U	100	0.764			
March 11....	U	100	0.741			
April 30.....	U	100	0.766			
June 21.....	U	100	0.752	0.756	0.548	0.712
January 31...	R	150	0.763			
February 5...	R	150	0.753	0.758	0.535	0.707
June 11.....	T	300	0.763			
June 21.....	T	300	0.766	0.765	0.564	0.737
Average.....			0.760			0.714
Evans Blue (E.K. number 3873)						
May 6.....	EB[2]	100	0.633		0.392	0.620
May 7.....	EB[2]	100	0.653		0.377	0.577
May 17.....	EB[2]	100	0.663		0.390	0.588
May 24.....	EB[2]	100	0.656		0.402	0.613
September 14.	EB[2]	100	0.687	0.658	0.433	0.630
June 10.....	EB[3]	300	0.655		0.412	0.628
June 11.....	EB[3]	300	0.670		0.409	0.611
September 14.	EB[3]	300	0.647		0.417	0.646
September 14.	EB[3]	300	0.670	0.661		
Average.....			0.660		0.408	0.614

is inserted, the galvanometer adjusted to 100 with the dye-free sample, and a new center setting obtained. All the hemolyzed samples are then read with the new center setting, and the hemolysis correction is made by means of the formula:

$$\text{Corrected } L_{620} = \frac{R_h \times L_{620} - L_{540M}}{R_h - R_d} = \frac{20 \times L_{620} - L_{540M}}{20 - 0.6}, \quad (2)$$

where R_h and R_d are the 540M : 620 ratios of oxyhemoglobin and Evans Blue respectively.

If the dye-free sample is hemolyzed, the galvanometer is set at 100 in the usual way; one of the non-hemolyzed dyed samples is read with both filters and the corresponding L values used in formula 2 to determine the corrected L_{620}. The L_{620} values of all other non-hemolyzed samples in the series are corrected for the error introduced by the hemolysis of the dye-free sample, by adding to each an amount equal to the difference between the observed and corrected L_{620} values of the first sample.

If any samples in the series are also hemolyzed, readings are taken with both filters, and the L values are calculated. In substituting in formula 2, the value used for L_{620} is the actual value obtained plus the same correction which was applied to the non-hemolyzed samples, while the value used for L_{540M} is the actual value obtained plus 20 times the above mentioned correction.

Comparison of results obtained by spectrophotometric and photoelectric methods

A summary of the values for plasma volume obtained by both spectrophotometric and photoelectric determinations on 28 patients is shown in Table II. In this series plasma volumes ranged from 2060 to 4900 cc. It will be noted that the

TABLE II

Comparison of plasma volumes as determined with the spectrophotometer and photoelectric microcolorimeter

Case number	Age	Sex	Date	Amt. of dye	Plasma volume		Percentage difference in plasma volume	
					Spectrophotometer	Microcolorimeter	+	−
	years		*1937*	*mgm.*	*cc.*	*cc.*	*per cent*	*per cent*
280	64	M	January 31	10	4900	4950	1.0	
288	50	F	February 5	10	2940	2980	1.3	
BL-1	37	M	February 6	10	3780	3815	0.9	
282	17	F	February 6	8	3100	3180	2.6	
283	60	M	February 8	10	3640	3680	1.1	
265	50	M	February 9	10	2780	2800	0.7	
271	72	M	February 10	10	3150	3155	0.2	
284	62	F	February 11	8	2510	2575	2.6	
285	48	F	February 11	10	2915	3000	2.9	
287	31	M	February 12	10	2715	2770	2.0	
288	21	M	February 12	10	2780	2860	2.9	
BL-2	65	M	February 13	10	2870	2895	0.9	
283	60	M	February 16	10	3590	3590	0	
289	37	M	February 17	10	2210	2160		2.3
BL-3	64	M	February 20	10	3140	3175	1.1	
BL-4	26	F	February 25	8	2780	2735		2.0
BL-5	21	F	February 25	10	3160	3140		0.6
BL-6	36	F	February 26	10	3300	3130		5.2
BL-7	26	F	March 5	10	2630	2590		1.5
BL-8	30	F	March 16	10	3910	3920	0.3	
BL-9	22	F	March 17	8	2060	2120	2.9	
BL-10	22	F	March 20	10	2470	2465		0.2
BL-11	22	F	April 29	8	2340	2370	1.3	
BL-12	36	F	May 14	8	2255	2265	0.4	
BL-13	25	F	May 19	10	2270	2265		0.4
BL-6	36	F	May 21	10	2670	2650		0.8
BL-14	22	F	May 21	8	3440	3460	0.6	
BL-15	30	F	June 16	10	4610	4600		0.2

values obtained with the photometer were within plus or minus 2.5 per cent of those obtained with the spectrophotometer.

TABLE III

Comparison of repeated plasma volume values as determined with the spectrophotometer and photoelectric microcolorimeter

Case number	Date and time	Amount of dye	Plasma volume		Percentage difference in plasma volume
			Spectrophotometer	Microcolorimeter	
	1937	*mgm.*	*cc.*	*cc.*	*per cent*
286 Male, 54 years	February 12	10	3430	3430	0
	February 25	10	2930	2980	+1.7
295 Male, 14 years	February 26	8	1670	1670	0
	March 2	10	1740	1700	−2.3
309 Male, 40 years	May 12 3:20 p.m.	30	2615	2600	0.6
	May 13 5:40 p.m.	10	2310	2350	+1.7
290 Male, 17 years	May 26 9:18 a.m.	8	2260	2240	−0.9
	12:19 p.m.	8	2265	2300	+1.5
	2:29 p.m.	8	2280	2200	−3.6

TABLE IV

Comparison of changes in plasma volume obtained by the short indirect method with the spectrophotometer and microcolorimeter

	Volume change during and after intravenous administration of 5 per cent glucose							
	Experiment 306. Normal male, 27 years, May 12, 1937				Experiment 304. Normal male, 33 years, May 12, 1937			
	Time after dye injection	Plasma volume		Percentage difference in plasma volume	Time after dye injection	Plasma volume		Percentage difference in plasma volume
		Spectrophotometer	Microcolorimeter			Spectrophotometer	Microcolorimeter	
	minutes	*cc.*	*cc.*		*minutes*	*cc.*	*cc.*	
Basal plasma volume	0	2305	2305	0	0	3100	3040	−1.9
Intravenous started.	57				43			
After 500 cc. of 5 per cent glucose in saline	68	2620	2630	+0.38	55	3720	3660	−1.6
After 1000 cc. of 5 per cent glucose in saline	85	2950	2900	−1.7	70	3760	3720	−1.1
	95	2775	2675	−3.62	76	3610	3540	−1.9
	115	2505	2430	−2.6	98	3250	3195	−1.7

Data on the agreement of 6 repeated volumes is given in Table III in which it is seen that the deviation is about plus or minus 3 per cent.

Several comparisons of results obtained with the indirect procedure indicate that the deviation of the two methods is of the same order as described above. (See Tables IV and V.)

TABLE V

Comparison of plasma volume changes obtained by the long indirect method with the spectrophotometer and microcolorimeter

Experiment 309, Male, 40 years	Plasma volume as determined during artificially induced fever		
	Spectrophotometer	Microcolorimeter	Percentage difference in plasma volume
	cc.	*cc.*	*per cent*
Initial plasma volume 3:49 p.m. May 12, 1937.....	2615	2600	−0.6
In fever cabinet 9:30 a.m. May 13, 1937			
Rectal temperature 103° F. at 10:59 a.m.....	2390	2365	−1.1
Rectal temperature 106° F. at 12:05 p.m.....	2215	2150	−2.9
Rectal temperature 102.4° F. at 12:52 p.m.	2150	2070	−3.7
Rectal temperature 99.2° F. at 3:02 p.m.....	2285	2380	+4.2

In our experience, correction for hemolysis is as accurate with the photoelectric method as with the spectrophotometer.

COMMENT

Several factors must be borne in mind in estimating the comparative accuracy of the spectrophotometric and photoelectric methods of estimating dye concentration of serum samples in the blood volume technique. For routine measurements the spectrophotometer is a tiring instrument to use, since the red color of the fields results in considerable eye strain and fatigue. As a result, the accuracy of the observer in obtaining precise matching of the color fields may be expected to fall off during the course of a large series of readings. This difficulty is obviated by the objectivity of the photoelectric photometer, with which the galvanometer readings are made with no abnormal lighting conditions. Since there is a probable error in even the best spectrophotometric readings of plus or minus 1 per cent, the actual deviation of results obtained in this study

by the two instruments probably is exaggerated by the figures given above.

CONCLUSIONS

1. A photoelectric method of determining the dye concentration of serum samples in the plasma volume method of Gibson and Evans is described.

2. Comparison of this method with that employing the spectrophotometer proves it to be accurate within a range of plus or minus 2.5 per cent in a series of plasma volume determinations.

3. For purposes of clinical research the simplified technique is as reliable as the spectrophotometric method and possesses the added advantages of greater simplicity, economy, speed and freedom from subjective errors.

BIBLIOGRAPHY

1. Gibson, J. G., 2d, and Evans, Wm. A., Jr., Clinical studies of the blood volume. I. Clinical application of a method employing the azo dye "Evans Blue" and the spectrophotometer. J. Clin. Invest., 1937, **16**, 301.

2. Gibson, J. G., 2d, and Evans, Wm. A., Jr., Clinical studies of the blood volume. II. The relation of plasma and total blood volume to venous pressure, blood velocity rate, physical measurements, age and sex in ninety normal humans. J. Clin. Invest., 1937, **16**, 317.

3. Gibson, J. G., 2d, and Evans, Wm. A., Jr., Clinical studies of the blood volume. III. Changes in blood volume, venous pressure and blood velocity rate in chronic congestive heart failure. J. Clin. Invest., 1937, **16**, 851.

4. Evelyn, K. A., A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., 1936, **115**, 63.

5. Evelyn, K. A., and Cipriani, A. J., A photoelectric microcolorimeter. J. Biol. Chem., 1937, **117**, 365.

6. Evelyn, K. A., and Gibson, J. G., 2d, A new type of absorption cell for the photoelectric microcolorimeter. J. Biol. Chem. (To be published).