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The Determination of Total Circulating Serum Proteins and Erythrocyte Volumes in Normal and Protein Depleted Rats.*

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(Introduced by Paul R. Cannon).

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In order correctly to evaluate the influence of variations in quality and quantity of ingested protein on the various blood components of an animal it is necessary to measure total quantities of circulating blood and plasma. Over a period of 2 years, during investigations on protein metabolism in this laboratory, we have performed approximately 700 blood volume estimations in rats. A method has been employed which differs sufficiently from other published methods to merit description. Previously used methods have been reviewed in detail by Metcoff and Favour.[1]

The use of diluted whole blood samples for estimation of dye concentration in blood volume determination is simpler and more economical of blood than the use of separated plasma or serum. One can use diluted whole blood for the dye determination, provided the dye concentration is high enough for accurate estimation, the volume of cells does not interfere with colorimetry, there is no significant hemolysis and the color of the dye is not altered by dilution.

With the method described, total blood volume may be determined on only 0.1 ml of blood, and plasma volume, red cell volumes, and hemoglobin on one sample of 0.2 to 0.3 ml of blood.

Methods and Materials. Adult male albino rats of the Sprague-Dawley strain weighing between 180 and 245 g were used. They were divided into 2 groups. One group was fed a ration containing 18% casein, the other a diet containing 1.8% protein (N $\times$ 6.25) derived mainly from carrots. The diets were isocaloric and offered in equal quantity each day. These diets and the method of depletion have been described in detail.[2]

At the end of 9 to 11 weeks on the diets, the animals were fasted overnight and the following determinations were then done: total blood volume, hematocrit, hemoglobin, and serum protein.

Dye Injection and Sampling. Blood volumes were determined using the dye T-1824. Under light ether anesthesia, using a calibrated tuberculin syringe and a ¾″ 24-gauge needle, one mg (0.30 ml of a 0.333% solution) of the dye was injected into a tail vein. The needle was held in place for approximately 15 seconds until dye had left the vein. Light pressure was applied over the point of injection, the needle was withdrawn, and pressure was maintained until bleeding ceased (usually within one minute). Four minutes following injection anesthesia was again begun and at 5 minutes $\pm$ 15 seconds, the animal was bled from a tail vein other than the one previously used for injection. The method of bleeding the rats has been described in detail.[3] Blood was collected directly from the tail into a 0.1 ml blood sugar pipette. Then 0.2 ml was collected in a small vial containing dry oxalate, (2.4 mg of ammonium oxalate and 1.6 mg of potassium oxalate). Finally, 0.5 ml was collected without anticoagulant for serum protein determination by the falling drop method.[4]

* This work was aided by grants from the John and Mary R. Markle Foundation, The National Livestock and Meat Board, The Douglas Smith Foundation of The University of Chicago, and Allen B. Wrisley and Co.

1 Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **141**, 695.

2 Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

3 Cannon, P. R., Humphreys, E. M., Wissler, R. W., and Frazier, L. E., *J. Clin. Invest.*, 1944, **23**, 601.

4 Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

Dilution of Samples. The 0.1 ml of blood in the sugar pipette was introduced immediately after withdrawal into 2.0 ml of a special diluting fluid contained in a colorimeter tube. The diluted blood was mixed, then centrifuged 10 minutes at 1500 r.p.m. in an ordinary centrifuge. The special diluting fluid was composed of 2.0 g of purified beef plasma albumin† dissolved by stirring into 100 ml of 0.86% NaCl solution containing .06% of ammonium oxalate and .04% of potassium oxalate. This solution was centrifuged 15 minutes at 4000 r.p.m. in an angle centrifuge. The decanted supernate was pale yellow and clear, and could be kept for 24 hours in the refrigerator.

Colorimetry. Readings were made in a Klett-Summerson colorimeter using a 620 mμ filter. The colorimeter has been adapted by us to take standard Wassermann tubes utilizing a 2 ml volume. For blank controls, blood from 3 depleted and 3 normal fasted rats was treated as described for the dye-stained samples. The average of the 6 values was used in the calculation of dye intensity.

With each set of determinations a standard was run. It was set up as follows: Three-tenths ml of the T-1824 solution used in the animals was injected from the tuberculin syringe, needle attached, into 10.0 ml of the albumin diluent. One-tenth ml of this was then introduced into 2.0 ml of diluent in a colorimeter tube. This and its blank, the diluting fluid, were read in the colorimeter along with the unknowns, and the K value was calculated. In 16 duplicate measurements the coefficient of variation of K was ±0.66%.

Hematocrit and Hemoglobin Determinations. Hematocrit values were determined in thick walled capillary tubes having a bore of 1 mm and a length of 10 cm. An approximately 8 cm column of blood was drawn into the tube. One end, sealed with plasticene, was seated in the same material in an empty .38 caliber revolver cartridge. They were centrifuged at 2000 r.p.m. with an effective head radius of 25 cm for 30 minutes. The lengths of the total blood column and the red cell column were measured with a millimeter rule and the hematocrit was calculated.

Hemoglobin was determined in the Dick-Stevens photoelectric hemoglobinometer,[5] which requires approximately .02 ml of oxalated blood per determination. A correction was applied for the effect of the dye in the samples.

Calculations. From the estimated dye concentration and the K value for the dye standard the total blood volume was calculated. Consideration was taken of the fact that the 2.1 ml volume in the colorimeter tube was composed of approximately .04 ml of cells which did not enter into the volume of the dye-containing fluid.[5] Therefore, the actual volume was 2.06 ml. We have usually disregarded the ±0.01 ml variation which would be caused by a 10% deviation in the hematocrit. The largest error thereby introduced was no more than ±0.5%.

Plasma and erythrocyte volumes were calculated from total blood volume and hematocrit values.

Repeated Determinations. Repeated blood volume determinations can be made on the same animal. If the interval is less than a week it is necessary to correct for residual circulating dye. This requires a preinjection bleeding of 0.1 cc which is diluted and read in the colorimeter. This value is substituted for that of the blank of diluted whole blood described above.

Discussion and Results. Evans blue is relatively non-toxic in doses of 20 mg or less per kg of rat.[6] The quantity of dye used here was less than 10 mg per kg and we have observed no untoward effects in animals so treated.

Injection of the dye via tail vein eliminates operative procedures used in other methods.[1] This can be accomplished after a little practice without significant loss of dye. The tuberculin syringe should have a clear glass plunger; if not, a white line placed on the tip of the plunger helps in setting.

A major problem in any dye dilution method is time of sampling in relation to complete-

† Kindly supplied by Armour and Co.

[5] Dick, G. F., and Stevens, D. S., *J. A. M. A.*, 1940, **114**, 1065.

[6] Gibson, J. G., and Gregersen, M. I., *Am. J. Physiol.* (proc.), 1935, **113**, 667.

TABLE I.
Comparison of Various Blood Components in Normal and Depleted Rats.*

	Controls	Depleted
Body wt g	303 ± 7.7	155 ±1.2
Surface area cm^2 [16]	386 ± 5.0	258 ±1.3
Total blood vol. ml	18.7 ± 0.33	10.1 ±0.12
" erythrocyte vol. ml	8.6 ± 0.21	4.4 ±0.091
" plasma vol. ml	10.1 ± 0.46	5.8 ±0.094
Serum protein g %	6.22± 0.57	4.07±0.075
Total circulating serum protein mg	630 ±12.2	235 ±5.3
Hemoglobin conc.	15.7 ± 0.11	13.4 ±0.19
Hematocrit %	45.8 ± 0.46	43.3 ±0.74
Mean corpuscular hemoglobin conc. %	34.3 ± 0.28	31.3 ±0.43
Unit blood vol. $\frac{ml}{100\ cm^2}$	4.8 ± 0.063	3.9 ±0.042
" erythrocyte vol. "	2.2 ± 0.044	1.7 ±0.11
" plasma vol. "	2.6 ± 0.046	2.2 ±0.037
" serum protein $\frac{mg}{100\ cm^2}$	163 ± 2.8	91 ±2.1

* The values given are the means and following each the standard error. There were 23 animals in the control group and 30 in the depleted group.

ness of mixing. It has been shown[1] that between 3 and 5 minutes after the injection of Evans blue into a rat one obtains the most constant relationship between dye concentration and surface area. This has been interpreted as indicating time of complete mixing. Serial determinations of dye concentration in several rats showed that it reached a maximum at approximately 3 minutes after injection, remained constant to within 2% up to 7 minutes, then began to decrease, 8% having disappeared in 30 minutes. It has been shown that in men and dogs the single sample modification of the dye dilution technic using T-1824 agrees well with the carbon monoxide method,[7] and in men with the multiple sample extrapolation technic.[8] Sampling 5 minutes following dye injection in the rat appears to give adequate time of mixing. The error introduced, assuming a linear loss and 8% disappearance in 30 minutes, is less than 1%. A variation in bleeding time of even ±1 minute has introduced a negligible error.

The type of anticoagulant used may interfere with the determination of hemoglobin and dye concentration. Heparin even in small amounts imparts sufficient color to the plasma to interfere with the estimations.[9] Oxalate can be used, preferably as a mixture of potassium and ammonium salts. The concentration needed to prevent coagulation is much greater than with human blood.

T-1824 is bound largely to the serum albumin and at low concentrations of albumin the light absorption of the diluted dye is markedly depressed.[10,11] The rat's serum albumin concentration may vary from 2 to 4 g %.[12] If diluted 1:21 with saline for dye determination, the values may range from 0.07 to 0.14 g %. From Rawson's data[10] one can compute that such a variation in albumin concentration at a dye concentration of 4γ/ml may produce a variation in light absorption of as much as 8%. We have found upon actual experiment that when the rat serum albumin concentration was increased from .06 to .12 g % at a T-1824 concentration of 4 γ/ml there was a decrease in light absorption of 5.8%. This effect can be obviated by maintaining a sufficiently high

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[8] Noble, R., and Gregersen, M. I., *J. Clin. Invest.*, 1946, **25**, 172.

[9] Gregersen, M. I., and Schiro, H., *Am. J. Physiol.*, 1938, **121**, 284.

[10] Rawson, R. A., *Am. J. Physiol.*, 1943, **138**, 708.

[11] Gregersen, M. I., and Gibson, J. G., *Am. J. Physiol.*, 1937, **120**, 494.

[12] Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. of Surg.*, 1944, **120**, 514.

TABLE II.
Blood and Plasma Volumes of Adult Normal Rats by Methods Utilizing T-1824.

Author	Plasma vol. ml/100 cm²	Total blood vol. ml/100 cm²
Beckwith and Chanutin[14]	2.71	5.37
Metcoff and Favour[13]	2.77	4.78
Hechter[15]	3.8	6.7
Benditt, Straube, Humphreys	2.6	4.8

concentration of albumin, *i.e.* greater than 1%. Therefore, we have used as a diluent for the dye containing blood samples the albumin solution described above.

There is an inaccuracy of approximately 4% in the hematocrit value as measured by packed cell volume.[9] This error should be relatively constant under constant conditions of packing, and not too large variations in hematocrit. In Table I there is seen a 10% difference between the mean corpuscular hemoglobin concentration of normal and protein depleted rats. From this it is evident that, on the average, a 10% error would be introduced in the erythrocyte volumes of the protein depleted animals if the hematocrits were computed from the hemoglobin as has been suggested.[1] To perform the hematocrit determination in duplicate, as described here, requires only about 0.13 ml of blood. The accuracy attained is adequate. A series of 12 duplicate measurements had a standard deviation of ± 0.85. Nine comparisons of the capillary hematocrits with standard Wintrobe hematocrits yielded an average difference of 0.3%.

In Table I are compared the values of the various blood components for adult male white rats, normal and protein depleted. The data show excellent consistency within each group, as evidenced by the standard errors. In all comparisons the protein depleted animals have values which are significantly lower than those of the controls.

In Table II are the mean blood and plasma volumes per unit surface area of adult white rats determined with methods utilizing T-1824. The values given in this paper agree well with those of Metcoff and Favour.[13] Beckwith and Chanutin's[14] plasma volume values also are in agreement with these, but their total blood volumes are greater. Hechter's[15] blood and plasma volumes both are much higher.

We have discussed above the effect of small quantities of protein on T-1824 color. An increase in apparent blood volumes as large as 15% may result from comparing diluted plasma samples containing small quantities of protein with a standard diluted in saline or water. This may explain the high values reported by Beckwith and Chanutin, and Hechter. They unfortunately do not describe their method of standardization.

Summary. A method is described using the dye T-1824 to measure total blood, plasma and circulating erythrocyte volumes in rats. The method employs a single sample of blood taken 5 minutes after the injection of the dye. Certain precautions necessary to obtain accurate measurements of dye in diluted blood samples are discussed. Data are presented for normal adult rats fed (1) an 18% casein diet, (2) and for adult rats fed a low protein diet for 3 months.

[13] Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **142**, 94.

[14] Beckwith, J. R., and Chanutin, A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 66.

[15] Hechter, D., *Endocrinol.*, 1945, **36**, 77.

[16] Lee, M. O., *Am. J. Physiol.*, 1929, **89**, 24.