Comment. Erythropoietin has not shown a consistent stimulatory effect in all *in vitro* experiments. However, in those experiments in which it has been effective, results suggest that its major site of action is at the stem cell level where it induces erythroid differentiation(7,8).

Failure to demonstrate erythropoietic effect in the present experiment might be interpreted as evidence against the stem cell as the site of action of erythropoietin. However, it seems more logical to conclude that the primitive cell that emerges as a result of phytohemagglutinin stimulation, even though derived from lymphocytes, may be completely lacking in potential for erythroid or any other differentiation. It may even represent a malignant transformation that is beyond control by normal humoral mechanisms(9).

1. MacKinney, A. A., Jr., Stohlman, F., Jr., Brecher, G., *Blood,* 1962, v19, 349.
2. Carstairs, K., *Lancet,* 1962, v1, 829.
3. Maximow, A., *Folia Haemat.,* 1909, v8, 125.
4. Yoffey, J. M., *Lancet,* 1962, v1, 206.
5. Smith, L. B., Popp, R. A., St. Amand, W., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 232.
6. Mocrhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., Hungerford, D. A., *Exp. Cell. Res.,* 1960, v20, 613.
7. Erslev, Allan J., *Erythropoiesis,* Leon O. Jacobson and Margot Doyle, Eds., Grune & Stratton, N.Y., 1962, p. 275.
8. Lajtha, L. G., *ibid.,* 1962, p. 140.
9. Berman, L, Stulberg, C. S., *Blood,* 1958, v13, 1149.

Received November 7, 1963. P.S.E.B.M., 1964, v115.

Determination of Evans' Blue in Plasma and Plasma-Dextran Mixtures By Protein Precipitation and Extraction.* (28970)

J. MAXWELL LITTLE AND CURTIS WILLIAMS†
Department of Pharmacology, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

A rapid method for determination of Evans' blue dye (T-1824) in plasma has been described by Constable(1). This method involves the precipitation of a portion of the plasma proteins by addition of M/15 Na_2HPO_4 buffer, pH 7.5, and 3 N NaOH. The precipitate is acidified with HCl and extracted with n-butanol. The color in a mixture of the butanol extract and ethanol is determined spectrophotometrically. Besides the shortness of the procedure, other advantages claimed for the method are a lack of interference by variations in inherent plasma color, hemolysis and turbidity of the plasma.

The upper range of the linear relationship between extinction and dye concentration in plasma was quite limited, being no greater than 0.02 mg/ml plasma. Furthermore, we found this method to be unsuitable for determination of the dye in mixtures of dextran and plasma.

Since the method of Constable yields a precipitate representing only 1-2% by weight of the total plasma protein, it occurred to us that the narrow range of dye concentration precipitable by this method and the failure of proportional precipitation in a dextran-plasma mixture might be due to the incomplete protein precipitation under the conditions of the procedure.

This report is concerned with a modification of Constable's procedure which extends the range of linearity between opitcal density and dye concentration and which is equally suitable for normal plasma and for plasma-dextran mixtures.

Procedure. We have adopted the $CdSO_4$ precipitation procedure for plasma proteins of Little(2). The procedure for determination

* Aided by grant from Am. Heart Assn.
† Supported by a medical student summer fellowship from Nat. Heart Inst., Nat. Inst. Health, U.S.P.H.S.

of blood volume follows. Withdraw 12 ml of heparinized blood for a standard and determine an hematocrit reading. Inject intravenously 0.6 mg/kg of body weight of T-1824. At appropriate, accurately timed intervals (10, 20, 30 minutes), withdraw 12 ml samples of heparinized blood. Centrifuge all blood samples at 3000 rpm for 15 minutes. To 15 ml glass stoppered centrifuge tubes add in sequence: 1 ml of plasma, 3 ml of $CdSO_4$ solution (34.667 g $3CdSO_4 \cdot 8H_2O$, 169.3 ml of exactly 1.0 N H_2SO_4 make to one l with H_2O), 9 ml distilled water and 1 ml of exactly 1.0 N NaOH added slowly with shaking. The standard is prepared from the control plasma by adding 1 ml of plasma, 1 ml of a standard solution of T-1824 in water (0.01 mg/ml), 3 ml of the $CdSO_4$ solution, 8 ml distilled water and 1 ml of 1.0 N NaOH. Shake both standard and unknowns for 5 minutes and centrifuge at 3000 rpm for 15 minutes. Decant and discard the clear, colorless supernatant fluid. Invert the tubes to drain. If necessary the procedure can be stopped at this point provided the stoppered tubes are stored in the refrigerator. Add 0.5 ml of concentrated HCl, insert stopper and shake vigorously until the precipitate breaks free of the sides of the tube. Add 4 ml of n-butanol, replace the stopper, and heat in a beaker under running hot tap water (approximately 65°C) for 1 minute and shake for 2 minutes. Repeat this procedure twice, heating for 30 seconds and shaking for 1 minute each time. Centrifuge the unstoppered tube for 5 minutes at 3000 rpm. Pipette 3 ml of the upper butanol layer into a tube containing 1 ml of ethanol and read the optical density at 620 mμ. For a blank use 3 ml of butanol and 1 ml of ethanol. The concentration of dye in the sample is read from a standard graph prepared by adding known quantities of the dye (0.01, 0.02, 0.03 0.04 mg) to 1 ml plasma as was the case with the standard above. A single standard is run with each blood volume determination as a control of the conditions of the procedure. We have used a Spectronic 20 to read the optical density of the solutions. From the extrapolated semilogarithmic graph of time *vs* concentration, the concentration at zero time is read and used for calculation of plasma volume.

TABLE I. Results of Constable's Method Applied to Plasma and a 1:1 Plasma-Dextran Mixture.

Dye added to 2 ml plasma or mixture (mg)	Optical density at 620 mμ	
	Plasma	Plasma-dextran
.01	.140	.085
.02	.260	.110
.04	.410	.130
.06	.450	
.08	.500	

Results and comment. Table I illustrates our experience with Constable's method when applied to plasma or a 1:1 plasma-dextran mixture. These results confirm the narrow range of linearity Constable reported with this method and also demonstrate the slight slope relating dye concentration to optical density (O.D.) in a plasma-dextran mixture. Constable reported that only 1-2% of the plasma proteins were precipitated by this method. It is obvious that only a small fraction of the total dye is precipitated for the supernatant remains colored. We considered the possibility that the slight precipitate might be a complex between calcium phosphate and protein, and that the quantity of protein precipitated might be increased by addition of $CaCl_2$ to the heparinized plasma. The possible role of calcium was suggested by the statement in Constable's paper that oxalated plasma would not precipitate, but a precipitate could be obtained if a small quantity of $CaCl_2$ were added to the plasma. We added an excess of $CaCl_2$ to normal plasma, but contrary to our expectations the dye extracted from the precipitate was considerably less than that extracted from Constable's precipitate without added $CaCl_2$. The nature of the precipitate with this method remains unknown and the failure of dye precipitation in the presence of dextran remains unexplained.

Table II summarizes the relationship between dye concentration and O.D. in plasma and plasma-dextran mixtures when the proteins are precipitated with $CdSO_4$ and NaOH. The plasma dye concentrations are twice those used by Constable; therefore, the range of linearity obtained is at least twice that of

TABLE II. Optical Density of Dye Added to 1 ml Plasma or Plasma-Dextran Mixtures by $CdSO_4$-NaOH Precipitation.

Medium	Optical density ± S.E. .01*	.02*	.04*
Plasma	.138 ± .001 (11)†	.269 ± .002 (10)	.559 ± .003 (11)
Plasma-dextran (1:1)	.140 ± .002 (19)	.271 ± .001 (20)	.561 ± .002 (20)
" " (1:2)	.139 ± .003 (10)	.268 ± .001 (10)	.564 ± .002 (10)
" " (1:7)	.140 ± .0004 (12)	.274 ± .0008 (13)	.574 ± .003 (16)

* mg dye/ml. † No. of experiments.

Constable. It will be seen also that even with an extreme plasma-dextran ratio the dye concentration extractable from the precipitate is quite comparable to that obtained from plasma alone. It is true that the mean O.D. value obtained from a 1:7 plasma-dextran mixture, when 0.04 mg of dye was added, is statistically significantly greater than that obtained with a 1:1 mixture, but the value is only 2.3% higher and would probably be of little significance in a measurement of blood volume after dextran infusion.

Table III summarizes experiments designed to check the validity of a single extraction of the precipitate with butanol. Parallel experiments were run. One precipitate was extracted once with 4 ml of butanol, and the other precipitate was extracted with two 2 ml portions of butanol. The double extraction procedure is not superior to the single extraction.

Fig. 1 compares the O.D.-dye concentration relationships for non-precipitated samples (A) and samples precipitated according to this procedure (B). The nonprecipitated dye solution was prepared as follows: a standard solution was prepared by diluting 1 ml of an aqueous solution of dye (1 mg/ml) with 1 ml ethanol, 2 ml concentrated HCl and n-butanol to 100 ml volume. The 3 final dye solutions were made as follows: 0.01 mg dye, 1 ml standard solution + 3 ml butanol, add 3 ml to 1 ml ethanol; 0.02 mg dye, 2 ml standard solution + 2 ml butanol, add 3 ml to 1 ml ethanol; 0.04 mg dye, add 3 ml standard solution to 1 ml ethanol. These final dilutions were read at 620 mμ. There is considerable difference between the non-precipitated and precipitated samples amounting to 33-42%. This difference could be due

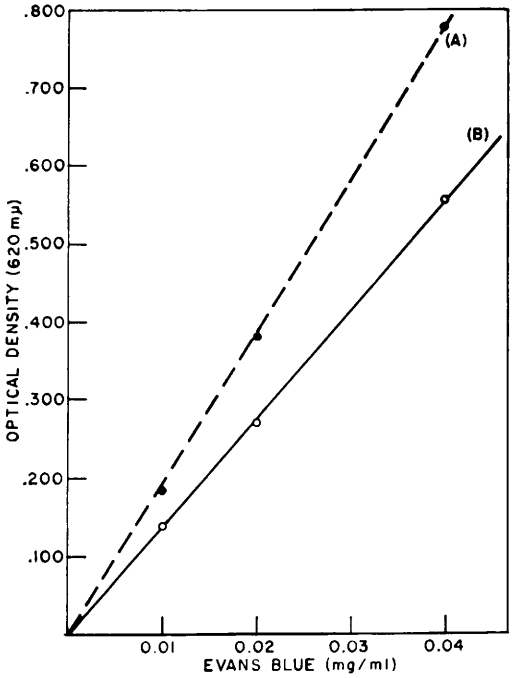

FIG. 1. Comparison of unprecipitated dye standards (A) with $CdSO_4$-NaOH precipitated standards (B). The mean ± S.E. optical density values for the unprecipitated standards in 9 experiments were respectively, .184 ± .001, .382 ± .004, .799 ± .006.

TABLE III. Comparison of a Single Butanol Extraction (4 ml) of Protein Precipitate with Two (2 ml) Extractions.

Dye (mg/ml plasma)	Mean optical density 1 extraction	2 extractions
.01	.148 (4)*	.142 (5)
.02	.270 (4)	.271 (4)
.04	.561 (5)	.555 (4)

* No. of experiments.

TABLE IV. Comparison of Dog Blood Volumes Using $CdSO_4$-NaOH Precipitation (Method I) and Harington's Method (Method II).

Dog wt (kg)	Blood vol (ml) Method I	Method II	Method I (ml/kg)	Method I as % Method II
10.5	664	697	63	95.3
11.0	881	990	80	90.0
12.5	1,018	1,090	81	93.4
8.5	818	816	96	100.2
9.0	1,029	1,055	114	97.5
14.5	1,321	1,332	91	99.2

to incomplete adsorption on protein and precipitation of the dye, to a change in the light absorption characteristics due to the conditions of precipitation and extraction(3), or to impurities, in the sample of dye, which absorb light at 620 mμ under the conditions of the solution but either are or not adsorbed to protein and are colorless under these conditions. A failure of precipitation of a colored component is not tenable since by visual inspection the supernatant from the precipitation procedure is colorless. Information concerning the second two possibilities is not available. However, colored impurities present in commercial preparations of Evans' blue have been reported(4).

Table IV summarizes data from some determinations of the blood volume in dogs using the extraction procedure from plasma of Harington *et al*(5) and our precipitation procedure. The plasma volume values were calculated from a single blood sample withdrawn 5 minutes after the dye injection. Blood volumes determined simultaneously with the two methods average agreement within 5%.

Over a period of months we have measured the recovery of 0.01 mg/ml dye added to the plasma of different dogs and determined by different technicians using this procedure. In 25 experiments on different samples of plasma mean concentration and standard error were 0.0099 ± 0.00015 mg/ml.

Allen(6) introduced a paper pulp adsorption and extraction procedure for determination of Evans' blue in plasma which eliminates many of the objections inherent in reading the optical density of plasma directly. There have been numerous modifications of Allen's procedure. The procedure reported here utilizes the naturally occurring adsorption on plasma protein for the same purposes.

Summary. A relatively rapid procedure for determination of Evans' blue in plasma or in plasma diluted with dextran is described. The procedure involves the total precipitation of plasma proteins by $CdSO_4$ and NaOH. After centrifugation and acidification of the precipitate it is extracted with butanol. The O.D. of the extract, with ethanol added, is read at 620 mμ, and the dye concentration is read from a standard graph.

1. Constable, B. J., *Clin. Sci.*, 1958, v17, 597.
2. Little, J. M., *J. Biol. Chem.*, 1949, v180, 747.
3. Gregersen, M. I., Rawson, R. A., *Physiol. Rev.*, 1959, v39, 307.
4. Cooley, G., *J. Physiol.*, 1954, v123, 16.
5. Harington, C. R., Pochin, E. E., Squire, J. R., *Clin. Sci.*, 1940, v4, 311.
6. Allen, T. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 145.

Received November 8, 1963. P.S.E.B.M., 1964, v115.

In vitro Synthesis of Progesterone by Ovaries and Adrenals of Snakes.* (28971)

IAN P. CALLARD AND JAMES H. LEATHEM
Department of Zoology and Bureau of Biological Research, Rutgers - The State University, New Brunswick, N. J.

Previous work has not established a relationship between the gonads and pregnancy maintenance in snakes. Clausen(1) found that castration of several species of a viviparous snake (Natrix) in early or mid-gestation caused resorption or abortion of the embryos,

* Supported by grants from Nat. Inst. Health and NSF.