Determination of Evans Blue in Avian Plasma by Protein Precipitation and Extraction. (30644)

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Evans Blue (T-1824) is perhaps the most widely used dye for plasma volume determinations. Analytical difficulties due to lipemia or hemolysis require the use of blanks and standards prepared with plasma from each experimental animal or extraction of the dye prior to analysis in a water solution(1,2). Changes in blank density *in vitro* prompted the development of a method for individual blank correction(3).

Recently Little and Williams(4) proposed precipitating the dye-protein complex with Cadmium sulfate (Cd SO_4) followed by extraction with n-butanol. Although the extraction procedure is relatively simple, the precipitation procedure must be adhered to rather closely for optimum precipitation. Young(5) simplified the precipitation procedure by using 10% trichloroacetic acid (TCA). His extraction procedure is, however, rather complex and time consuming.

The purpose of this study was to develop a simple procedure for analysis of Evans Blue in plasma based on precipitation of the dye-protein complex with TCA followed by extraction with n-butanol.

Methods and results. TCA-Butanol procedure. (Method 1). Precipitation and extraction procedures recommended are as follows: Pipette 0.2 ml of dyed plasma into a round bottomed glass or plastic test tube (approx. 15 mm diam). Add 0.5 ml of physiological saline, 5.0 ml of 10% TCA, mix well and let stand for about 5 minutes. Centrifuge at 200 $\times$ *g* for 5 min, decant supernatant and stand tube upside down on paper toweling to drain for a few minutes. Break up the pad of precipitate by vigorous shaking or with a mechanical mixer.† Add 0.5 ml of concentrated HCl and mix again. Add 4.0 ml of n-butanol, mix and hold in a water bath at about 65°C (hot tap water) for 2 minutes. Centrifuge at 1000 $\times$ *g* for 10 minutes and decant butanol-dye extract directly into cuvettes. Read optical density at 620 mμ.

Prepare standards and blank by adding 1.0 ml of standard Evans Blue solutions (10-40 μg/ml) or water to 0.2 ml of undyed plasma. Precipitate and extract as described above.

Evans Blue dye was obtained from British Drug House Ltd. Optical densities were determined with a Spectronic 20 colorimeter. At least one standard Evans Blue solution was run with each series of unknowns.

The relationship of optical density to dye concentration is shown in Fig. 1. Curve A is representative of the standard curves obtained with the TCA-butanol procedure.

Efficiency of precipitation and extraction. Efficiency was determined by comparing the optical density values of TCA precipitated-butanol extracted dyed plasma samples with those of acidified-butanol solutions containing equivalent concentrations of dye. The latter solutions, termed non-precipitated, were prepared as follows: Three solutions containing 10, 20 or 30 μg/ml dye were prepared by diluting 0.2, 0.4 or 0.6 ml respectively of an aqueous solution of dye (5.0 mg/ml) with 2.0 ml concentrated HCl and n-butanol to 100 ml volume. An aliquot of each solution was read directly in the colorimeter and the data plotted as curve B, Fig. 1. Plasma samples containing 10, 20, 30 or 40 μg/ml dye were precipitated with TCA and extracted with butanol as outlined above. These data are plotted as curve A, Fig. 1.

Differences between the precipitated and non-precipitated samples were within 4%. In a second trial the optical density values for non-precipitated groups were about 3% lower than those for the precipitated groups.

Comparison of TCA-butanol (Method 1), with $CdSO_4$-butanol (Method 2) procedure. To compare the precision of our procedure

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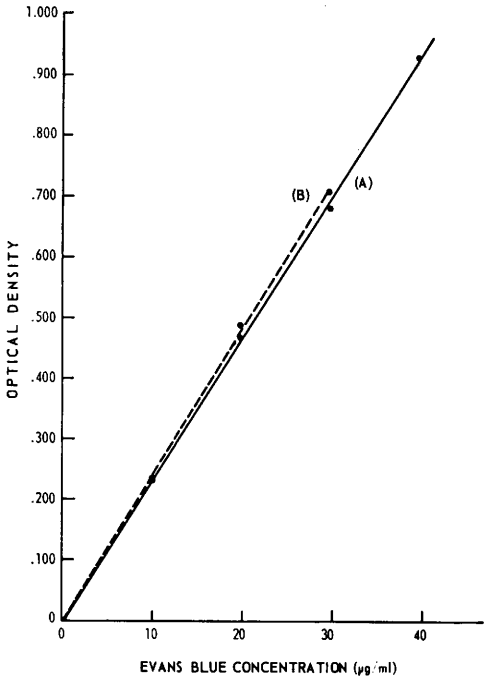

FIG. 1. Comparison of TCA precipitated, butanol extracted Evans Blue standards (A) with butanol-dye solutions of equivalent concentration (B)—unprecipitated standards.

with that of Little and Williams, 10 replicates of a dyed plasma sample were analyzed by each procedure. In a third group, 1.0 ml of ethanol was added to 3.0 ml of the dye-butanol extract from method 1. Results are given in Table I.

Slightly better precision was obtained with Method 1 than with Method 2. Addition of ethanol to Method 1 did not improve the precision. The mean O.D. values are, of course, not comparable because of differences in dilution.

As a further comparison, plasma volumes of 14 geese were determined using both procedures. For Method 2, 0.2 ml of plasma rather than 1.0 ml was used. Blood was withdrawn into a heparinized syringe 5 minutes after dye injection. Plasma dye concentration was determined in duplicate by both methods simultaneously. Data are summarized in Table II.

TABLE I. Precision of TCA and Cd SO_4 Precipitation Procedures.

Procedure	No. of replicates	Optical density Range	Mean	± S.D.
TCA-butanol	10	.450-.461	.459	.003
TCA-butanol + ethanol	10	.350-.358	.352	.003
Cd SO_4-butanol	10	.272-.305	.296	.011

Plasma volumes agreed, on the average, within 2%. Analyses of variance showed no significant difference between groups.

Discussion. The present procedure simplifies the precipitation procedure of Little and Williams(4) and the extraction procedure of Young(5). The close agreement between the precipitated and non-precipitated samples (Fig. 1) indicates extraction of virtually all of the dye from the plasma. In a similar comparison Little and Williams(4) noted a difference of 30-40% between the two groups.

TABLE II. Comparison of Plasma Volume of Geese Using TCA Precipitation (Method 1) and Cd SO_4 Precipitation (Method 2).

Bird No.	Body wt (kg)	Plasma volume (ml/kg) Method 1	Method 2	Method 1 as % Method 2
1	5.50	52.2	53.3	98.0
2	5.05	53.3	52.7	101.1
3	4.11	42.8	44.8	95.7
4	3.55	47.9	46.5	103.0
5	4.16	42.3	47.6	88.9
6	3.64	48.6	55.5	87.6
7	5.43	38.5	37.2	103.5
8	5.86	34.1	34.2	99.5
9	3.52	47.2	46.6	101.2
10	6.00	34.3	33.2	103.5
11	5.16	35.7	34.3	104.0
12	6.60	30.0	31.7	94.7
13	5.64	32.8	34.4	95.4
14	4.75	40.4	42.3	95.5
			Mean =	98.0

Ethanol is used in the Little-Williams procedure to eliminate cloudiness in the dye-butanol-extract due to the presence of water. This step is not required in our procedure because of the small amount of water remaining in the precipitate provided the tubes are well drained and any liquid remaining on the lip of the test tube is wiped off (Table I). In a limited number of trials using 1.0 rather than 0.2 ml of plasma, some cloudiness was observed which could be eliminated by addition of 1.0 ml of ethanol.

Repeated extraction with butanol or additional heating and mixing during the initial extraction did not increase the O.D. values obtained with the procedure outlined above.

This study was conducted with avian plasma only. There is no reason to suspect, however, that the procedure would not be applicable to mammalian plasma. Whether it could be used for extraction and analyses of Evans Blue in other tissues or in plasma-dextran mixtures requires further investigation.

Summary. A simple procedure is described for determination of Evans Blue in plasma based on precipitation of the dye-protein complex with trichloroacetic acid and extraction of the acidified precipitate with n-butanol. The standard curve is linear to at least 40 μg/ml of dye.

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A Method for the Isolation of Pyroglutaminyl Peptides from Glycoproteins.* (30645)

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A considerable number of human plasma proteins including α_1-acid glycoprotein(1), Zn-α_2-glycoprotein(2), 3S γ_1-globulins(3) and fibrinogen(4) are known to possess N-terminal amino acids that do not react with dinitrofluorobenzene. Other blood proteins such as the 7S γ-globulins(5,6) when subjected to the same analysis yield only small amounts of N-terminal aspartic and glutamic acid. In view of the importance of the terminal amino acids of proteins for elucidation of the chemical structure of these macroglobulins, a procedure was developed that permits determination of the N-terminal amino acids of the mentioned glycoproteins. As model compound α_1-acid glycoprotein(7) was selected.

Extensive studies of proteolytic digests of the native and denatured form and of several derivatives of α_1-acid glycoprotein revealed the following: (a) Initial removal of the sialic acid is a prerequisite for separation of the peptides resulting from the subsequent hydrolysis. If sialic acid was not cleaved off, any N-terminal peptides with substituted α-amino groups and the sialic acid-containing glycopeptides were found together in the effluent of the Dowex-50 column mentioned below. Removal of sialic acid was achieved with a neuraminidase or by mild acid hydrolysis (0.025 N H_2SO_4, 80°, 1 hour). (b) Reduction of the disulfide bonds and alkylation of the formed sulfhydryl residues appear necessary for the complete and permanent unfolding of the polypeptide chain. (c) Succinylation of the free ϵ-amino groups(8) and benzylation of the guanidinium groups (9) were carried out to make possible the isolation of any N-terminal peptides that may contain lysine and/or arginine.

The hydrolysis of the glycoprotein modified as described above was carried out with pronase. The obtained hydrolysate was fractionated on a Dowex-50 column (X2, H-cycle) in the cold(10). The acidic peptides together with a small amount of glycopeptides present in the effluent were next separated from each other by high voltage electrophoresis at pH 3.5 in pyridine acetate buffer.

The investigation of α_1-acid glycoprotein by this procedure showed that high voltage electrophoresis of the acidic peptides yielded a major and 4 minor fractions. The main

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