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Determination of Evans Blue Dye in Blood and Tissues.* (22155)

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To our knowledge there is only one report in the literature of a method for determining Evans blue dye in whole blood(1) and only 3 for determining the dye in tissues(2-6). The present paper describes a new procedure for its estimation in tissues, of which blood may be considered a special case. In addition, by employing this method, data have been obtained on the fate of the dye for up to 140 hours after its injection into the rat.

Method. Description of method. The method is based upon 3 fundamental operations: (1) homogenization of tissue in a concentrated solution of urea, (2) splitting of the Evans blue-protein complex and partial precipitation of chromogens with acetone, and (3) complete precipitation of chromogens by addition of Somogyi reagents. Details of the method as employed for tissues are as follows: (1) A 5-g sample of the dye-containing tissue to be analyzed and 2 additional 5-g dye-free samples of similar tissue obtained from another animal were weighed. To one of the latter was added a known amount of dye in approximately the amount to be found in the unknown tissue sample. The 3 samples (tissue plus unknown dye, tissue plus known dye, tissue without dye) were minced. (2) Concentrated urea solution (equal weights of A.R. urea and distilled water) was added to each tissue sample until the total volume was 100 ml and the mixture was homogenized in a Waring Blendor for 10 minutes. The homogenate was poured into a flask in a cold tap water bath and allowed to cool for 5 minutes. (3) Two hundred ml of A.R. acetone added to each sample and each was shaken briefly. Ten ml of Somogyi reagent I (10% zinc sulfate • $7H_2O$) was added to each sample which was then mixed well by shaking. After further addition of 10 ml of Somogyi reagent II (0.5 N NaOH) to samples, the latter were mechanically shaken for 10 minutes and allowed to stand 15 minutes. (The 2 Somogyi reagents should be standardized by titration against each other to the end point of phenolphthalein). (4) The samples were filtered through Whatman No. 42 paper. Optical density of filtrates from the 2 dye-containing samples was determined at 620 mmu in colorimeter which has a 20 cm cell length. The tube containing dye-free tissue was used for the I_0 setting. *Procedure for analysis of blood.* The method was applied to blood as follows: (1) one-half to 2 ml of whole blood was taken and 0.9% NaCl was added where necessary to bring the final volume to 2 ml. Two ml of the urea solution used for tissue analyses was added followed by 8 ml of A.R. acetone and 0.5 ml each of the 2 Somogyi reagents. The samples were shaken between each addition. (2) After centrifugation at high speed for 15 minutes the supernatant liquid was taken for colorimetry at 620 mmu.

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TABLE I. Recovery of Added Evans Blue from Dog Tissues.

Tube No.	Contains	Optical density	% yield
I_o	Water only	.0	0
1	All reagents (a check on water as I_o)	.0	0
2	Dye + reagents (except Somogyi reagents)	.218	100
3	Heart muscle, no dye	.002	1
4	" " + "	.222	102
5	Intestine, no "	.0	0
6	" + "	.218	100
7	Brain, no "	.002	1
8	" + "	.215	98.5
9	Liver, no "	−.002	−1
10	" + "	.215	98.5
11	Kidney, no "	.002	1
12	" + "	.218	100

The standard solution was made with known amounts of dye added to an amount of whole blood equal to that taken for analysis of the unknown. The solution used for the I_o setting was made from dye-free blood. With less than 0.5 ml of blood the optical density is a function of amount of blood because of the effect of blood colloids and salts on absorption of light by the dye. *Fate of dye in rats.* 1. While under sodium pentobarbital (Nembutal) anesthesia each of 8 fasted rats was injected with 0.25 ml of 5% Evans blue dye into the exposed right jugular vein. Twenty minutes later was taken as zero time, and concentration of dye in a 0.2-ml sample of tail blood at this time was taken as 100%. The animals recovered from the anesthetic 4 to 8 hours later. The animals sacrificed within 16 hours were placed in cages without food but with water. The animals sacrificed after 16 hours were placed in cages with food and water but were taken off food for 12 hours before they were sacrificed. At various time intervals up to 140 hours individual rats were stunned by blow on head and 0.2 ml blood was taken from the still beating heart. Blood and whole rat carcass were analyzed for dye by methods previously described using a 6% correction for weight loss of rat tissues caused by evaporation of water during grinding. Excreta were included in the carcass analysis only in the case of rats sacrificed before recovery from anesthesia. To judge from color of urine and feces, their dye content was relatively trivial.

Results. Recovery of dye from various tissues. Two experiments were run, each on a set of 5 different tissues obtained from dogs. In the first experiment 0.0376 mg of Evans blue was added per gram of experimental tissue and a colorimeter equipped with a 20 cm cuvette was used. In the second experiment 0.10 mg of dye was added per gram of tissue, and the Coleman Spectrophotometer equipped with a 1 cm cuvette was employed. Since data from both experiments were essentially the same, only those from the second experiment (0.1 mg dye per g of tissue) are given (Table I). Recoveries of added dye ranged from 98-102%. Blood was added to dye-containing tissue samples to determine the contribution of blood pigments to non-dye color in the final solution. The results (Table II) indicate that added blood up to 50% of weight of the tissue sample does not result in a positive error. More blood than this yields erroneously high dye recoveries.

Recovery of injected dye by analysis of the whole animal. Evans blue was administered intravenously to each of 14 rats and the whole

TABLE II. Effect of Added Blood on Dye Recovery from Tissues.

Sample	g of tissue	ml of blood added	% yield
Control	0	0	100.0
1	5	0	101.5
2	5	2.5	100.0
3	5	5.0	106.5
4	5	7.5	111.9
5	5	10.0	117.5

TABLE III. Assay of Whole Rat for Evans Blue Content.

Sample	Optical density	% yield on basis of raw data	% yield corrected for wt loss during grinding
Rat	.444	102.8	98.8
Control	.432	100.0	—

animal was taken for analysis. The following is a typical protocol: A rat weighing 409 g was fasted overnight and anesthetized wtih sodium pentobarbital (Nembutal), 35 mg/kg given intraperitoneally. One ml of 1.0% Evans blue dye was injected into the exposed jugular vein. It was necessary to fast the animals because fox chow in the gastrointestinal tract contributed errors of 7 to 28% due to a green color which is extractable from the chow and which appears in the final solution used for colorimetry. One ml of the same 1% dye solution was added to 409 ml of water to serve as a standard. The rat was killed by a blow on the head following which it was quartered and ground in a meat grinder 6 times. The tissue mass was blended with spatulas between the several grindings. After grinding, the tissue weighed 393.6 g, a loss of 15 g from evaporation of water. The final result was corrected for this weight loss. Five g of the tissue were taken for analysis. A second rat which received no dye was ground in the same manner and 5 g of its tissues were added to 5 ml of the standard solution. Another 5 g of tissue from the second rat was employed for preparation of the solution used for the I_0

TABLE IV. Recovery of Added Evans Blue from Whole Rats.

Rat No.	% yield (corrected)*
1	103.2
2	97.7
3	97.1
4	97.5
5	102.5
6	101.1
7	101.2
8	103.2
9	101.0
10	97.1
11	99.5
12	98.6
13	102.9
Rat from Table II	98.8

* For wt loss during grinding.

setting. All samples were homogenized in concentrated urea solution and treated with acetone and the Somogyi reagents as described above. Colorimetry was done at 620 mmu using a colorimeter equipped with a 20 cm cuvette. The results are given in Table III. In Table IV are given the results for the remaining 13 rats. In this series the method was accurate within 3% of the true value. In preliminary trials, proportions of urea solution and tissues, amounts of the 2 Somogyi reagents and the amount of acetone were varied independently. The method as specified is based upon optimal reagent amounts as established in these trials.† It was necessary to add dye-free tissue to the standard dye sample to prevent the shift of dye color from dark blue to light purple, as may occur in acetone-water solutions in the presence of Somogyi reagents.

Fate of injected dye in rats. The data are presented in Fig. 1, in which the lines were fitted to the plotted points by visual inspection. Because of this and because each point for a given time interval was obtained from a single animal, only the general features of the curves may be considered as meaningful. Furthermore, the points for percentage of dye in the body which is extravascular have been calculated on the assumption that blood volume has remained constant, *i.e.*, that the amount of intravascular dye is directly proportional to blood dye concentration. For large changes in blood dye concentration, errors introduced by this assumption may be considered of secondary importance. Proceeding on the basis of this assumption, one finds that 40 hours after its administration, about 90% of the dye has left the blood but approximately 83% is still in the whole carcass. Even after 140 hours, when about 92% has left the blood, some 67% can be still recovered from the carcass. Disappearance of the dye from the body is most rapid 10-24 hours after injection, and is attributed mainly to decolorization within the body rather than loss via the excreta. Thus most of the dye which presumably disappears from blood in colored form can be recovered from the whole animal even at 140 hours. This finding might be ex-

† Details of these trials are available elsewhere(8).

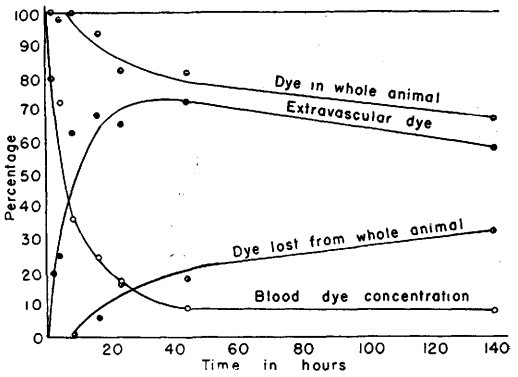

FIG. 1.

plained by the presence in blood of material which yields color by carcass analysis but not by blood analysis. This is highly improbable and, moreover, no blue color was observed in Somogyi precipitates of blood. The conclusion appears justified that the dye in the carcass not accounted for by blood analysis is in the extravascular spaces.

Discussion. Sellers *et al.*(7) have reported serum Evans blue/serum protein concentration ratio as a function of time up to 70 hours after intravenous injection into rats of 20 mg of Evans blue. They make no mention of employing anesthesia except for terminal sampling. If serum protein concentration is assumed to remain constant, their curve can be considered a blood dye concentration curve, and in fact their curve is almost identical with that for blood in Fig. 1. This similarity suggests that the present findings were not greatly influenced by the ether anesthesia.

Summary. 1. A method is described for determination of Evans blue dye in various animal tissues. In brief it consists of (1) homogenization of tissue in a concentrated solution of urea, (2) splitting of the Evans blue-protein complex and partial precipitation of protein and chromogens with acetone, and (3) complete precipitation of chromogens by addition of Somogyi reagents. 2. The method has been employed to follow the fate of the dye after intravenous injection into rats. After such administration of 12.5 mg of Evans blue, about 90% of the injected dye apparently left the blood but only about 15% could not be recovered from the body in 40 hours. At 140 hours after injection about 67% of the injected dye could still be recovered from the body.

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Plasma Prothrombin Time and Hematocrit Values of Blood of Dairy Cattle.* (22156)

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Hemostasis is a complicated mechanism of blood coagulation composed of many coordinated reactions(1-3). According to Quick (2), coagulation of blood occurs in 3 stages, none of which are complete when considered individually. These stages must be combined in a definite arrangement before a complete explanation of hemostatic mechanism can be offered.

Determination of plasma prothrombin time

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