QUANTITATIVE MEASUREMENT OF EVANS BLUE SPACE IN THE TISSUES OF THE RAT: INFLUENCE OF 5-HYDROXYTRYPTAMINE ANTAGONISTS AND PHENELZINE ON EXPERIMENTAL INFLAMMATION

VIVIAN L. BEACH AND BERNARD G. STEINETZ

Warner-Lambert Research Institute, Morris Plains, New Jersey

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Several investigators have used Evans Blue dye as an indicator of changes in capillary permeability. Thus, West (1957) and Parrat and West (1958) have explored the effects of histamine and serotonin liberators and inhibitors on capillary permeability in rats, by subjective assessment of bluing of the skin. Subjective criteria have also been used in similar investigations of the anaphylactoid reaction in rats (Fries, 1958). Spector (1958) has recently pointed out the need for an efficient quantitative technique for the determination of Evans Blue dye concentration in soft tissues.

Caster *et al.* (1954, 1955) have described a quantitative procedure for determining Evans Blue space in tissues, but this method, though precise, is far too laborious for routine use when large numbers of analyses are required.

Accordingly, a method has been developed which circumvents the difficulties encountered in the procedure of Caster *et al.* (1954, 1955) and permits analysis of 40 or more tissue samples in less than a day by a single technician.

The method has been useful for estimation of (1) plasma volume in tissues and (2) changes in capillary permeability.

MATERIALS. Evans Blue dye, 1% aqueous (Warner-Chilcott Laboratories Div.); 15 and 50 ml conical centrifuge tubes; 0.25, 2 ml tuberculin and 5 ml syringes; 26 gauge 1″ and 17 gauge 4″ needles; chloroform, reagent grade (Baker's); hydrochloric acid, reagent grade, concentrated; benzalkonium chloride (Zephiran), 12.8% (Winthrop Laboratories); 25- or 50-ml burettes (Teflon stopcock); filter paper, Whatman #1, 4.25 and 9.0 cm; funnels, 1″, 1½″ or 2″; spectrophotometer, preferably adaptable to microanalysis.

METHODS. A. *Injection of Evans Blue and dissection procedure.* Choice of anesthesia should be governed by the nature of the particular experiment, but ether is suitable for determination of plasma volume in tissues (Caster *et al.*, 1955), and for studies of the anaphylactoid reaction.

One per cent Evans Blue dye is injected into the exposed jugular vein of anesthetized rats at a dose of 0.5 ml/100 g body weight. At time intervals meaningful to the investigation,[1] the chest is opened, heparinized blood samples are obtained (heart puncture) and the large vessels of the heart are clamped with a hemostat. Desired tissue samples are rapidly excised, trimmed, blotted on moist toweling and weighed. For routine work, it is convenient to freeze tissues immediately on dry ice and store them in capped bottles in a deep freeze until ready for analysis. Alternatively, tissues may be dropped directly into conical centrifuge tubes containing concentrated HCl.

B. *Digestion of tissues.* Tissues are digested in concentrated HCl in conical centrifuge tubes: 3 ml for most tissue samples (100 to 1000 mg) or 0.5 ml for small tissues such as adrenal and pituitary. (The amount of HCl is not critical, and should be decided by preliminary trial. The only purpose is solubilization of the tissue, so that the smallest quantity which will accomplish complete digestion in a reasonable period of time should be used.)

The digestion step is most conveniently accomplished by allowing tissues to stand in HCl overnight at room temperature. Certain tissues (*e.g.*, testes, uterine cervix) require mild heating (37°C in an incubator or bath) for approximately 18 hours. Higher temperatures cause charring, and should be avoided. Most tissues will digest in 1 hour at 37°C if overnight digestion is not expedient.

C. *Extraction of Evans Blue from digests.* When digestion is essentially completed (*i.e.*, the next morning) 3 ml of benzalkonium chloride (Zephiran) are added from a burette, the mixture is shaken and allowed to stand for approximately 30 minutes with interval agitation. Zephiran com-

[1] For example, for tissue plasma volume determinations, a 3- to 5-minute interval is optimal; for changes in capillary permeability, 2 to 3 hours may be required.

plexes with the Evans Blue, and this complex is selectively soluble in chloroform (Caster *et al.*, 1954).

Accurate amounts of chloroform from the burette are added to the tubes and mixed (1.1 ml of chloroform for microdeterminations, 5 or 10 ml for most samples). The exact amount of chloroform to be used is determined by rough estimation of the quantity of Evans Blue in the tissue. Thus, for obviously dark blue samples more chloroform is used than for pale blue tissues.

The chloroform will tend to layer at the bottom of the centrifuge tube. Agitation of this mixture by alternate vigorous aspiration and ejection of the chloroform layer with a 5-ml syringe (17 gauge 4″ needle) will complete the separation. The tube is allowed to stand with intermittent reagitation until the aqueous (acid) phase is devoid of any blue color. In general, this requires 3 to 4 agitations with the syringe over a period of approximately ½ to 1 hour. On long standing without agitation, the Evans Blue complex will concentrate at the chloroform-aqueous interface and meaningful aliquots will not be obtained. Centrifugation will also induce migration and absorption of Evans Blue at the interface and should be avoided.

The chloroform layer is withdrawn and filtered with the same syringe and needle. The filtrate is collected directly in a spectrophotometer tube or cuvette. Tubes or cuvettes must be capped immediately to prevent evaporation of chloroform.

Readings are made at 620 mμ against a chloroform blank. The optical densities of Evans Blue solutions obey Beer's Law from a concentration of 0.5 to 20 μg/ml, so that only one concentration of a standard need be run. A 100 μg standard is always carried through the entire procedure. (Stock standard = 100 μg in 0.1 ml or 1 ml HOH.)

Evans Blue concentration in plasma and hematocrits is determined according to conventional methods. Because of the large dose of Evans Blue required to produce a measurable quantity in tissues, the precautions described by Caster *et al.* (1953) for determining plasma dye concentration did not prove to be necessary. The plasma is diluted 1:100 with distilled water and plasma blanks at this dilution have no greater optical density than distilled water alone.

Plasma volume in tissues is calculated according to Caster *et al.* (1954): ml plasma/g tissue = $\frac{\text{dye concentration in tissue}}{\text{dye concentration in plasma}}$. In studies of Evans Blue accumulation in tissues, results may be expressed as μg Evans Blue per gram of tissue. Alternatively, comparable groups of animals may be sacrificed at 5 minutes (to obtain tissue plasma volume) and at some later interval (*e.g.*, 2 hours) after injection. With these data, an approximation of the extravascular Evans Blue is readily obtained, providing an estimate of capillary leakage. It must be emphasized that this value is a rough approximation, for the dynamics of Evans Blue in the animal body are very complex. Caster *et al.* (1955) have discussed the various factors influencing the dye concentration in plasma and tissues at any instant of time.

TABLE 1

Recovery experiments

40 μg Evans Blue added to each tissue.

Tissue	No. of Analyses	Average Recovery		Range of Values
		%	±S.E.	
				%
Brain	10	97.8	0.8	94.7–100.0
Lung	10	98.5	0.8	92.0–101.3
Heart	10	99.0	0.6	94.4–101.1
Skin	10	93.9	0.8	89.8– 98.3
Paw	10	97.0	0.3	93.5– 98.0

RESULTS. A. *Recovery of known amounts of Evans Blue from tissue digests.* In order to establish the reliability of the method, fresh and frozen rat tissues were digested in concentrated HCl to which had been added 40 μg Evans Blue dye. The entire extraction procedure was performed and optical densities of the chloroform extracts compared with appropriate standards. Recovery data are shown in table 1. It is apparent that the maximum error to be expected is 10% and the average error is 3%.

B. *Duplicate tissue samples; within and between animal variations.* Whereas whole pituitaries, adrenals, ovaries, thyroids, etc. may be handled conveniently, large organs and tissues such as skin present a sampling problem. Accordingly, experiments were performed to determine whether or not random tissue sampling introduced a significant error in assessing the Evans Blue uptake in a given organ or tissue in homogeneous groups of rats. Adult female (230 to 260 g) and male (360 to 490 g) rats were injected with 0.5 ml 1% Evans Blue/100 g body weight and sacrificed at 1½ hours. Two samples each of skin, lung, heart, brain and paw were obtained, and the Evans Blue concentrations determined. Within-group variation was expressed as the standard error of the mean Evans Blue concentration; the sampling error was

expressed as the ratio of the duplicate samples × 100. The data are shown in table 2. It is obvious that the between-animal variation was not much greater than the between-sample variation. However, with groups as small as five animals each, the sampling error was canceled, as the means of the duplicates were in good agreement. The mean ratios of duplicate determinations ranged from 95 to 107%, and the error was less than ± 10% in the case of paw, skin and lung. The larger variations (± 14 to 15%) observed in duplicate analyses of heart and brain segments are a reflection of the difficulties encountered in trying to obtain equivalent samples of these organs. To eliminate this difficulty, whole brains and hearts are analyzed.

C. *Chloroform-soluble interfering substances.* A brilliant blue chloroform extract is obtained from digests of most tissues. However, kidney, spleen, liver and placenta have been found to contribute a significant amount of a yellow contaminant to the extract, which, in combination with Evans Blue, imparts a greenish color to the chloroform layer. Spectrographic analysis showed a maximum absorption of this contaminant at 420 mμ and only little absorption above 500 mμ. It has been estimated from the spectrogram that interference at 620 mμ (absorption peak of Evans Blue) does not amount to more than 10%. If desired, the corrections described by Caster *et al.* (1953) for interfering substances in plasma may be applied, or tissue blanks obtained from animals not injected with Evans Blue may be run concomitantly.

TABLE 2

Duplicate determinations of dye concentrations

Tissues of rats injected (i.v.) with 0.5 ml/100 g 1% Evans Blue 1½ hours previously.

Tissue	μg Evans Blue/g Tissue ± S.E.				
	Females (5)		Males (5)		(Females + Males)
	A	B	A	B	A/B
					%
Paw	16 ± 2	16 ± 2	28 ± 3	25 ± 2	106 ± 9
Skin	14 ± 2	15 ± 1	26 ± 1	25 ± 1	98 ± 4
Lung	171 ± 23	171 ± 18	217 ± 20	209 ± 13	107 ± 4
Heart	50 ± 9	49 ± 8	75 ± 6	79 ± 15	106 ± 14
Brain	8 ± 2	10 ± 1	16 ± 1	16 ± 1	95 ± 15

D. *Plasma volume in tissues of normal male and female rats.* The described method was used to determine the plasma volume in tissues of young (6 weeks of age) and old (14 to 18 months of age) male and female rats (Budd Mountain, Wistar).

Total plasma volumes of young rats of both sexes were significantly higher (25 to 30%) than those of older animals. Plasma volume decreased in skin (30 to 61%) and increased in heart (48 to 62%) and the female rat brain (38%) with advancing age. There were also observed increases in plasma volume of stomach and duodenum in the older groups (table 3).

E. *Changes in tissue Evans Blue concentration at longer time intervals following dye injection.* In

TABLE 3

Plasma volume in tissues of young and old rats

Sex	Male		Female	
Age	6 wks	14–18 mos	6 wks	14–18 mos
No. rats	10	8	10	12
Body wt	140–165 g	436–640 g	115–140 g	307–418 g
	ml/100 g	*ml/100 g*	*ml/100 g*	*ml/100 g*
Total	5.74 ± 0.13	3.82 ± 0.18	5.82 ± 0.09	4.33 ± 0.14
Skin	1.89 ± 0.05	1.32 ± 0.12	1.53 ± 0.08	0.94 ± 0.08
Lung	17.51 ± 0.61	19.30 ± 1.38	15.61 ± 2.19	15.30 ± 0.84
Heart	3.76 ± 0.11	6.10 ± 0.36	3.47 ± 0.20	5.15 ± 0.46
Paw	1.50 ± 0.20	1.56 ± 0.11	1.80 ± 0.20	1.52 ± 0.09
Brain	1.50 ± 0.20	1.81 ± 0.23	1.40 ± 0.08	1.94 ± 0.09
Duodenum	3.88 ± 0.22	4.50 ± 0.49	3.31 ± 0.13	4.85 ± 0.25
Stomach	3.11 ± 0.17	5.50 ± 0.35	—	5.14 ± 0.22

TABLE 4

Changes in dye concentration

Tissues of rats killed 5 min and 1½ hr after injection of Evans Blue.

	Males (440–640 g)			Females (195–418 g)		
No. rats	5 min 8	1½ hr 9	Signif. %Change at P = 0.05	5 min 12	1½ hr 13	Signif. %Change at P = 0.05
	μg/g	μg/g		μg/g	μg/g	
Total plasma	1179 ± 21	821 ± 34	−30	1116 ± 31	807 ± 36	−28
Skin	15 ± 1	24 ± 1	+60	10 ± 1	17 ± 1	+70
Lung	224 ± 14	184 ± 12	−18	170 ± 10	168 ± 9	0
Heart	71 ± 2	99 ± 6	+39	57 ± 5	70 ± 5	0
Paw	18 ± 1	35 ± 3	+94	17 ± 1	32 ± 1	+88
Brain	21 ± 3	16 ± 2	0	22 ± 1	14 ± 1	−36
Duodenum	51 ± 4	100 ± 3	+96	54 ± 2	111 ± 2	+106
Stomach	64 ± 3	74 ± 4	0	57 ± 2	83 ± 4	+45

TABLE 5

Evans Blue concentration in tissues of rats treated with histamine and serotonin liberators and inhibitors

Evans Blue concentration—μg/g fresh tissue.

Treatment	No.	Skin	Heart	Lung	Paw	Brain
None	5	20 ± 1	36 ± 1	151 ± 1	24 ± 2	9 ± 1
Egg white controls 2.4 ml (50%)/100 g, i.p.	4	60 ± 9	22 ± 4	95 ± 8	158 ± 10	4 ± 0.6
Egg white + BOL-148 4 mg/kg, i.v.	5	23 ± 2	46 ± 9	143 ± 12	106 ± 35	9 ± 1
Egg white + py. mal. 8 mg/kg, i.v.	5	18 ± 1	33 ± 3	109 ± 8	251 ± 12	7 ± 0.6
Egg white + chlorpromazine 10 mg/kg, s.c.	5	18 ± 1.5	42 ± 4	143 ± 11	24 ± 1	7 ± 0.4

order to study the movement of dye across capillary walls, rats sacrificed at longer time intervals were compared with similar animals killed 3 to 5 minutes after injecting Evans Blue. In one experiment (table 4) male and female rats (14 to 18 months old) were sacrificed 5 minutes and 1½ hours after dye administration. Twenty-eight to 30% of injected dye had left the circulation at the later time. Skin, paw and duodenum showed marked accumulation of dye. Concentrations of dye in brain roughly followed the decrease in plasma dye concentration, suggesting that under these conditions, little or no Evans Blue had escaped from the vascular beds of this organ.

F. *Changes in Evans Blue space of tissues of rats treated with serotonin and histamine liberators and inhibitors.* The anaphylactoid reaction was studied in 130 to 160 g female rats by quantitative measurement of Evans Blue concentration in lung, heart, paw, skin and brain.

Edema was induced by intraperitoneal injection of 50% egg white in 0.9% saline, 2.4 ml/100 g body weight (West, 1957). At the same time 1% Evans Blue was injected intravenously (jugular vein) at a dose of 0.5 ml/100 g. Attempts were made to inhibit edema formation by injection of chlorpromazine (10 mg/kg s.c.), pyrilamine maleate (8 mg/kg i.v.) or 2-brom-D-lysergic acid diethylamide (BOL-148[2]; 4 mg/kg i.v.) ½ hour *before* administration of egg white. Rats were sacrificed 1½ to 2 hours after egg white injection and tissues were analyzed for Evans Blue content.

Egg white injection was observed to induce a significant increase in Evans Blue concentration

[2] Sandoz Pharmaceuticals.

TABLE 6

Evans Blue concentration in lungs of rats treated with serotonin and histamine liberators and inhibitors

Treatment	No. Rats	Evans Blue Concentration in Lung μg/g Fresh Tissue	P Value	
			vs. Control	*vs.* Egg white
None (control)	28	151 ± 3	—	0.01
Egg white 2.4 ml (50%)/100 g i.p.	15	106 ± 6	0.01	—
Egg white + phenelzine sulfate 50 mg/kg i.p.	10	130 ± 7	0.02	0.02
Egg white + chlorpromazine 10 mg/kg s.c.	5	143 ± 10	N.S.	0.01
Egg white + BOL-148, 4 mg/kg i.v.	10	143 ± 6	N.S.	0.01
Egg white + pyrilamine maleate 8 mg/kg i.v.	9	125 ± 12	N.S.	N.S.
Phenelzine sulfate, 50 mg/kg i.p.	13	177 ± 18	N.S.	
Reserpine acetate 5 mg/kg i.v.	7	156 ± 7	N.S.	
Phenelzine sulfate + reserpine acetate	18	325 ± 27	0.01	
Chlorpromazine 10 mg/kg, s.c., ½ hour before phenelzine sulfate and reserpine acetate	9	141 ± 7	N.S.	
Serotonin depleted* before phenelzine and reserpine	8	188 ± 16	N.S.	
Histamine depleted† before phenelzine and reserpine	6	267 ± 34	0.01	
Serotonin and histamine depleted‡ before phenelzine and reserpine	9	147 ± 15	N.S.	

* Serotonin was depleted by intraperitoneal injection of reserpine acetate, 10 mg/kg daily for 3 days before the experiment.

† Histamine was depleted by intraperitoneal injection of polymyxin B, 1 mg/kg once on the first day, and in the morning and afternoon of the second and third days before the experiment.

‡ Histamine and serotonin were simultaneously depleted by combining treatments 1 and 2.

of skin and paw (table 5), confirming qualitative data of previous investigators (West, 1957; Parrat and West, 1958; Fries, 1958). However, a *reduction* in dye accumulation was observed in heart, lung and brain of egg white-treated rats (table 5).

Pretreatment with an antihistamine, pyrilamine maleate, afforded little protection against effects of egg white administration, whereas the anaphylactoid reaction was essentially inhibited by chlorpromazine (serotonin and histamine inhibitor). BOL-148 (serotonin inhibitor) partially protected rats from the consequences of egg white injection (table 5).

The mechanism whereby lung concentration of Evans Blue is decreased during anaphylactoid reaction was investigated further. The experiments just described were repeated, and in addition, the monoamine oxidase inhibitor, phenelzine sulfate,[3] was evaluated (table 6). All compounds tested, except pyrilamine maleate, afforded some protection against the effects of egg white. Since evidence in hand suggested that serotonin was primarily responsible for the effects observed following egg white injection, it was reasoned that injection of reserpine (serotonin liberator) in rats treated with a monoamine oxidase inhibitor might mimic the anaphylactoid reaction. Accordingly, groups of rats were treated with phenelzine sulfate (50 mg/kg i.p.), reserpine acetate (5 mg/kg i.v.) or their combination, ½ hour before injection of Evans Blue dye.

Neither reserpine nor phenelzine influenced lung-dye concentration when the compounds were administered separately but the combined treatment significantly *increased* accumulation of dye in lungs (table 6). This increase was prevented 1) by concomitant treatment with chlorpromazine (10 mg/kg s.c.); 2) by pretreatment with reserpine (10 mg/kg i.p. daily for 3 days); 3) by combined pretreatment with reserpine and polymyxin B (histamine liberator; 1 mg/kg i.p. in 5 doses over a 3-day period); but 4) not by polymyxin B alone. (The dosing schedules for reserpine and polymyxin B were reported by West (1957) to deplete animals of endogenous serotonin and histamine, respectively.)

Discussion. The technique described for measuring Evans Blue dye concentration in

[3] Nardil, Warner-Chilcott Laboratories Division.

tissues appears to retain good precision while providing the speed and simplicity necessary for routine laboratory use.

The handling of animals, however, is open to the same questions raised in regard to other techniques of this type (*i.e.*, deep anesthesia, surgery and the pharmacological effects of Evans Blue dye itself). Despite these obvious drawbacks, useful information may be obtained by careful control and by remembering that conditions of this type of experiment are far from physiological. As pointed out by Caster *et al.* (1955), data obtained under these conditions differ little from results observed when animals are fast frozen in liquid nitrogen (Friedman, 1955; Kallis and Pressman, 1950).

Tissue plasma volumes determined by the present method agree reasonably well with those reported by Caster and associates (1954, 1955) using a more difficult and elaborate technique. In addition, the data obtained in concomitant experiments with young and old male and female rats may explain much of the disagreement in the literature over correct plasma volumes of rats. Thus, total plasma volume was significantly higher in young rats than in older animals of the same strain. Plasma volumes of tissues likewise varied with the age, weight and sex of the animals.

The method was used to study egg white-induced anaphylactoid reaction in rats. A significant increase in dye accumulation was observed in skin and paw of edematous rats, agreeing with previous qualitative studies (West, 1957; Parrat and West, 1958; Fries, 1958). However, a *decrease* in Evans Blue space was observed in lung, heart and brain of egg white-treated animals. The anaphylactoid reaction is thus complex. Endogenous substances released by egg white injection may increase Evans Blue space in some tissues (*e.g.*, skin) presumably by increasing capillary permeability. In tissues such as lung, dye space is decreased, possibly due to vasoconstriction or bronchoconstriction. There is considerable evidence that serotonin induces vasoconstriction in the lung of the dog (Rudolf *et al.*, 1959; Kabins *et al.*, 1959; Shepherd *et al.*, 1959). Pulmonary vasoconstriction would, in turn, preclude observation of secondary effects on capillary permeability when Evans Blue dye is used as indicator. The data presented, as well as those of West (1957) and Parrat and West (1958), suggest that serotonin liberation is primarily responsible for the effects observed following injection of egg white in the rat. Thus, serotonin inhibitors such as chlorpromazine or BOL-148 largely prevented the anaphylactoid reaction. However, phenelzine sulfate, a potent amine oxidase inhibitor, which theoretically should favor accumulation of serotonin, also significantly *inhibited* the effects of egg white. Furthermore, treatment with phenelzine and reserpine, which combination might be expected to increase serotonin levels, actually greatly *increased* Evans Blue space in lung. Since this increase was prevented by treatment with chlorpromazine (serotonin inhibitor) or by prior depletion of serotonin stores (reserpine pretreatment) the effect of the combination of phenelzine and reserpine presumably involved liberation of serotonin. Thus, serotonin released under different experimental conditions may have opposite effects on Evans Blue space of rat lung. Evidence is required concerning participation of the catecholamines, and the possible direct action of phenelzine on lung, before any definite conclusions may be reached regarding the mechanism of reversal of serotonin effects.

SUMMARY

A simple procedure for estimating Evans Blue concentration in rat tissues is described. Forty samples, fresh or frozen, may be processed in less than one day by one technician. No special equipment is required; usual laboratory materials are used. When known amounts of Evans Blue were added to tissues, recoveries averaged 97%. The method described yielded results comparable to those obtained with more elaborate and difficult procedures appearing in the literature.

The techniques presented provide a practical means of assessing changes in plasma volume and capillary permeability in tissues of the rat. Original data illustrating these applications have been presented.

Plasma volume in tissues of the rat was investigated using the Evans Blue method described. Total plasma volume was higher in younger than in older animals, and tissue plasma volume varied with sex and age.

Evans Blue concentration was measured in tissues of rats treated with egg white to produce anaphylactoid reactions. Whereas dye accumulation was elevated in edematous tissues such as

skin and paw, Evans Blue space in lung, heart and brain was significantly decreased. These effects were reversed by chlorpromazine and (partially) by 2-brom-D-lysergic acid diethylamide. Pyrilamine maleate afforded slight protection.

Factors influencing dye accumulation in lung were further studied. An amine oxidase inhibitor, phenelzine sulfate, partially reversed the effects of egg white on lung dye concentration. A combination of phenelzine and reserpine induced a marked increase in Evans Blue space of rat lung. This effect was prevented by chlorpromazine or prior depletion of serotonin stores. The data suggest that the effects of serotonin on lung vasculature vary greatly with the conditions under which the amine is liberated.

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