ing 21 days. The data indicated that mammary gland development and secretory activity increased as the number of suckling young per mother rat increased.

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A Method for Extraction of Evans Blue from Plasma and Tissues.* (29207)

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Dilution of Evans Blue (T-1824) following intravenous injection is the simplest method for estimation of plasma volume(1). The difficulties encountered with the method are principally associated with determination of the dye in plasma, and include turbidity due to lipaemia, occasional haemolysis, and the inherent color of the plasma, which may be exaggerated as in ruminants. The spectral characteristics of the dye, moreover, change on conjugation with plasma, and the changes are not the same for the plasmas of all species(2). This necessitates comparison with standards prepared by adding dye to the plasma of the same species. It is obvious therefore, that determination of the dye following its extraction from the plasma would have distinct advantages. Extraction is in fact obligatory if Evans Blue is used as a marker for plasma proteins for quantitative determination of the changes in the retentive properties of capillaries. One method of extraction from plasma was developed by Allen (3,4) which involves adsorption of the dye on cellulose, followed by prolonged elution (4-16 hr) with aqueous acetone. However, though consistently good recoveries (97%) were obtained with plasma, extraction of the dye from albumin in tissues gave only 60-80% recoveries(5). For this reason, a simpler and more versatile method was developed.

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The conjugation of Evans Blue with albumin involves the ionic interaction of the sulphonate anions of the dye with quaternary nitrogens of this protein, most probably the ϵ-amino group of lysine (pK 9.4-10.6) and possibly the guanidinium group of arginine (pK 11.6-12.6)(6). Suppression of the ionization of these amino groups by a sufficient rise in the pH should lead to disconjugation of the dye. Concentrated ammonia solution (S.G. 0.880) proved satisfactory for this purpose.

Methods. Human plasma was mixed with Evans Blue, and precipitated by addition of an equal volume of 10% (w/v) trichloracetic acid. The precipitate was washed twice with absolute ethyl alcohol (5 min at 70°C) followed by extraction with 1 ml of concentrated ammonia solution (25%, d^{20}_4 0.91) for 10 min. Four ml of alcohol was then added with thorough mixing and the supernatant decanted. This extraction with ammonia was repeated twice more, and the combined supernatants mixed with 3 ml of glacial acetic acid and made up to 25 ml with ethyl alcohol. Addition of sufficient alcohol to the ammoniacal extract is essential if a clear supernatant is to be obtained. The time and speed of centrifugation at every stage must be similarly adjusted to give clear extracts. A bench-type centrifuge (International Equipment Co., Boston, Mass., model HN) was used. Five minutes at 1000 times gravity sufficed for separation of the precipitate from the

TABLE I.

	Quantity of dye	No. of observations	Recovery	Standard deviation
			(%)	(%)
Plasma	50 μg/ml	4	99.4	.6
	10 " "	4	97.9	1.4
Heart	40 μg/g	4	99.7	.4
Liver	40 " "	6	92.3	3.5

trichloracetic acid solution and from the alcohol; 10 minutes at 2000 times gravity was required for clarification of the ammoniacal extracts.

To test the efficiency of the extraction of Evans Blue from plasma proteins in tissues, 1 ml of the dye-plasma conjugate was added to 1 g of rat heart or liver and the tissue homogenized in a glass homogenizer. The subsequent extraction was carried out as described for plasma.

The Evans Blue used (British Drug Houses Ltd) was found to be free of the red impurity described by Cooley(7), when examined by paper chromatography using the organic phase of the mixture butanol (4): acetic acid (1):water (5) as the solvent. The dye was determined spectrophotometrically at a wavelength of 600 mμ in a Beckman DU spectrophotometer. The standard solutions of the dye prepared for comparison with the extracts were made up in ethyl alcohol.

Results. Extraction of plasma. As is shown in Table I, 2 ml of plasma containing 50 μg/ml of Evans Blue gave on extraction a mean recovery of dye of 99.4%. Plasma containing 10 μg/ml of dye gave a mean recovery of 97.9%.

Extraction of tissues. Table I also shows that heart tissue containing 40 μg/g of Evans Blue conjugated with 0.1 ml of plasma, gave on extraction a mean recovery of 99.7%, and liver containing the same quantity of dye gave on extraction a mean recovery of 92.3%.

Discussion. That the same excellent recoveries of Evans Blue are obtained from the albumin conjugate both in plasma and in tissues, not only facilitates the more exact measurement of the dye in blood, but also allows improvement in one of the existing methods of determining an increase in capillary permeability to albumin. The leakage from the vasculature of plasma proteins conjugated with dye, with a resulting coloration of the surrounding tissue, is well established as a method for detection of increased capillary permeability(8); and has been used in the study of increased permeability following inflammation(9), and following the release of permeability factors by drugs(10,11). As a method of extraction was not then available, changes in capillary permeability could only be estimated by either measuring the area of color or comparing intensity of color by eye. Though a method has been developed for extraction from tissues of trypan blue(12), the dye used in some of these investigations, the use of this dye as a plasma marker for such permeability studies is subject to the same qualification as its use in determining blood volume; that is, it is much less firmly bound to plasma albumin than Evans Blue(13), being lost 5 times more rapidly from the plasma. It is evident, therefore, that with a method of extraction available, Evans Blue is the most suitable non-radioactive plasma marker for determination of changes in capillary permeability.

Summary. Evans Blue (T-1824) was successfully extracted from human plasma and from plasma in tissues using concentrated ammonia solution. The recoveries were over 98% for plasma and heart tissue, and 92% for liver. Ability to extract quantitatively Evans Blue suggests this dye as the non-radioactive marker of choice for determination of changes in capillary permeability.

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Serum Enzyme Levels in Lines of Chickens Differing in Susceptibility to Leucosis.* (29208)

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Genetic differences in susceptibility to leucosis in chickens are well established. The purpose of this study is to find out if the chemical basis for such differences is manifested in differences in the level of enzymes in serum. It is recognized, however, that there are many other possibilities besides enzyme level which might be responsible for differences in susceptibility to leucosis.

The stocks employed were a resistant and a susceptible line developed at Cornell University(1). These lines differ markedly in mortality resulting from leucosis, especially the neural form. Studies were conducted at an early age because of evidence indicating the first fortnight posthatching to be a critical period in exposure to the disease(2). Only females were used since they show greater mortality from the disease than do males(3). Enzymes studied were of a wide variety. Their selection was based partially on the availability of routine assay methods. Leucosis is caused by viruses(4), therefore protease and nuclease assays were included, as such enzymes might conceivably inactivate virus either in the vascular system or in the organ(s) from which the serum enzymes originate.

Materials and methods. A susceptible line and a resistant (K) line in respect to leucosis were obtained as hatching eggs from Cornell University. Chicks were reared separately from adults in batteries; no attempt was made to expose them to leucosis. Blood was sampled from females and serum poured off after incubation at 37°C for 1½ hours. Assays for acid phosphatase were performed on the day blood was sampled; a decline of 20% in serum acid phosphatase activity had previously been observed after storage for one week at —20°C. Deoxyribonucleases I and II were also assayed on fresh serum. Other enzyme assays were performed within 9 days of blood sampling. Serum was stored at —20°C. A separate experiment showed no change in cathepsin activity after one week at —20°C. All other enzymes studied have been previously shown to be stable under conditions of this study.

An incubation temperature of 41°C and a Coleman Universal spectrophotometer were used for all assays except for glutamic-oxalacetic transaminase and lactic dehydrogenase, which were done at room temperature (29-35°C) with a Beckman DU spectrophotometer. Acid phosphatase, alkaline phosphatase, and cathepsin were assayed by the methods of Andersch and Szczypinski(5), Bessey *et al*(6), and Anson(7), respectively. In assays for deoxyribonucleases enzyme activity was computed using a standard curve of known amounts of purified enzyme (Worthington). Deoxyribonuclease II activity was measured essentially by methyl green method of Kurnick and Sandeen(8), and deoxyribonuclease

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