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Quantitative Extraction of Pontamine Blue from Skin; Its Application for Measurement of Increased Capillary Permeability.* (28314)

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The capillary wall is a barrier freely permeable to water, electrolytes and small molecules but only slightly permeable to proteins. The term "increased capillary permeability" therefore refers to an alteration in the capillary wall leading to an accelerated rate of passage of plasma proteins into the extravascular tissues. This phenomenon is one of the cardinal features of acute inflammation. It has been accepted that some vital dyes, such as trypan blue or pontamine sky blue, given intravenously, become bound to plasma proteins, particularly to albumins; therefore their accumulation in inflamed skin sites indicates exudation of plasma proteins. However, evaluation of experimental results in such tests often lacks precision(1), although there have been attempts to estimate quantitatively the amounts of accumulated dye in the inflamed or treated skin sites for instance, in terms of "mean lesion diameter"(2) or by comparing the color intensity with a series of comparator cards(3). The present paper describes a method of quantitative extraction and estimation of pontamine blue in the skin.

Materials and methods. Adult male albino rabbits were used.

(a) Solutions of pontamine blue at graded concentrations in physiological saline were injected intradermally in the depilated skin of the flanks. The volume injected was 0.1 ml. The skin sites were excised 30 minutes after injections, immediately after killing the animals. After removing the fat and muscle, the skin was cut into several pieces with scissors. The skin pieces were immediately placed into 10 volumes of a mixture of absolute ethanol and dioxane (1:1) at about 20 mm Hg of vacuum at room temperature. When bubbling stopped, after 20 to 30 min., the solvent was removed by centrifugation and the skin pieces were placed into an incubator at 37°C. After incubation of 15-20 minutes, the skin pieces became rubbery in consistency, and could be minced into fine pieces with scissors.

The minced skin was placed into 10 volumes of dioxane for one hour at room temperature, with constant stirring. After removal of the dioxane by centrifugation, the skin pieces were placed into 10 volumes of a mixture of chloroform and methanol (2:1) for 30 minutes at room temperature, with constant stirring. At this point ¼ volume of absolute ethyl ether was added and 10 minutes later the supernatant was removed by centrifugation.

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TABLE I. Recovery of Pontamine Blue Given Intradermally.

Dye injected,* μg	Dye recovered,† μg
2.0	1.45 ± .2‡
5.0	4.50 ± .2
10.0	9.00 ± .1
20.0	18.20 ± .8
50.0	44.70 ± 1.2
100.0	89.10 ± 2.5
200.0	187.00 ± 3.5

* Volume injected was 0.1 ml; dye being dissolved in saline.
† Skin removed 30 min after injections.
‡ Mean ± standard deviation.

TABLE II. Extraction of Dye from Kallikrein-Treated Skin Sites.

Concentrations* of kallikrein, unit/ml	Dye extracted,† μg
Saline	0
.005	2.2 ± .1
.01	5.2 ± .3
.05	11.1 ± .3
.1	14.0 ± .4
1.0	35.4 ± 1.6‡

* Volume injeced was 0.1 ml, kallikrein being dissolved in saline.
† Skin removed 30 min after intravenous injection of dye.
‡ Mean ± standard deviation.

To the residual skin, 2.5 ml of 30% aqueous pyridine was added, and heated in a water bath at 80°C for 30 minutes to 1 hour. After centrifugation, the clear but colored supernatant was made up with the aqueous pyridine to 3 ml for spectrophotometric estimation. If necessary, the last procedure with aqueous pyridine can be repeated, until the blue color of the skin completely disappears. The supernatants are pooled for spectrophotometric estimation. The dye was estimated at 620 mμ by means of a Shimazu spectrophotometer (RQ-50). The total amount of dye was computed by means of the standard calibration curve shown in Fig. 1, taking into account the volume of the supernatant containing the dye.

(b). Solutions of kallikrein (Bayer) at graded concentrations in physiological saline were injected intradermally into the depilated skin of the flanks. Immediately after injections, the animals received an intravenous injection of pontamine blue, 60 mg/kg as a 5% solution in 0.425% of saline. The animals were killed 30 minutes later and the dye extracted from the skin following the method just described.

Results. Table I demonstrates that the dye, given intradermally, can be recovered from the skin with a satisfactory yield. Table II indicates the correlation between the amounts of extracted dye and the effect of increasing concentrations of kallikrein, showing that the method is useful for estimating increased vascular permeability in the skin.

Discussion. The first step of the method is to denature tissue proteins to facilitate the subsequent steps; the use of dioxane was suggested by the extraction procedure for leukotaxine(4). The second step removes completely the tissue lipids which interfere with the extraction. The third step is extraction of the dye adsorbed on tissue proteins by means of aqueous pyridine. No other solvent tried seemed able to dissolve and elute the dye from the skin.

The successful application of the method for estimating increased capillary permeability was shown by the use of kallikrein which is one of endogenous permeability factors; its effect is particularly intense in the skin of rabbits(5). The method also allowed accurate measurement of the increased capillary permeability in the cutaneous Arthur reaction in thermal injury and in assessing the effects

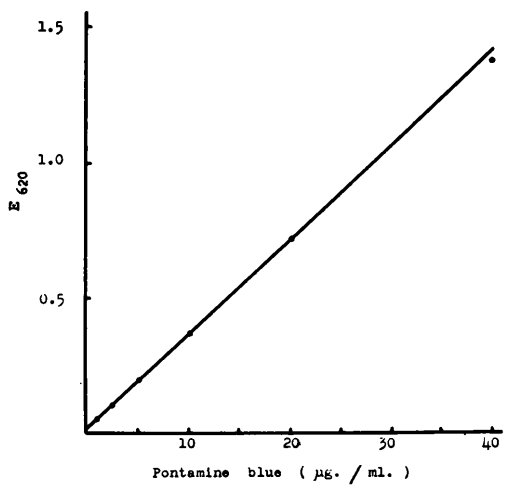

FIG. 1. Calibration curve for estimation of pontamine blue. The dye was dissolved in 30% aqueous pyridine.

of endogenous permeability factors affecting the response(6).

Summary. A method of quantitative extraction of pontamine sky blue from the skin was described, allowing a quantitative measurement of increased capillary permeability to plasma proteins in the skin.

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Determination of Chloride in Serum and Urine by a Modified Mercuric Thiocyanate Method. (28315)

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Determination of chloride ion by reaction with mercuric thiocyanate and complexing liberated thiocyanate with ferric ions has been reported by Iwasaki *et al.*(1). These workers applied this method to determination of chloride in water. Bergmann and Sanik(2) extended the method to determination of chloride in naphthas. The precision and simplicity of the procedure make it attractive for use in clinical chemistry. The present paper extends the use of the method to the determination of chloride in serum and urine specimens.

Methods. Reagents. 1. Mercuric thiocyanate ¾ saturated solution. Shake about 400 mg mercuric thiocyanate with 100 ml ethanol (ethanol denatured with methanol is satisfactory) for about 10 minutes at 25°C. Filter and dilute 90 ml of the filtrate with 30 ml of ethanol. 2. Acetic acid, 0.5 M. 0.3 ml glacial acetic acid diluted to 100 ml with distilled water. 3. Nitric acid, 0.8 M. 5 ml concentrated nitric acid diluted to 100 ml with distilled water. 4. Ferric nitrate. 5 g $Fe,(NO_3)_3 \cdot 9H_2O$ is dissolved in 100 ml of reagent 3. 5. Stock standard, 0.1 M NaCl. Working standard is prepared by diluting 2.0 ml stock to 20 ml with distilled water. *Procedure.* To 0.5 ml serum or urine in a test tube are added, with mixing, 4.0 ml distilled water and 0.5 ml reagent 2. The tube is stoppered and placed in a boiling water bath for 4 - 5 minutes, cooled in tap water, the condensate on the walls mixed with the contents of the tube and the tube then centrifuged. Glass-stoppered tubes or cork stoppers covered with Saran wrap may be used. Blank, standard and unknown are then set up by diluting 1.0-ml aliquots of water, working standard and supernate from above to 10 ml with reagent 3, respectively. After mixing, 1.0-ml aliquots are transferred to suitable test tubes or photometer tubes, 4.0 ml reagent 4 added and mixed, and 1 ml reagent 1 added and mixed. Absorbances are measured against a water blank at 460 mμ or with a filter with nominal wavelength in this region, except that with the Klett photometer the no. 42 filter should be used. Calculation:

$$\text{meq } Cl^-/1 = \frac{A_{Unknown} - A_{Blank}}{A_{Standard} - A_{Blank}} \times 100.$$

Results. Beer's Law. Several instruments were used. Table I shows the absorbance for each instrument tested below which a linear relation between chloride concentration and absorbance was observed. The color obtained showed a maximum at 460 mμ and was stable for at least 5 hours.

Recoveries. Mean recovery for chloride added to 3 urine and 3 serum specimens at a level of 50 meq/l was 103% and 98%, re-