Simple Method for Quantitation of Enhanced Vascular Permeability[1] (34695)

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The wall of certain vessels (venules and capillaries) of the microcirculation represent the blood–tissue barrier. This barrier is freely permeable to water, electrolytes, and small molecules, but only slightly permeable to proteins. The term "increased vascular permeability" refers to an alteration of this barrier, leading to an accelerated rate of passage of plasma proteins into the extravascular tissues: exudation. This phenomenon leads to swelling, which is one of the cardinal features of acute inflammation. Exudation is closely linked to other vascular phenomena, such as hyperemia and stasis. It has been generally accepted that some vital dyes, such as Evans blue, trypan blue or pontamine sky blue, given intravenously, become bound to plasma proteins, particularly to albumin. Therefore, the accumulation of such dyes in inflammatory lesions indicates exudation of plasma proteins. However, evaluation of experimental results in such tests often lacks precision. The present paper describes a simple physicochemical assay for the quantitative measurement of enhanced vascular permeability.

Material and Methods. Adult male albino rabbits, both male and female albino guinea pigs and female Wistar rats were used.

Dye extraction method. Evans blue was injected intravenously in concentrations of 60 mg/kg for rabbits and rats and 20 mg/kg for guinea pigs, respectively. Inflammatory skin lesions were produced by intradermal injections of various inflammatory agents. The skin lesions were punched out with a standard steel punch (1.5–2.5 cm in diameter. To each piece of skin containing the lesion, 4.0 ml of formamide (Fisher Scientific Co. Ltd.) was added and incubated at 45° for 72–96 hr or at 65° for 24–36 hr. If necessary, the incubation time was prolonged, until the blue color of the skin completely disappeared. After filtration with glass filter (Pyrex, coarse; 1.0 cm in diameter), the optical density of the filtrate was measured at 620 mμ in a Zeiss PMQ II spectrophotometer. The total amount of dye can be calculated by means of a standard calibration curve.

Simultaneous radioassay and dye extraction. Evans blue (doses as above) were injected intravenously mixed with ^{125}RISA (radioiodinated human serum albumin; Charles Frosst and Co., Montreal, Canada). The ratio of ^{125}RISA to Evans blue was 1 μCi/mg for studies in rabbits and guinea pigs and ⅓ μg/mg for experiments in rats. The punched out pieces of skin, containing the lesion, were placed in tubes containing 4 ml of formamide. First the radioactivity was measured in a γ-scaler (Model 4204 Nuclear Chicago), calibrated with cesium (44,000 counts ± 500/min) and known amounts of ^{125}RISA. Subsequently, the Evans blue was extracted and the amount of dye was determined as described above.

Experiments to test vascular permeability. As known chemical mediators, synthetic bradykinin (Sandoz, Montreal, Canada), histamine (histamine base, Fisher Scientific Co., Toronto, Canada) and serotonin (serotonin sulfate, Upjohn Co., Kalamazoo, Michi-

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gan) were selected. Each of them was suspended in buffered (pH 7.4) saline at the following concentrations: bradykinin, 10 μg/ml; histamine, 100 μg/ml; and serotonin, 1 μg/ml. They were further diluted if necessary before use. Volumes of 0.1 ml of each sample were injected intradermally with 27-gauge hypodermic needles into the abdominal skin of rabbits and the back of guinea pigs and rats which had received Evans blue with ^{125}RISA intravenously. Unless otherwise stated, 30 min later, the animals were killed with sodium Nembutal (Upjohn Co., Kalamazoo, Michigan) and the extravasated dye and the radioactivity of the punched out skin were determined.

As an experimental model of inflammation, the following two types of inflammation were used. (i) The Arthus reaction: This was elicited in the abdominal skin of BSA-immunized rabbits according to methods previously described (1). Unless otherwise stated, 0.1 ml of antigen (2.5 mg of bovine serum albumin (BSA); Mann Research Lab., New York) was injected intradermally at 48, 24, 12, 6, 4.5, 3, 2, 1 hr, 30, 10, and 5 min before injecting Evans blue and ^{125}RISA. (ii) Thermal injury was induced in the abdominal skin of rabbits at 56° ± 0.25 for 20 sec, by using the burning apparatus of Sevitt (2), slightly modified. Lesions were induced at 6, 5, 4, 2.5, 1 hr and at 20 and 5 min before injecting dye and ^{125}RISA. Thirty min later, the animals were killed and the extravasated dye and radioactivity were measured as described above.

Results. The relationship between extravasated dye and radioactivity of skin lesions. First, the radioactivity of skin lesions of varying intensities was determined. Then following formamide extraction, the total amount of extravasated dye (μg) in a particular skin lesion, was calculated by means of the standard calibration curve. As shown in Fig. 1, a linear response was obtained between about 1000 and 13,000 counts, corresponding to about 0–18 μg of Evans blue.

Recovery of Evans blue given intradermally. 0.1 ml of Evans blue was injected intradermally at various concentrations. The skin

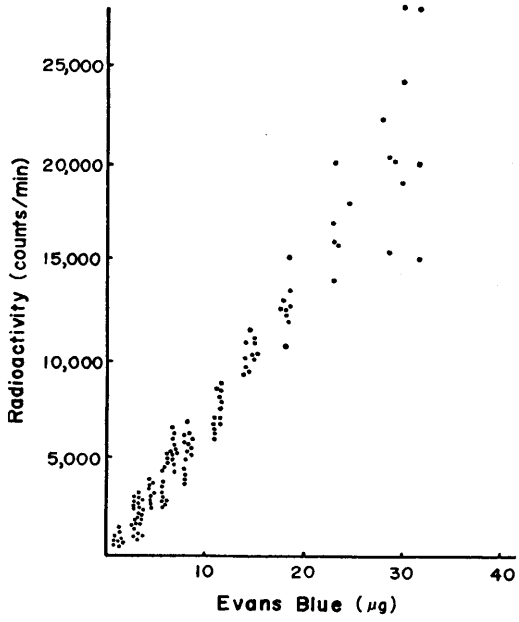

FIG. 1. The dots show radioactivity of skin lesions of varying intensity expressed as counts per minute per lesion. They also show the relationship between radioactivity of the individual skin lesions and the amount of Evans blue extracted from the same lesions.

was removed 30 min after injection. The dye was extracted and measured.

As illustrated in Table I, the recovery of dye was over 95% in all animals tested (rabbits, guinea pigs, and rats). This shows that the dye given interdermally can be recovered from the skin almost completely.

Extraction of dye from skin sites treated with bradykinin, histamine, and serotonin.

TABLE I. Recovery of Evans Blue Given Intradermally.

Dye injected (μg)	Dye recovered (μg)[a]			Yield (av; %)
	Rabbits	Guinea pigs	Rats	
5.0	5.0	4.9	4.8	Over 96
10.0	10.1	9.8	9.8	Over 98
30.0	29.6	29.8	29.3	Over 97
50.0	48.8	49.2	48.1	Over 95
75.0	74.4	74.5	73.6	Over 98
100.0	98.2	97.6	—	Over 97

[a] Mean values of 5 experiments.

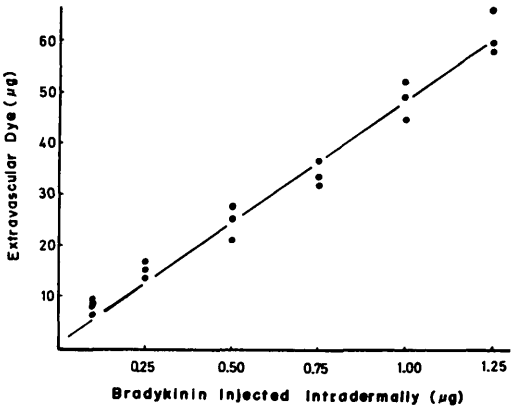

FIG. 2. Dose–response curve of bradykinin: volume injected was 0.1 ml; skin was removed 30 min after intradermal injections; Evans blue and ^{125}RISA were injected intravenously as described in the text.

0.1 ml of synthetic bradykinin was injected intradermally, in graded concentrations, into the abdominal skin of rabbits and the back of guinea pigs which had received Evans blue and ^{125}RISA intravenously. As shown in Fig. 2, the dose–response relationship shows a straight line between concentration of 0.10 and 1.25 μg/ml of bradykinin.

Similar injections, using histamine and serotonin, were given to guinea pigs and rats, respectively. The same relationship between concentrations of 0.3 and 10 μg/ml of histamine and 0.1 and 0.5 μg/ml of serotonin were obtained. These results indicate that this assay is useful for estimating increased vascular permeability in the skin induced with known chemical mediators.

Enhanced vascular permeability in experimental models. The time courses of vascular permeability in cutaneous Arthus reactions and moderate thermal injury in rabbits were tested. As shown in Fig. 3, the general pattern of vascular response appeared to be biphasic. The early response appeared in 5 min, lasted 20–30 min and decreased thereafter in both responses. The late response reached its maximum in 4–5 and 2 hr, respectively, and disappeared in 10–12 and 4–5 hr, respectively. This indicates that the assay allows accurate measurement of increased vascular permeability in cutaneous Arthus reaction and in thermal injury.

Discussion. The earliest attempts to estimate quantitatively the amounts of accumulated dye in inflamed skin sites were based on "mean lesion diameter" (3) or by comparing the color intensity with a series of color standards (4). However, evaluation of experimental results in such tests often lacks precision. Attempts to extract the dye from the skin were cumbersome in most instances. Beach and Steinetz (5) used acid digestion, Judah and Willoughby (6) pounded the skin frozen at —70°, Carr and Wilhelm (7) homo-

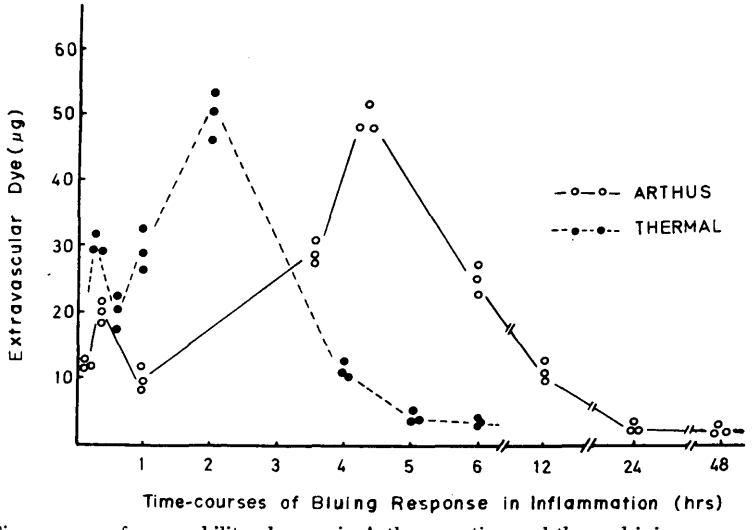

FIG. 3. Time-course of permeability changes in Arthus reaction and thermal injury.

genized the skin mechanically, whereas Nitta *et al.* (8) extracted pontamine sky blue from the skin in two steps: (a) denaturation and dehydration of tissues with dioxane, and (b) elimination of the tissue lipid with organic solvents such as methanol, ethanol, and ether.

In 1962, Frimmer and Müller (9) demonstrated that the extravasated dye could be extracted from the skin with formamide and estimated quantitatively by colorimetry. The extraction being performed at 80° for 24 hr induced a color change ranging from green to dark brown. When extracted under the conditions described in this report, no color changes were observed. Especially at 45°, the incubation time can be prolonged until the blue color of the skin completely disappears without any color changes taking place. Good results were obtained with this method in two studies in which enhanced vascular permeability has been measured (10, 11). In these studies large amounts of animals had to be used because of considerable variation in bluing from animal to animal obtained with intense bluing reactions. As shown in Fig. 1 not much scattering is obtained within a certain range. This means that the material to be tested has to be prepared in such a way as to give a bluing response not exceeding 20 μg of Evans blue or about 13,000 cpm/lesion. If a certain standard (*e.g.*, synthetic bradykinin or histamine) which falls within the linear dose–response is used in each experiment, one can compare it visually with the bluing induced by the unknown permeability factor. If the latter gives too intense a reaction it can be further diluted. The assay with the ^{125}I-labeled serum albumin is simple, sensitive and rapid. It allows quantitation within minutes after completion of the experiment.

In addition to permeability tests with known chemical mediators (Fig. 2) it was shown that this assay is applicable to the time course study of enhanced vascular permeability in cutaneous Arthus reactions and thermal injury (Fig. 3). These results show that this assay permits quantitation of enhanced vascular permeability in studies dealing with certain immune reactions and of inflammatory lesions induced with various chemical mediators and of other phlogistic agents.

Summary. A simple physicochemical assay for the quantitation of enhanced vascular permeability in inflammation was described. It was shown that the assay is applicable to the study of inflammatory lesions induced with known chemical mediators, to the study of enhanced vessel permeability associated with the Arthus reaction, and that associated with thermal injury.

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