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STUDIES ON VASCULAR PERMEABILITY

I. DESCRIPTION AND EVALUATION OF METHODS

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Summary.—Three methods for assessing the extravasation of serum albumin in tissues (skin) and intended to demonstrate increased vascular permeability (IVP) in (immunopathological) inflammation sites have been compared.

The radioassay (extravasated ^{131}I-serum albumin counting) gave the values theoretically expected and was chosen as the reference technique. Two colorimetric techniques based on extravasation of Evans' blue bound *in vivo* to endogenous serum albumin were compared with it at cutaneous sites in which the vascular permeability had been increased by graded histamine reactions and delayed hypersensitivity reactions. The first technique makes use of a densitometer and has been found more precise than the second. A correlation curve was established between extravasated blue serum albumin (densitometric units) and extravasated radiolabelled serum albumin (c.p.m.). The second technique assesses the area of the blue spot, and the concentration of blue (by naked eye comparison with a carefully prepared and calibrated reference skin). It was found to be reliable and reasonably satisfactory, especially for studies including a great number of IVP sites.

THE modifications of vascular permeability in tissue reactions of an immunological nature play an important role in their development and mechanism. The measure or appreciation of these modifications, particularly that of increased vascular permeability (IVP), gives rise to difficult problems. Two main methods have been used by many investigators. Both involve intravenous injection of a substance bound to serum albumin and a search for this substance at the site where IVP is looked for (usually a site of experimental cutaneous inflammation).

The first method consists of injecting radiolabelled serum albumin (^{131}I-HSA for instance) i.v. and counting localized radioactivity at the selected site, with proper corrections from normal symmetric sites and taking into account the blood concentrations (Ascheim, 1965). This method is accurate but it requires radioactive material and equipment and takes some time, especially when dozens or hundreds of spots are to be read.

In the other method, the substance is a dye (Ramsdell, 1928) which binds *in vivo* the blood serum albumin. Those mainly used are trypan blue, pontamin sky-blue (Miles and Miles, 1952) and Evans' blue and similar preparations (Ovary, 1958; Voisin and Toullet, 1960, 1963; Steele and Wilhem, 1966) as well as

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fluorescent dyes which have to be looked for with ultraviolet light (Criefs and Levine, 1949).

The quantitation has been attempted by measuring the mean diameter of the spot of extravasated coloured albumin or its surface. Sometimes the intensity of the blueing seems to be more important than the surface; an attempt has therefore been made to evaluate it by comparison with reference skins in which various quantities of blue have been directly injected (Wilhem and Mason, 1960). A real quantitation of extravasated dye would necessitate the time consuming operation of extracting the blue from the skin and then measuring it (Nitta, Hayashi and Norimatsu, 1963; Toullet and Voisin, 1969). However, one may consider that the quantity of extravasated dye is related both to the diameter of the blue spot and to the colour intensity.

An attempt has been made to simplify the technique by multiplying the surface area of the spot by the intensity of its colour expressed in terms of dye weight per surface unit (Voisin and Maillard, 1967).

This paper describes two colorimetric methods. The first one is based on naked eye comparison with reference spots; the second one makes use of a colorimeter operating in reflected light. These two methods have been compared and also compared (in order to evaluate their accuracy) with the radiolabelled serum albumin technique.

MATERIAL AND METHODS

Animals.—Hartley strain guinea-pigs weighing 250–300 g were used in all experiments.

Purified and radiolabelled albumins.—Radioiodinated human serum albumin (^{131}I-HSA from CNTS Paris) and radioiodinated guinea-pig serum albumin (^{131}I-GPSA) were injected mixed with Evans' blue (National Anilin Division). Guinea-pig serum albumin was isolated from whole normal guinea-pig serum by Sephadex G100 and by preparative agar gel electrophoresis. Iodination was performed following the method of Dixon and Conahey (1966) using chloramine T. The half-life of radioiodinated guinea-pig serum albumin was found to be 3 days in the guinea-pig.

Measurement of permeability effects.—This was attempted in 3 ways: (a) Estimation of blue (bound to serum albumin) by the naked eye. Briefly, the spot area S was estimated with a transparent plastic rule on which circles of various diameters were drawn (this has been found to be both more rapid and more accurate than measuring 2 perpendicular diameters and calculating the area).

The colour intensity I (blue concentration) was estimated by comparison with a reference skin bearing blue spots of known concentration. The amount of blue (μg) present in the unknown spot was obtained by multiplying the area S (mm^2) and the intensity I (μg/mm^2).

The establishment of a reference skin with spots of increasing intensities of known values constituted the essential part of the method (Fig. 1): intracutaneous injections of graded concentrations of Evans' blue (256, 128, 64, 32, 16, 8, 4 and 2 μg in 0·1 ml of guinea-pig serum) were made on the depilated back of a guinea-pig. Thirty minutes later (assuming normal diffusion of blue serum albumin in the tissues), the animal was killed, skinned and the subcutaneous and muscular tissues were cleaned out from the skin. After 3 days, the skin was dry and ready for study.

Each one of the spots of the reference skin was designed with a number that was similar to the calculated concentration of dye:

$$\text{I calcul} = \frac{\text{Quantity of injected dye Qi}}{\text{Area of the spot S}}$$

These values were corrected to obtain mean values for several animal skins.

A first correction factor was obtained by comparing each one of the spots with those of 9 skins treated in the same way as the reference skin.

The colour intensity Ie of blue in each spot was estimated blindly (without previous knowledge of the localization of the spots placed at random on the skin) by 3 different persons and

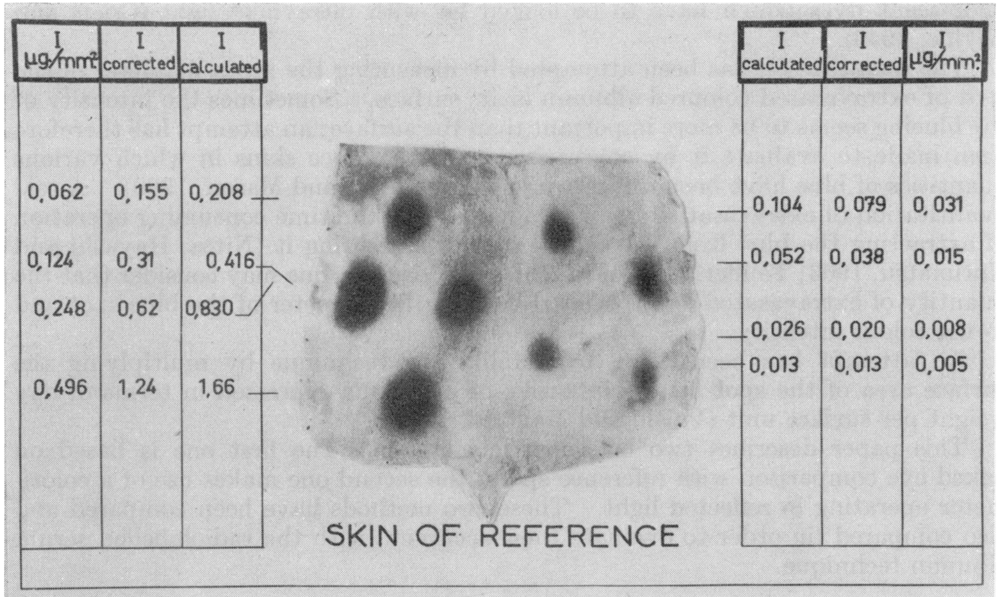

FIG. 1.—Establishment of a reference skin for the appreciation of Evans' blue extravasation. Dry skin from a guinea-pig having previously received i.d. injections of Evans' blue serum dilutions. From above to below, left; 32, 64, 128 and 256 μg: right; 16, 8, 4 and 2 μg of Evans' blue.

$$\text{I calculated} = \frac{\text{quantity of locally injected blue}}{\text{surface of the spot}}$$

$$\text{I corrected} = \text{I calcul.} \times f_1$$

(f_1 is a correction factor taking into account the small difference observed between the reference skin and 10 similarly treated guinea-pig skins; f_1 varies slightly from one spot to another).

1 $\mu g/mm^2 \times$ I correct. $- f_2$ = definitively adopted value for the corresponding reference spot.

(f_2 is a correction factor compensating for the loss of dye due to cleaning the skin of subcutaneous tissues. It has been obtained by comparison with 10 uncleaned skins, otherwise similarly treated to the reference skin and was found to be uniformly very close to $1/2{\cdot}5 = 0{\cdot}4$).

the arithmetical means of these values were calculated. The (slight) differences between calculated and estimated concentrations were compensated by associating a correction factor f1 to the calculated colour intensity of the reference skin.

A second correction factor is necessary since reference and standard skins have been thoroughly cleaned of subcutaneous and soft tissues from the deep dermis, allowing good preservation but occasioning a loss of some dye. For this purpose, 10 guinea-pigs were injected in the same way but their skins were not cleaned of subcutaneous tissues. The colour intensities I′e were appreciated by comparison with the reference skin. We found for each graded spot I′e = 2·5 Ie.

At the conclusion of these operations, each spot of the reference skin was given a definite value I, that was the best estimation of its concentration of Evans' blue.

$$\text{I} = \frac{\text{I correct}}{2{\cdot}5} = \frac{\text{f1} \times \text{I calcul}}{2{\cdot}5}$$

Estimation of blue with densitometer (*Chromoscan, Joyce and Loebl*).—The chromoscan employs an optical electromechanical system in which a recording pen traces a bell-shaped

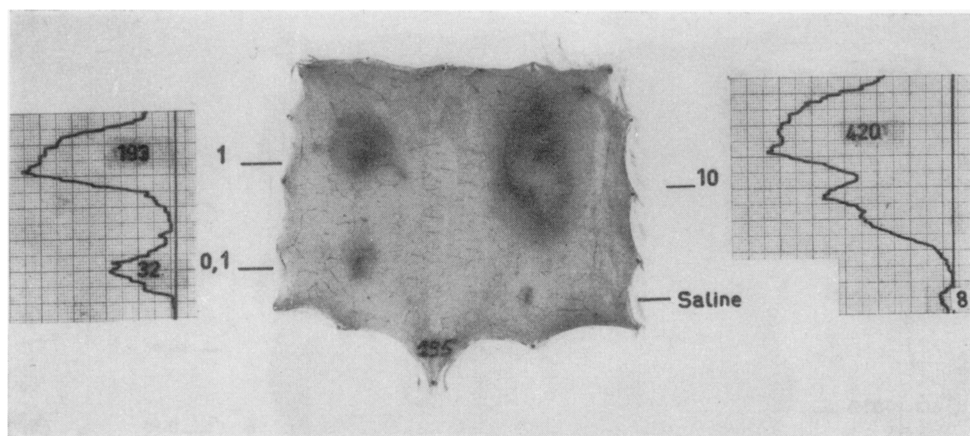

FIG. 2.—Cutaneous reactions to histamine. Measure of extravased blue by the densitometer. To each quantity of injected histamine (0·1, 1 and 10 μg) corresponds a spot of blue and the densitometer gives a curve and a number (densitometer units) proportional to the surface of the associated peak.

curve for each blue spot on a dry skin passing under the experimental beam of light ($\lambda = 592$ μm) compared with a reference curve.

An integrating system gave a number proportional to the area of the curve and function of the intensity of the blue spot. This number was taken to represent the quantity of Evans' blue present in the spot and is expressed in densitometric units (Fig. 2 and 3).

Measurement of radioactivity (bound to serum albumin) with a gamma spectrophotometer.—Skin samples were removed from inflamed and normal skin areas. Their radioactivity was counted in a gamma spectrometer and expressed as counts per minute (ct/min).

The radioactivity of normal areas was subtracted from the radioactivity of inflamed areas to obtain the extravasated radioactivity.

In order to compare the responses of different animals, it was necessary also to take into account the blood levels of the radioactive serum albumin. For this reason, the extravasated radioactivity was divided by the radioactivity of 1 g of venous blood.

Induction of increased vascular permeability in the guinea-pig skin.—Two experimental models of inflammation were used: (a) immediate, intense and transitory (histaminic) reactions: Histamine (0·1 ml) was injected intradermally on the back skin of normal guinea-pigs; and 10 min later intravenous Evans' blue and ^{131}I-serum albumin. Each animal received 4 doses between 0·1 and 10 μg (Fig. 2); (b) delayed, moderate and durable reactions of delayed hypersensitivity: BGG (fraction II, B grade from Calbiochem) was injected intradermally on the back skin of guinea-pigs sensitized 7 or 8 days previously, using the Dienes-Salvin technique (Salvin, 1958) to obtain a rather pure delayed hypersensitivity reaction.

Each animal received 4 test doses between 3·16 and 100 μg 23 hours before the injection of intravenous Evans' blue and ^{131}I serum albumin (Fig. 3).

Experimental procedures to compare colorimetric with radiolabelled assays.—Two experimental models were used: (a) direct intracutaneous injections of blue and radiolabelled serum albumin. A solution of Evans' blue and ^{131}I-SAH was prepared in HSA (Evans blue, 50 mg/0·1 ml and radioactivity of 0·04 μCi per 0·1 ml). Five double dilutions were made in HSA; 0·1 ml of each solution was injected intradermally in the skin of 20 guinea-pigs. The animals were killed 30 min later. The skins of 10 of them were cleaned of subcutaneous and muscular tissues and allowed to dry. The 10 others were dried without cleaning. The amount of blue present in the coloured spots was measured by means of the 2 colorimetric techniques. The radioactivity was counted afterwards at the same spots. (b) Extravasation of similar preparations injected intravenously, at cutaneous sites with induced inflammation. An IVP was induced in the skin either by the injection of histamine into 26 normal guinea-pigs or by the injection of antigen (GGB) into 8 sensitized guinea-pigs. A solution of blue and ^{131}I-SAH or ^{131}I-SAC (0·24 ml of Evans' blue at 0·5% and 2μCi per 100 g weight) was

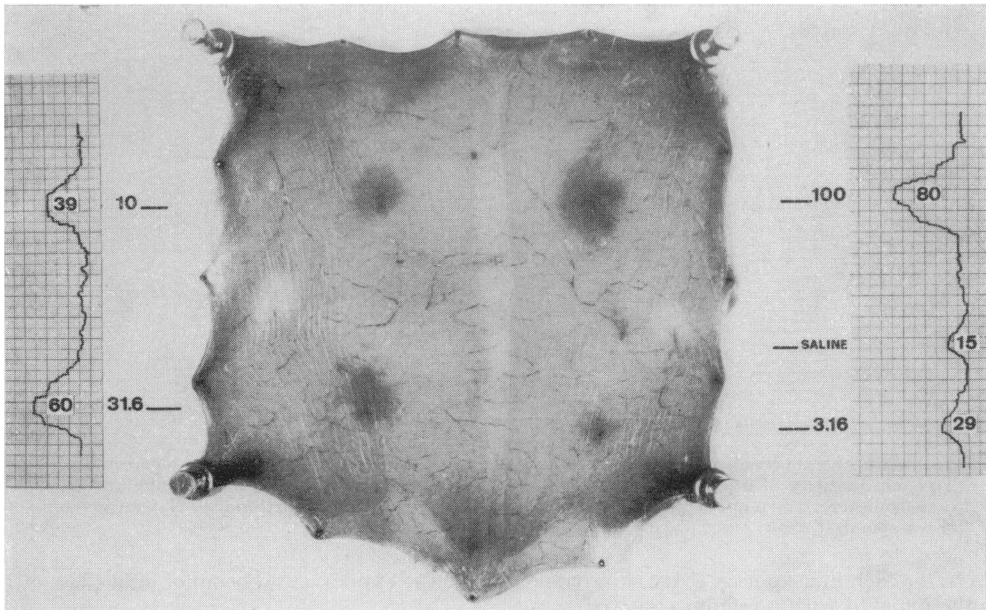

FIG. 3.—Cutaneous reactions of delayed hypersensitivity to BGG. To each quantity of injected BGG (3, 16, 10, 31, 6 and 100 μg) corresponds a spot of blue and the densitometer gives a curve and a number (densitometer units) proportional to the surface of the associated peak.

injected intravenously 10 min before the histamine injections or 23 hours after the antigen injections.

The animals were bled 30 minutes and 2 hours after the i.v. injections in case of histamine induced IVP and in case of delayed-hypersensitivity-reaction-induced IVP respectively. Blood samples and skins were kept for radioactivity counting and blue assessment.

RESULTS

Evaluation of the 2 colorimetric methods compared with radioassay in cutaneous sites where blue HSA and ^{131}I-HSA were directly injected

First, the blue of colour spots of varying intensities was assessed by naked eye and with a densitometer. Then the skin was cut around each spot for radioactivity counting.

As shown in Fig. 4, a linear curve was obtained by radioassay and by estimating blue by naked eye. With the densitometer a regular curve was obtained, but not linear. The radioactivity curve corresponded with the theoretical curve. This justified the choice of radioassay as a reference technique to evaluate the 2 colorimetric methods.

The results obtained by naked eye were somewhat high. This over-estimation was uniformly of the order of about $\times 1{\cdot}17$; using the reference skin, it might therefore be corrected by a correction factor $f3 = 0{\cdot}85$. However, since this over-estimation was probably due to a lightening of the particular reference skin blue utilized, it was not integrated in the calculations. Otherwise, the amounts assessed in the cleaned skins were 2·5 times weaker than those for the uncleaned skins. This was as valid with the first colorimetric method as with the isotopic

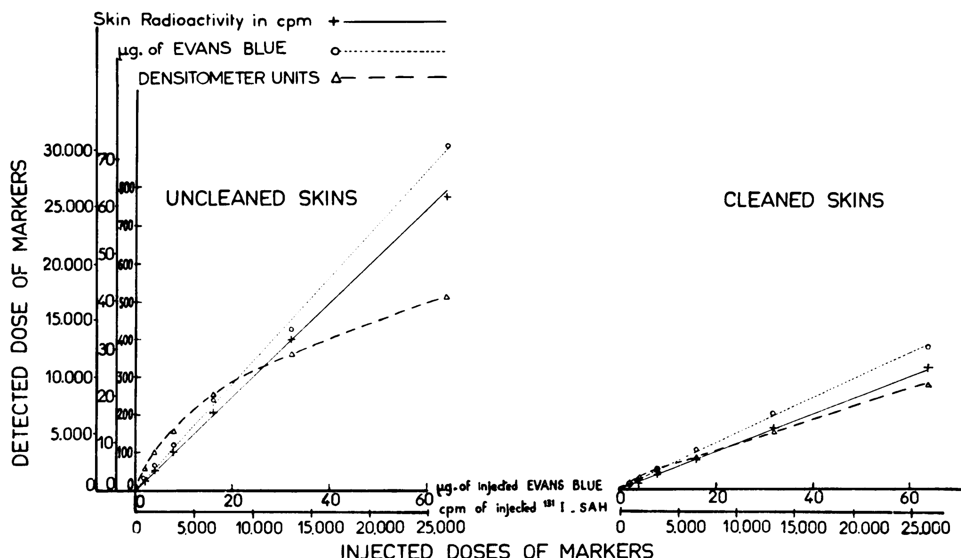

Fig. 4.—Comparative appreciation of serum albumin present at sites of direct cutaneous injection by 3 different techniques. Serum albumin was labelled with both Evans' blue and ^{131}I. The quantities of blue and ^{131}I-HSA injected on the back of 20 guinea-pigs are respectively: Blue: 64, 32, 16, 8, 4 and 2 μg; ^{131}I: 26700, 13350, 6675, 3338, 1669 and 834 ct/min. The skins of 10 guinea-pigs were cleaned of subcutaneous tissues; the skins of 10 others were not.

methods. These results justify the second correction factor for the reference skin.

The results obtained with the densitometer were objective but not linear. This could be prevented by the establishment of a correlation curve between extravasated densitometric blue and radioactivity in the skin lesions.

Application and comparison of colorimetric and isotopic methods in cutaneous sites with IVP

The albumin extravasation was measured by means of the 3 techniques in the sites treated with intradermal injections of histamine in normal animals or BGG antigen in sensitized animals.

The isotopic assay was taken as the reference method to correlate the 2 colorimetric methods.

As illustrated in Fig. 5, the first blue dye technique (naked eye) gave a good appreciation of the studied phenomenon: the extravasation curves of blue albumin were approximately similar to the extravasation of radiolabelled albumin; the histaminic reaction led to a much more important extravasation of blue albumin than did the delayed hypersensitivity reaction.

On the other hand, the second blue dye technique (densitometer) underestimated the extravasation in very intense inflammatory reactions and overestimated it in moderate inflammatory reactions. However, a good correlation curve (Fig. 6) could be obtained between extravasated blue (densitometric units) and the rate of radiolabelled albumin. (It must be noted that the mean results of different experiments are indistinct with the correlation curve.)

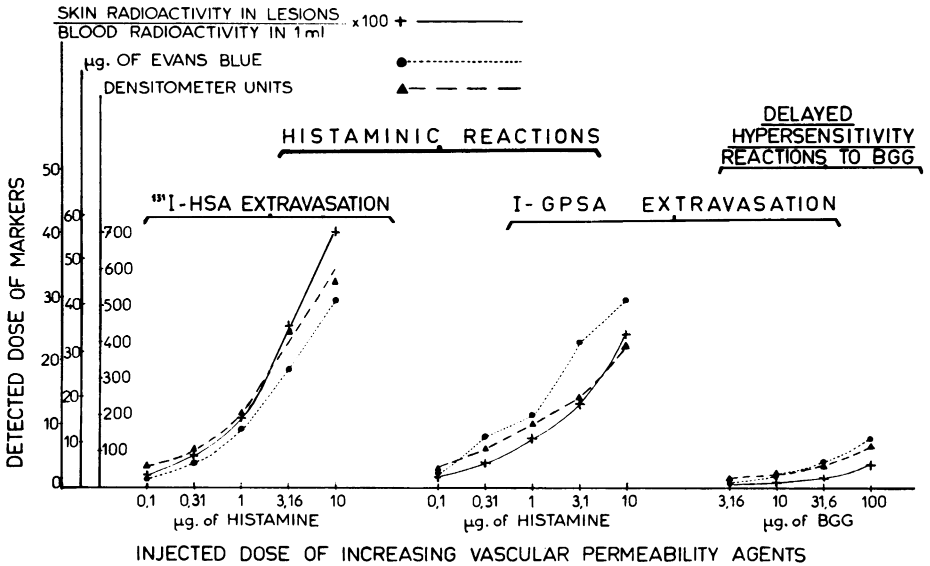

FIG. 5.—Comparative assessment of serum albumin extravasation by 3 different techniques according to the type of inflammation. The increased vascular permeability was induced either by intradermal injection of histamine in normal guinea-pigs, or by injection of GGB antigen in sensitized guinea-pigs. The radiolabelled albumin injected intravenously with Evans' blue was either ^{131}I-HSA or ^{131}I-GPSA.

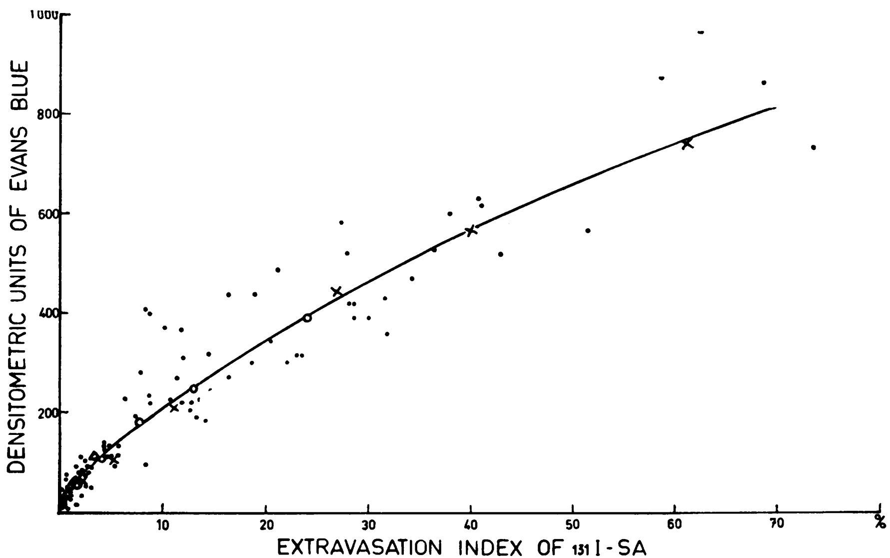

FIG. 6.—Correlation between densitometry and radioactivity counting of extravasated serum albumin. Each dot represents an individual value. The mean values obtained in the 3 experiments described in Fig. 5 are represented respectively by +, ○ and △.

DISCUSSION

The isotopic method, with no difference observed between injected and counted radiolabelled albumin in the first protocol, was the best method to appreciate extravasated albumin in inflamed skin or to measure an IVP, in spite of some disadvantages: (1) it necessitated the use of radioisotopes; (2) it necessitated radiolabelled purified proteins which could be denatured or modified by manipulation; (3) it was time consuming. So this method was not satisfactory, especially when many measurements have to be made.

For these reasons, we have compared with it 2 easy colorimetric methods in which the autologous albumin was labelled *in vivo* by Evans' blue without any risk of denaturation and with minimum preparation time. Since simplicity and rapidity were sought, no measurement was made of the concentration of circulating blue albumin and only the blue accumulated in the skin was taken into consideration. However, previous studies (Voisin and Toullet, 1963) have shown that no very important variations take place in healthy animals and the agreement here established with the radioiodinated albumin extravasation justifies this simplification.

The first naked eye method was not objective since it depended on the observer and on the time interval between experiments, but it appeared to be reasonably reliable and it was by far the fastest method and very useful in studies, including a great number of IVP sites. The main requirement was to have a carefully prepared and calibrated reference skin.

The second (densitometric) method appeared to be the best method, objective and reproducible, for accurate studies on IVP. However, 2 critical remarks must be made: (1) the correlation curve blue-albumin/radiolabelled albumin was not strictly established according to mathematical criteria; (2) the experimental beam of light used in the densitometer was not strictly monochromatic, so that one cannot prevent the possibility of over-estimating the results by the presence of haemorrhagic phenomena in the inflamed sites studied.

In spite of these remarks, the densitometric method was more objective than the naked eye methods; it was also easier and faster than the isotopic method and the method extracting blue.

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