J. Physiol. (1952) 118, 228–257

VASCULAR REACTIONS TO HISTAMINE, HISTAMINE-LIBERATOR AND LEUKOTAXINE IN THE SKIN OF GUINEA-PIGS

By A. A. MILES and E. M. MILES

From the National Institute for Medical Research, Mill Hill, London

(*Received* 3 *April* 1952)

A substantial increase in capillary permeability is a feature of acute inflammation in bacterial infections. The present investigation is part of an attempt to prove an old hypothesis, namely, that this increase in permeability is mediated by histamine. A comparative study was made of histamine, of the histamine-liberator 48/80, a condensation product of *p*-methoxyphenylethylmethylamine and formaldehyde (Baltzly, Buck, de Beer & Webb, 1949), and of leukotaxine (Menkin, 1936, 1938*a*, *b*).

In animals with a recently injected vital dye in their blood, the intradermal injection of substances that increase permeability of the blood vessels is followed by an accumulation of dye at the site of injection, presumably due to the passage of an excess of dye-stained plasma into the tissue spaces. When the blood flow and the vascular bed of the skin are relatively constant, differences in the size and intensity of stained areas of skin reflect differences in vascular permeability, and may be used to investigate the properties of substances that increase permeability in this way. Our work was confined to the skin of guinea-pigs, partly because much is already known about skin reactions to toxins and other inflammatory agents, and partly because it is a tissue readily studied in the intact and unanaesthetized animal—an important consideration with phenomena which, like the passage of dye through vascular endothelium, are peculiarly dependent on the state of the blood vessels in the tissue under test.

MATERIALS AND METHODS

Albino guinea-pigs, 300–450 g in weight, were used throughout. The skin of the trunk was depilated, after clipping away the hair, by a paste consisting of wheat flour, 350 g; talcum powder, 350 g; barium sulphide, 250 g; Castile soap powder, 50 g; and water. The depilated area was thoroughly washed with warm water.

Detection of increased permeability. Neither the intradermal injection nor the pricking-in of histamine or 48/80 produce in the depilated skin any measurable reaction indicating change in

vascular permeability; and there is no trace of the wheal that characterizes the reaction of human skin to histamine. In the skin of animals with circulating dye, however, both substances induce remarkably constant effects. A 5% solution of pontamine sky blue 6X ('pontamine blue') was used, given intravenously in the leg in doses of 65–75 mg/kg body weight. Animals so injected are referred to below as 'blued'. A few minutes after the injection of the dye the sites of wounds, of too-long application of depilating paste, and of recent careless or even firm manipulation of the skin, become blue. Though traumatic blueing of this kind commonly results from depilation, with nice judgement it is possible to depilate cleanly without damage to the skin. Owing to the sensitivity to trauma of blued animals, injections cannot be made into the skin pinched into a fold between thumb and finger; the skin must be steadied by gentle stretching over the underlying tissue.

In the absence of further interference, the skin of a blued animal becomes generally and maximally stained in 10 hr or more; but up to 6 hr after the intravenous dye, the intradermal injection of a substance that increases permeability results in a local increase in the intensity of blueing. The best contrasts were obtained within 1 hr of giving the dye. Unless otherwise stated, all our injections were given into the skin of the trunk posterior to the shoulder blade and anterior to the knee joint in the sitting animal, and omitting the thin skin about 30–40 mm on each side of the ventral mid-line; all solutions for injection were made up in 0·85% saline, which by itself induces no blueing. The volume injected was usually 0·1 ml., which initially raises a bleb 9–11 mm in diameter. With short-bevel no. 26 gauge needles, a small area of traumatic blueing 1–3 mm in diameter develops at the centre of the bleb.

Skin reactions in the ears of blued animals were induced either by free-hand intradermal injections with a no. 28 needle, or by injections with a mechanically manipulated glass micro-needle into animals under light bromethol (avertin) anaesthesia. The micro-injections were either intradermal or made directly into the lymphatic plexus of the ear, which is readily entered via one of the numerous anastomosing lymphatic channels at the margin of the ear. The ears were transilluminated and observed under $\times 20$ and $\times 40$ magnification.

Materials. The acid phosphate of histamine was used; amounts are cited as the weight of the base. The specimen of 48/80 (Wellcome Research Laboratories, U.S.A.) was probably in the form of the dimer, trimer and tetramer (Paton, 1951), with an average molecular weight of about 540 The leukotaxine was a single batch prepared by Dr J. H. Humphrey, by a combination of the methods of Cullumbine & Rydon (1946) and Spector (1951), and corresponded to the fractions described by Spector as active in inducing capillary permeability, containing eight to fourteen amino-acid residues. On this basis its molecular weight is of the order of 1500.

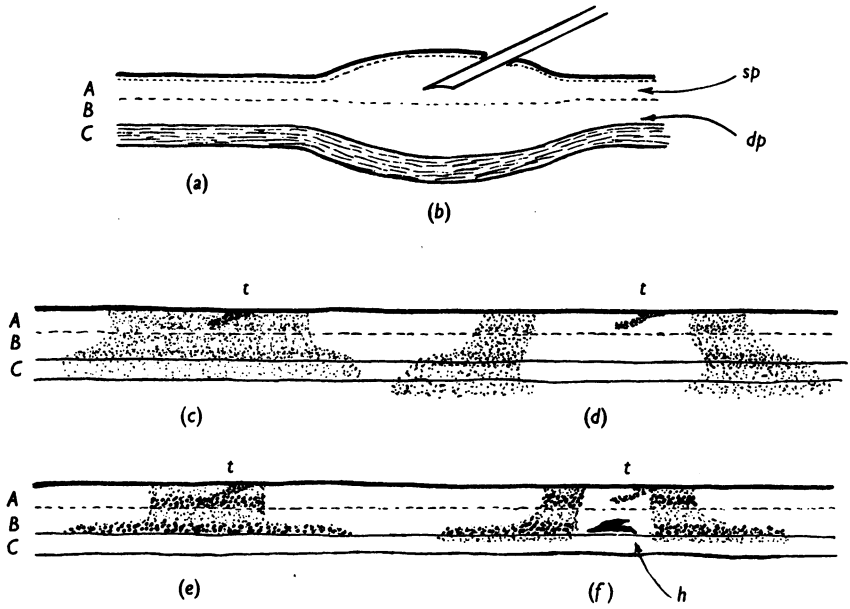
RESULTS

The mechanics of intradermal injection

The behaviour of injected drugs is determined in part by the mechanics of intradermal injection. The skin of the trunk moves loosely over the underlying abdominal and thoracic muscles. For our purpose this movable skin may be described as three layers of about equal thickness (Text-fig. 1*a*). (*A*) Epidermis and dermis which together are about 1·0 mm thick, appearing on cross-section as a dense whitish layer, whiter in the deeper part; the dermis contains an extensive plexus of blood vessels (*sp*) round the main bodies of the glands and hair follicles, and a fine plexus of lymphatic channels, detectable when indian ink is injected into this region by a micro-needle. (*B*) A looser connective tissue about 1·0 mm thick, appearing on cross-section as a grey gelatinous layer; at its base, immediately above *C*, the panniculus carnosus, is a plexus

of blood vessels (*dp*) and a coarse scanty plexus of lymphatic channels, each joined to the corresponding upper plexus in *A* by relatively few vessels. (*C*) The panniculus carnosus, a muscle layer about 1·0 mm thick.

In making an intradermal injection, the depth of the needle-tip to some extent determines the depth at which the bulk of the injected fluid will spread, but not as completely as is generally supposed. The fluid spreads outwards, upwards and downwards to form a lenticular mass of wet tissue (Text-fig. 1*b*). When the needle tip is as low as the deepest part of *B*, the swelling of the skin

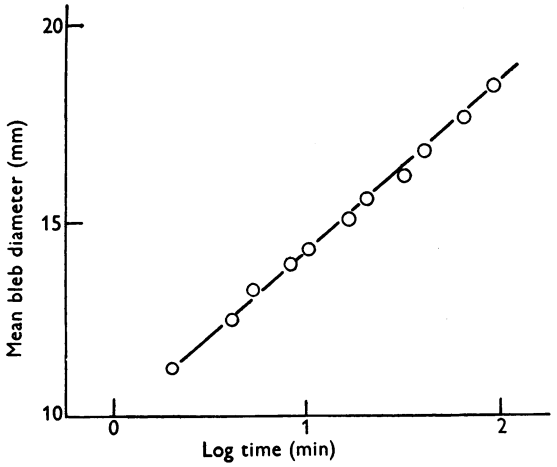


Text-fig. 1. Schematic sections of guinea-pig skin. (*a*) normal; (*b*) intradermal injection bleb; (*c*) blueing with low dose of histamine, showing traumatic blueing *t*, due to needle; (*d*) blueing with high dose of histamine showing central inhibition; (*e*) blueing with low dose of 48/80; (*f*) blueing with high dose of 48/80, showing central inhibition, and haemorrhage, *h*. *sp*, *dp* = superficial and deep plexus of blood vessels. For definition of layers *A*, *B* and *C*, see p. 229.

is due mainly to the distension of that layer, but when it lies in the middle of *B*, or in the dermis, both *B* and the dermis are equally permeated by the injection fluid. The initial diameter of the bleb is a simple function of volume injected; being linearly related to log. volume (Text-fig. 3*a–d*).

The initially domed injection-bleb of saline is scarcely visible after 3–4 hr. Some of the fluid is doubtless taken into the blood stream and some into the lymphatic channels; though, judging by the results of intradermal injection of dyes and indian ink, only a very small proportion of the injected substances escapes by the lymphatic channels during the first 2 hr. Other forces must be at work to account for the gradual disappearance of the bleb, which both decreases in thickness and spreads outwards. The diameter of a 0·1 ml. bleb,

measured under illumination by a very oblique beam of light, grows linearly with respect to log. time (Text-fig. 2); and on the average, blebs starting at 10·5 mm are 19·5 mm in diameter after 2 hr and 20·5 mm after 4 hr. The rate of spread is much the same in freshly killed animals. The decrease in thickness is partly due to seepage through the muscle layer into the subcutaneous tissue, which becomes noticeably wet; and partly to the outward spread of the fluid. Of the forces responsible for the outward spread, diffusion is unlikely to play any large part, because the intradermal invasion of diffusible substances applied to the cut edge fo normal skin is small and slow (e.g. the enzyme hyaluronidase, Hechter, 1946). Spread probably takes place by mass movement of the fluid either by reason of the hydrostatic pressures engendered in the tissue during injection or because the tissue has an affinity for water, which moves therefore from the hydrated bleb to the surrounding less hydrated tissue.



Text-fig. 2. Growth of an intradermal bleb in the guinea-pig formed by 0·1 ml. saline. Each point the mean of three blebs.

Large syringe pressures are required to initiate an intradermal bleb. In a series of measurements on ten guinea-pigs, this pressure varied between 80 and 140 cm Hg; once the bleb was begun, it increased rapidly in size under syringe pressures of 60–100 cm Hg. Only 1–2 cm Hg were required to expel fluid rapidly from the unimpeded syringe needle. The tissues do not, however, store the energy to a degree represented by these pressures, because when the bleb is cut vertically across its middle, fluid oozes only very slowly from the cut surface. Indeed, the pressure within the bleb soon after it is made cannot be higher than that in the small vessels involved, because exudation from the vessels takes place in blebs only 3 min old (see p. 236). At this time the maximum pressure must therefore be less than that in the largest vessels from which exudation takes place; and probably does not exceed 1–2 cm Hg. That the occlusion of the vessels during injection is only short-lived can be seen when

the formation of an intradermal bleb is watched under low-power magnification in a transilluminated hyperaemic ear. The hyperaemia is apparently restored after a few seconds. In arteries 0·4–0·8 mm in diameter, the flow returns in 5–10 sec; and in veins 0·5–2 mm in diameter flow returns in 30–120 sec, and initial diameter is restored in under 3 min. The high injection pressures are apparently needed only to tear apart the tissues at the advancing edge of the bleb.

Fluid may be expressed more rapidly by gently pinching the cut edge of a bleb between finger and thumb. A great deal of the fluid therefore cannot be held by hydration of the tissues. It is presumably held in innumerable small, distended, interconnecting loculi in the connective tissues, and is forced outwards to the periphery of the bleb by contraction of the distended tissue fibres. Hydration, however, may well account for the retention of some of the fluid in a cut bleb, as the following experiment shows. The average water content of pieces of muscle-free, intact skin from the trunk was about 50%; i.e. one part of dry matter holds about one part of water. Pieces of fresh guinea-pig skin were cut into 10 μ slices on a freezing microtome, washed in saline to remove damaged cells and the contents of the cells cut open during section, and the washed slices allowed to imbibe water from 0·85% saline for 30 min at 32° C. They were then deposited as a hard cake by centrifugation, and excess fluid removed from the deposit by firm pressing between sheets of filter-paper. The average water content of these masses was about 70%, i.e. one part of dry matter can hold $70/30 = 2{\cdot}3$ parts water. A 0·1 ml. bleb occupies about 160 mg of intact skin, which, if it attained the same degree of hydration as the sliced skin, could hold $80 \times 2{\cdot}3 = 184$ mg water; i.e. not only the 80 mg of natural water, but the injected 100 mg as well.

The dosage-response to intradermally injected substances

The distribution in the skin of an intradermally injected substance will depend on its concentration and the rate at which it is adsorbed or 'fixed' by the tissues during the outward flow of injection fluid from the needle. A substance that was adsorbed very little would spread with the injection fluid, and, according to the evidence in Text-fig. 2, would eventually produce lesions whose diameter was directly proportional to the diameter of the injection bleb; i.e. to the volume of fluid injected. Most substances, however, are adsorbed in some degree, and accumulate at and around the centre of the bleb. The relation of the bleb-diameter to lesion-diameter with different drugs can be used to characterize their adsorption to the tissues. It was explored for histamine, 48/80 and leukotaxine by two methods of injection in blued animals: (*a*) graded concentrations in a constant injection-volume, and (*b*) graded injection-volumes containing a constant dose.

'*Constant-volume*' *measurements.* Four doses of the substance under test in

0·1 ml., were randomized in a 4 × 4 Latin square on the trunk, two rows on each side of the spinal mid-line. Histamine, 48/80 and leukotaxine all produced round blue lesions in the skin and in each case the lesion-diameters measured 30 min after injection were linearly related to the log. dose (cf. Text-fig. 5*a*, *b*). The responses were subjected to analysis of variance, with the results exemplified in Table 1, which records a titration of histamine in three blued animals. The departure from linearity of the regression line of lesion-diameter on log. dose is insignificant. The variation between columns and rows is also insignificant, so that for practical purposes the skin of the more dorsal

TABLE 1. Analysis of variance of a titration of histamine in a 4-fold Latin square in three blued guinea-pigs

	Degrees of freedom	Sum of squares	Mean square	Variance ratio	*P*
Between animals	2	3·2279	1·61395	1·66	>0·05
Between columns	3	3·1275	1·0425	1·07	>0·05
Between rows	3	2·0174	0·6725	—	—
Between doses	3	312·7425	104·2475	107·46	<0·001
Linearity	1	72·8017	72·8017	75·05	<0·001
Curvature	1	3·2939	3·2939	3·40	>0·05
Response	1	235·8784	235·8784	243·15	<0·001
Error	36	34·9239	0·9701	—	—
Total	47	356·0392	—	—	—

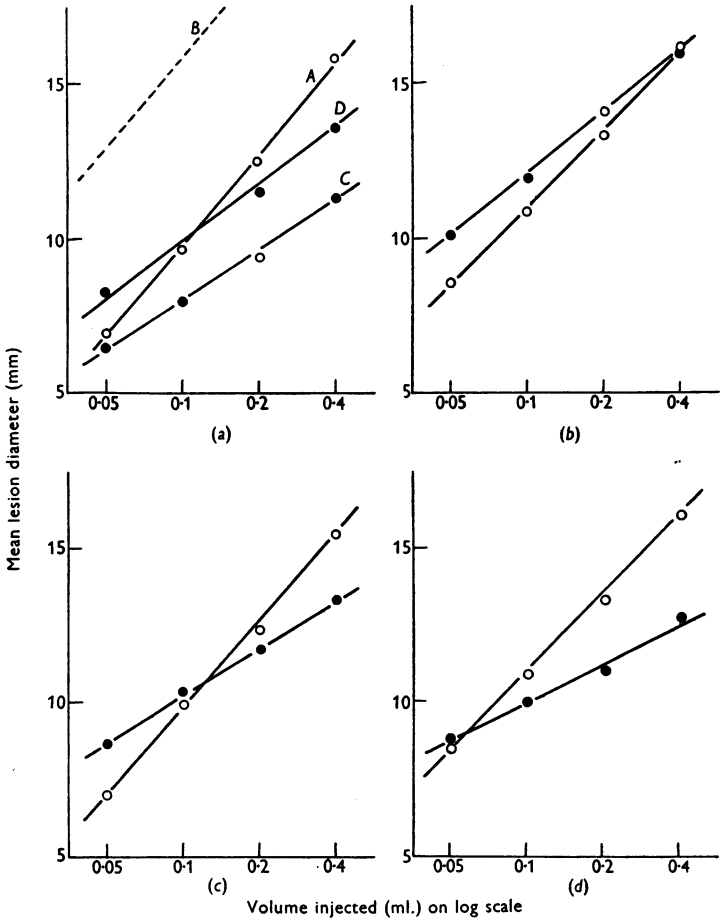
part of the guinea-pig's trunk may be considered homogeneous in its sensitivity to histamine. (This is not true of the thinner ventral skin, where lesions tend to be larger.) Each animal yields a mean of four responses per dose. The error term for inter-animal variation is large, but reliable comparisons between various treatments were obtained in other tests by using four to six animals per group. This linear relationship differs from that found by Bain (1949) in the human skin, where *area* of histamine whealing was proportional to log. dose. Linearity of diameter against log. dose holds for many substances in guinea-pig skin: diphtheria toxin (Miles, 1949), tuberculin in tuberculous animals (Wadley, 1949) and appears to hold for other bacterial antigens in hypersensitive animals, and for the lesions produced in the blued animals by various toxins such as the exotoxins of *Clostridium welchii*, *Cl. oedematiens* and *Staphylococcus aureus*, and for cobra venom (unpublished work). The difference in Bain's titration may lie in the modification of histamine spread or of wheal-diameter, by the copious exudation during whealing that occurs in man but not in the guinea-pig.

Full 4 × 4 Latin-square titrations were usually made only when the significance of a result was in doubt; in most of the tests fewer replications of doses were used, partially randomized among three to six animals per group. In these titrations, 48/80 and leukotaxine differed from histamine mainly in the slope of the dosage-response lines. Slopes varied with each experiment, and particularly with the amount of dye injected. Moreover, since the concentration of circulating dye falls rapidly during the first 2 hr, slope decreases with lapse of

time between blueing the animal and the intradermal titration. In guinea-pigs receiving 65 mg/kg tested soon after its injection, the slopes for histamine lay between 2·8 and 3·5, for 48/80 between 1·5 and 2·0 and for leukotaxine between 1·5 and 4·0. The shallow slope of 48/80, which indicates an increase of only 1·5–2·0 mm in lesion-diameter for a 10-fold increase in concentration, suggests that, compared with histamine, it is strongly adsorbed to the tissues. It should be noted that the susceptibility of this linear dosage-response to statistical analysis makes possible an accurate though not very precise measure of the potency of each of the three substances. The method is too insensitive for routine assay, but was well adapted to our investigations of skin reactions. Thus where parallel regression lines can be fitted to two sets of log. dose-diameter responses, the horizontal distance between the two lines is the log. ratio of drug potency; and, for a given specimen of a drug, it is the log. inverse ratio of sensitivity to the drug. For example, in Text-fig. 5*b*, 3 mg neoantergan has shifted the slope to the right by 0·43 = antilog. 2·7. That is, the neoantergan has decreased the 48/80-sensitivity of the animals 2·7-fold, since 2·7 × the dose in normal animals is required to produce the same effect in the treated animal.

'*Constant-amount*' *measurements.* In this method a fixed amount of the drug was injected in volumes of 0·05, 0·1, 0·2 and 0·4 ml.; i.e. the concentration of the drug is varied. The diameter of the blebs, measured immediately after the injections, was linear with respect to log. volume (Text-fig. 3*a*, *A*); and, according to the data in Text-fig. 2, after a given period (e.g. 30 min, Text-fig. 3*a*, *B*) the expanded bleb-diameters would be linear, on a line parallel to that for the immediate bleb-diameters (Text-fig. 3*a*, *B*). The slope of the initial bleb-diameters, irrespective of any lesion produced by the drug injected, is relatively constant in the region of 9·0. When the drug has acted the resulting lesion-diameters are also linear with respect to log. injection volume. Text-fig. 3*a*, *C* and *D*, records results with 12·5 and 50 μg pontamine blue in unblued animals. The 'lesion' here is the area of skin coloured by the injected dye. The slope of lesion-diameter is not parallel to that for initial bleb-diameter, which would have indicated no adsorption during injection. Nor is it horizontal, which would have indicated an adsorption so strong that it was independent of concentration within the range tested. The slope of *C* and *D* is in fact 5·5, compared with 9·7 for the initial bleb-diameter. Pontamine blue has a good affinity for skin tissue (Evans, Miles & Niven, 1948), and we would therefore expect slopes like *C* and *D*, which show that as the solutions are forced outwards during injection, adsorption decreases with decreasing concentration of the injection fluid. The magnitude of the slope, compared with that for the initial bleb, is a reasonable inverse indicator of the affinity of the tissues for the drug. Text-fig. 3*b–d*, and Table 2 summarize similar titrations on histamine, 48/80 and leukotaxine; the slopes indicate that the tissue affinity is least for histamine, and greatest for leukotaxine.

The relative position of the two slopes is also informative. In Text-fig. 3*a*, the 50 μg line *D* crosses *A* at about 10 mm, i.e. the skin occupied by the initial bleb from 0·1 ml. just adsorbs the dye. The 50 μg of dye is clearly insufficient



Text-fig. 3. Constant-dose intradermal titrations. Lesion-diameters plotted against log. volume injected. Each point the mean of twelve to sixteen lesions. In all the graphs, ○ — ○, mean diameter of the initial blebs raised by the *fluid* injected; ● — ●, diameter of lesion produced by the *substances* injected. (*a*) pontamine blue: *A*, initial bleb-diameter; *B*, bleb-diameter after 30 min calculated from data in Text-fig. 2. *C* and *D*, lesion-diameters of 12·5 and 50 μg dye. (*b*) Histamine, 8 μg; (*c*) 48/80, 13 μg; and (*d*) leukotaxine, 40 μg, in blued animals (see pp. 234–6 and Table 2).

to fill the 0·2 ml. bleb. It oversaturates the 0·05 ml. bleb so that as the fluid spreads outwards from the region initially filled by the injection, the excess of dye is carried some way with it; at 30 min, when the reading was taken, the dye has spread over 8 mm and the fluid (line *B*) over 12·5 mm. The intersect of initial bleb-diameters and lesion-diameters is thus a convenient measure of the

adsorbing power of the skin for a given dose of drug, though clearly it can give no information about the concentration gradient of the drug within the bleb. It is the volume in which the adsorption of the drug is so balanced that the minimal effective concentration is produced at the edge of the initial bleb; and may be called the critical volume. In Text-fig. 3*a*, the critical volume for 50 μg of pontamine blue is 0·105 ml.

TABLE 2. Slopes and critical volumes in 'constant-amount' titrations of histamine, 48/80 and leukotaxine

Substance	Approx. equimolar dose (μg)	Slope mean diameter		Critical volume (ml.)
		Initial bleb	Lesion	
Histamine	8	8·6	6·6	0·400
48/80	13	9·3	5·2	0·012
Leukotaxine	40	8·6	4·1	0·058
(Pontamine blue	—	9·8	6·2	0·105)

For a valid comparison, the critical volumes of equimolar concentrations of histamine, 48/80 and leukotaxine were determined from the data in Text-fig. 3*c–e* (Table 2). 8 μg of histamine was tested, and the approximate molecular equivalent, 13 μg, of 48/80. The result for an equimolar amount of leukotaxine (40 μg) was extrapolated from the slope for 20 μg using the value 4·0 for the slope of a constant-volume titration. The values both for slope and critical volume must be regarded as very approximate. Histamine (Table 2) has a high 'constant-amount' slope, and a high critical volume; it is thus relatively poorly adsorbed during injection, and the skin is relatively poor in adsorbing sites. Both 48/80 and leukotaxine are more strongly adsorbed, and the skin is richer in adsorbing sites.

Skin reactions to histamine

Histamine even as strong as 1·6% will not induce blueing in dyed animals when applied to undamaged skin. When the skin of blued animals is damaged by rubbing (cf. Matolsty & Matolsty, 1951) or scratching sufficient to produce patches of traumatic blueing, the application of 1% histamine will increase the size and intensity of such blueing, presumably because the drug, having penetrated the damaged skin, diffuses inwards from the damaged area. It is also possible to induce blueing by the electrophoresis of 1% histamine; the histamine is not however driven in uniformly, but produces irregular patches of blueing. In none of these tests was whealing ever observed.

Intradermal injection in the trunk. Histamine injected intradermally into blued animals produces a round area of blueing that appears in 3–5 min and increases slightly in diameter and intensity during the next 7 min. In a volume of 0·1 ml., as little as 0·1–0·2 μg is effective; with increasing dose the blue increases in intensity and area. With amounts greater than 1–3 μg the colour first appears at the edge of the bleb, but gradually fills the centre. With doses

of 4 μg and more, the centre remains uncoloured except for the traumatic blueing round the site of needle-entry, and colour develops at the edge. More than 10–20 μg produces only a narrow band of very faint blue at the edge of the bleb.

On section after 10 min, a 1 μg histamine lesion is seen to be uniformly stained at the skin surface, more widely stained in layer *B* (Text-fig. 1*c*), and feebly stained in the muscle. Occasionally the staining is slightly deeper in the region of the two plexuses of blood vessels. With stronger doses of histamine (Text-fig. 1*d*), the drug has in 10 min spread downwards and outwards so that there is blue exudate in the underlying subcutaneous tissues, and the abdominal and thoracic muscles may be stained. The other feature of the stronger dose, the central inhibition of blueing, is also evident in cross-section, where the colourless zone reaches down to the muscle layer.

The linear response, lesion diameter on log. dose, holds with histamine for diameters between 5 and 17 mm: below 4 mm histamine blueing may be confused with traumatic blueing. In reading the diameters, inhibition of blueing at the centre is ignored even though with the higher concentrations of the drug only faint, thin rings of blue may be produced. The lesion-diameters measured on the under-surface of flayed skin are larger than those on the upper surface, by about 40%. This ratio is approximately constant. There is, however, no advantage in the measurement, because the lesions are less well defined and, with the bigger doses, obscured by subcutaneous blue exudate.

Intradermal injections in the ear. Blueing of the ear, as in the trunk (see below), was partly inhibited by bromethol anaesthesia; but under light anaesthesia it was sufficiently strong for a number of useful observations, though lesion diameters were not such a consistent guide to drug-sensitivity. The ear was found to be less sensitive to histamine than the skin of the trunk. In unblued animals, concentrations of 5–1000 μg/ml. caused immediate flushing of the skin of the bleb, which lasted about 10 min. Stronger histamine, up to 10,000 μg/ml. also caused immediate flush, and after $1\frac{1}{2}$ min an intense vasoconstriction, lasting 3–5 min, of the arteries traversing the bleb area.

In blued animals, the permeability effect is visible in $1\frac{1}{2}$ min. The minimal blueing concentration was about 0·5 μg/ml. Central inhibition of blueing occurred at 50 μg/ml., lasting only 3 min, after which the centre of the bleb became blue. At 1000 μg/ml. it lasted about 16 min, unlike the central inhibition by 50 μg/ml. in the skin of the trunk, which persisted for several hours. The results in Table 6 were obtained from blebs of 3 mm initial diameter; the injection volume was not measured exactly.

With concentrations of 100 μg/ml. the area of blueing of a 3 mm bleb was 10 mm in diameter after 10 min. Histamine in stronger concentrations, 1000–10,000 μg, leaked into the lymphatic plexus, spread both distally and proximally through the freely anastomosing channels, passing back into the

tissues to produce an oedematous wedge-shaped area of intense blueing with its apex at the base of the ear where the main lymphatic ducts leave the ear (Pl. 1A, B). This is a characteristic lymphatic spread of strong histamine in the ear, and is also produced by strong histamine injected directly into the lymphatic plexus. It is evident therefore, that histamine readily passes both ways through lymphatic endothelium.

Factors that change skin-reactivity to histamine in the trunk

Temperature. Depilated animals held at 10° C react poorly to histamine; those at 20° C react well; and those at 37° C poorly and irregularly. Thus, the mean lesion-diameters for 3, 9 and 27 μg histamine were 6·7, 8·3 and 9·6 mm at 20° C, and 4·7, 5·7 and 5·9 mm in animals held at 37° C. The intensity of blueing was considerably less at 37° C. In this reaction to heat the guinea-pig is similar to man in his whealing reaction to histamine (Lewis & Grant, 1924).

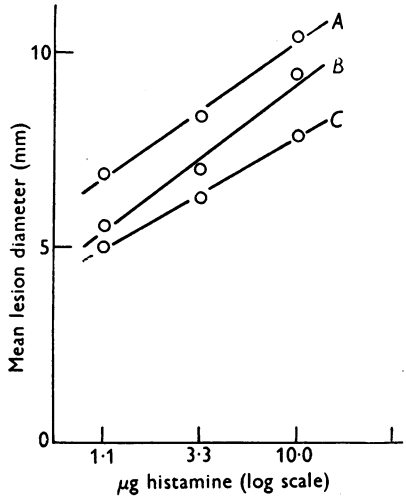
Anaesthesia. Under ether, chloroform, chloralose, bromethol, pentobarbitone and urethane anaesthesia, blueing is greatly diminished or even abolished. In general, the deeper the anaesthesia, the greater the inhibition of blueing. After a short period of anaesthesia with ether, bromethol or chloroform, reactivity is sometimes restored on recovery. Loss of reactivity is accompanied by a decline in the pressure of the central arteries of the ear, measured by a modified Grant's capsule (Miles & Niven, 1950), from 40 to 70 mm in the unanaesthetized state, to 20–40 mm Hg. This suggests that in the skin of the trunk also there is in anaesthesia a decline in blood pressure to the point where dye is no longer forced out into the tissues, though the permeability of the vessels may be increased by the histamine. However, in many recovered animals, in which the blood pressures in the ear have returned to normal, the skin of the trunk remains unresponsive; either the anaesthetic has modified histamine-sensitivity, or the circulation is in these areas restored less quickly than in the ear.

Shock. In guinea-pigs given sublethal shocking doses of *Proteus vulgaris* and *Bacterium coli* endotoxins, of adenosine-triphosphate, insulin or intraperitoneal hypertonic glucose, reactivity to histamine is diminished; and, as in anaesthesia, the loss is associated with low blood pressure in the ear, and with low skin temperature (Miles & Niven, 1950). In shocked or anaesthetized blued animals, whose skin does not respond to histamine, it is possible to demonstrate an increase in capillary permeability by indirectly raising the intracapillary pressure. When a suction cup is applied to the skin of these animals, substantial blueing of a histamine-treated area is produced within 5 min, by a suction of 10 mm Hg applied intermittently for 2–3 sec every 10 sec.

Infection and malnutrition. Guinea-pigs suffering from spontaneous chronic infective abscesses, or in poor health because of heavy infection with B.C.G.

(Bacillus of Calmette and Guérin) or as a result of long-term partial deficiency of vitamin C, react poorly to histamine.

Cortisone, preparations of adrenocorticotrophic hormone (ACTH) and posterior pituitary lobe extract (P.P.E.). In an attempt to relate the anti-allergic effect of cortisone and ACTH to an effect on histamine sensitivity, histamine was titrated in blued animals, 2–3 hr after doses of cortisone (2 mg) or ACTH (1 i.u.). These doses had proved effective in diminishing tuberculin allergy in the guinea-pig (Long & Miles, 1950). The cortisone had no effect. The diameters of the lesions were not substantially changed by the ACTH, but intensity of staining was greatly diminished. At this time, the skin of the ACTH-treated animals was slightly colder than those of controls. Nearly all current preparations of ACTH contain some posterior lobe extractives, and these may have been responsible for the ACTH effect we observed. Certainly both 2 and 0·2 i.u. P.P.E., given subcutaneously 2½ hr before the titration, diminished reactivity to histamine (Text-fig. 4); and here again the diminution was probably due to poor blood supply, because the skin was cooler than that of the controls.

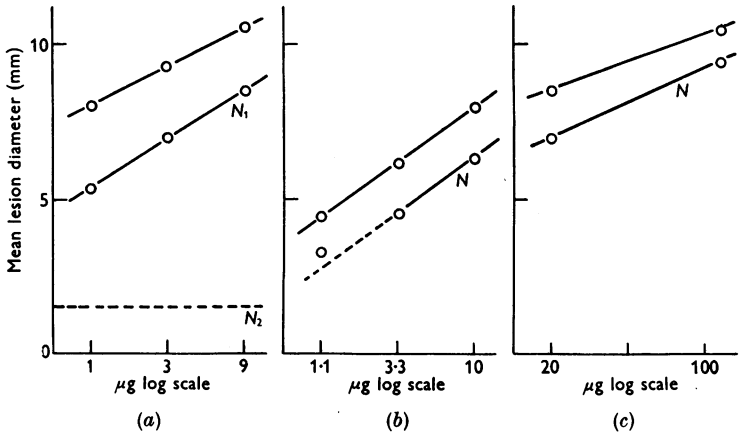


Text-fig. 4. The depressant effect of intramuscular posterior pituitary extract (P.P.E.) on histamine blueing in the guinea-pig. Each point the mean of twelve lesions. *A*, untreated animals; *B*, 0·2 i.u. P.P.E., 2·5 hr earlier; *C*, 2·0 i.u. P.P.E., 2·5 hr earlier.

Neoantergan. 20 μg neoantergan (mepyramine maleate) given intradermally in 0·1 ml. itself induced slight blueing of the skin; 5 μg histamine induced intense blueing 11 mm in diameter; and 20 μg neoantergan mixed with 5 μg histamine gave a pale blue area 10 mm in diameter. Local neutralization by neoantergan is therefore possible, but not very effective. Intravenous neoantergan, on the other hand, is most effective; 0·1 mg/kg almost abolished histamine blueing, and 0·02 mg/kg diminished the efficacy of intradermal histamine about 9-fold (Text-fig. 5*a*).

Excepting inhibition in animals held at high atmospheric temperatures, and by intravenous neoantergan, most of the effects on blueing described above can be attributed to a decline of intravascular pressure, rather than to insensitivity to histamine. The insensitivity of an abnormally warm skin may be due, as Lewis & Grant (1924) suggested for the human subject, to the more rapid removal of histamine in the more physiologically active tissue. The variety of the states in which there is inhibition of blueing is a warning that

absence of local blueing cannot safely be interpreted as absence of increase in capillary permeability unless there is good reason to believe that the blood supply to the skin and the state of the skin vessels have not been altered by the experimental procedure. On the other hand, it is reasonable to assume that a substance like histamine, which rapidly induces a deep blueing in healthy animals held at an atmospheric temperature of about 20° C, does so by an abnormal increase in capillary permeability. Histamine can act as vasodilator, but it is unlikely that the increased blueing in the guinea-pig is due to increased flow and exudation of dye as a result simply of vasodilatation, as suggested by Dekanski (1949). The rate of histamine blueing is too rapid to be attributable to this cause. Histamine induces in 3 min an intensity of blueing reached by untreated skin in 10 hr or more; i.e. the rate of accumulation of the dye is increased by over 200-fold.

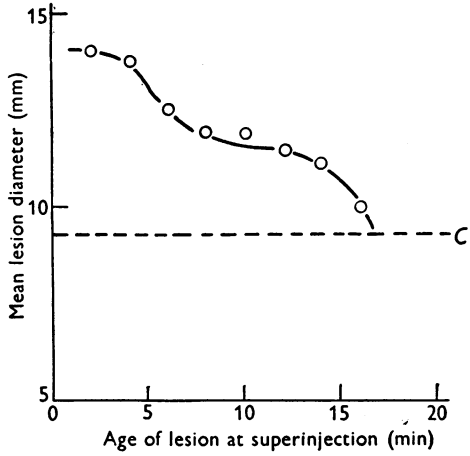


Text-fig. 5. The effect of neoantergan (N) on blueing in the guinea-pig. Each point the mean of twelve or sixteen lesions. (*a*) Histamine, $N_1 = 0{\cdot}02$ mg/kg, N_2 = average diameter of lesion with 0·1 mg/kg; (*b*) 48/80, $N = 8$ mg/kg; (*c*) leukotaxine, $N = 3$ mg/kg.

The time-course of the histamine effect

The rate of 'fixation' of histamine. The 'constant-amount' titration of histamine (Text-fig. 3*b*, Table 2), measuring the blue area developing in 10 min, shows that during injection the drug is lightly adsorbed. Within 5 min, however, some reaction with tissue must have occurred, because the increased permeability is by that time almost fully developed. The rate of that reaction may be estimated by the technique of superinjection (Miles, 1949) in which a substance is injected through a needle painted with a trace of indian ink so that the site of needle entry is exactly marked; and after an interval an injection of saline made into the same site. If any of the injected substance is free, it will be displaced outwards by the superinjected saline beyond the periphery of the initial bleb, with a consequent increase in the size of the lesion; if there is no increase, the injected substance has already been held fixed or destroyed by the tissues.

In applying the method to histamine and histamine-liberators the evidence from increase in lesion-diameter is not unambiguous because the superinjected saline may push, not free histamine, but already-formed blue exudate, beyond its original confines. The ambiguity may be resolved in part by determining the rate at which 'exudate' is fixed to the tissues. To this end, guinea-pig serum was coloured with pontamine blue so that in an unblued animal 0·1 ml. stained the skin to the intensity produced by 3 μg histamine in 0·1 ml. injected into a blued animal. Into the sites of injection of this fluid, 0·2 ml. saline were superinjected after graded intervals of time. The results were variable but indicated that some of the dyed serum was fixed within 3–4 min and the rest could be dislodged by superinjection up to 20 min. later. The best that can be

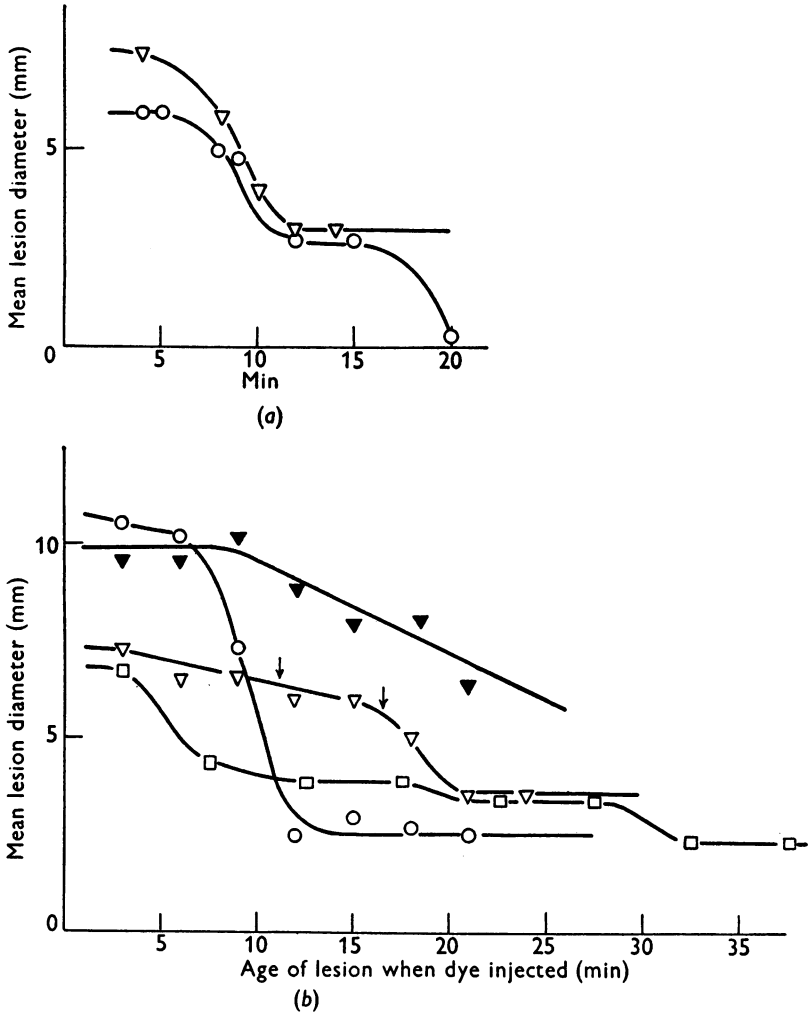


Text-fig. 6. The fixation of 3 μg histamine by guinea-pig skin. Superinjection of histamine lesions by 0·2 ml. saline. *C* = average diameter of control lesions. Each point the mean of four lesions.

hoped for from the test is that with time the lesion-diameters decline to a steady value, but this will not necessarily be the same as the diameter of lesions left to develop without superinjection. When saline was superinjected into blebs made in blued animals with 3 μg histamine (Text-fig. 6) little was fixed in 3–4 min, a period corresponding to the latent period before blue exudation starts, the greater part was fixed in 4–12 min, and nearly all was fixed in 16 min old lesions. If we allow 3 min for the fixation of the blue exudate, then all the histamine may be fixed in as little as 13 min, and most of it is fixed, as might be expected, during the period when the histamine-induced exudation is taking place.

Duration of increased permeability. The duration of the histamine effect was measured by injecting 2 μg in 0·1 ml. at regular intervals for 30 min in an animal, and giving the dye intravenously immediately after the last intradermal injection; there are thus lesions varying in age from 1 to 30 min when the dye is injected, and lesions in which the vessels are no longer permeable do

not blue. It is clear from Text-fig. 7*b* that after 10 min the capillaries have recovered; only the traumatic blueing caused by the needle remains. A similar result was obtained in the ear (Text-fig. 7*a*) Here the residual traumatic blueing due to the fine micro-needle is minimal.



Text-fig. 7. The duration of increased permeability in guinea-pig skin. In each case there is a decline to the level of traumatic blueing induced by the injection needle. (*a*) The ear: histamine, approx. 2 μg in 0·02 ml., ○ — ○; 48/80, 2 μg in 0·02 ml., ▽—▽. (*b*) The trunk: histamine, 1 μg in 0·1 ml., ○ — ○; 48/80, 20 μg in 0·1 ml., ▼—▼; 2 μg in 0·1 ml., ▽—▽; leukotaxine, 10 μg in 0·1 ml., □ — □.

Immunity to histamine. It follows that the site of a histamine injection remains colourless if it is 10–15 min old when dye is given. When more histamine is superinjected into such a site, blueing is either feeble or absent. The

injection site has become refractory—or immune—to further histamine. The immunity is neither solid nor regular in its manifestation; it may take the form of diminished area of feeble staining, as though all the tissue were partly immunized; or of small patches of blue from 1 to 4 mm in diameter, as though the blood vessels in certain areas of the injection site had escaped immunization. This irregularity of response partly spoils measurement of immunity in terms of lesion-diameter, but the differences between immune and non-immune tissues are usually so large that mean lesion-diameters are good enough indices of the change. It is clear, for example, from Table 3, that after 1 hr 9 μg

TABLE 3. Immunizing action of intradermal histamine to injections of histamine made 1 hr later. Means of three lesions

Immunizing dose of histamine (μg)	Mean diameter (mm) and intensity* of reaction to test dose of histamine		
	1 μg	3 μg	9 μg
Nil	8·5 + +	10 + +	11·3 + +
9	0	6 f.	7·8 f.

* f., ±, +, + + = faint, moderate, marked, intense blueing.

induces a solid immunity to 1 μg, and a substantial immunity to 9 μg. A number of similar experiments established that though some immunity is present after 10 min, when the capillaries have recovered from the immunizing dose, it is greatest in lesions 1½–2½ hr old, and is passing off after 4 hr.

Histamine in concentrations of 10–100 μg/ml. immunized the ear vessels to histamine. Immunity was irregular in lesions up to 35 min old, well established after 40 min, maximal at 2–3 hr and lasted up to 5 hr. After 40 min, 50 μg/ml. had immunized to a test dose of 50 μg/ml. so that the diameter and intensity of blueing was reduced from 9 mm + +, in a control area to 4 mm ±, in the immunized area; 20 μg/ml. did not immunize to 50 μg/ml., and the immunity induced by 10 μg/ml., though it protected against 10 μg/ml., lasted only 60 min.

The inhibition of skin-reactivity by high concentrations of histamine

The restriction of blueing to the periphery of blebs containing 4 μg histamine or more may be explained in terms of the recovery of normal impermeability and of immunity, if we also postulate that the histamine at the centre of the bleb was strong enough to induce vasoconstriction of the arterioles for at least 10 min. As a result, the centre of the bleb would not blue during this time because the blood supply is cut off—a state corresponding to the inhibition of whealing in man by pressure-occlusion of the vessels—and by the time the blood supply is re-established, the central capillaries would have recovered from the histamine. Alternatively, the reaction of the vessels with high concentrations of histamine may proceed so rapidly that the immune stage is reached before the preceding stage of permeability can manifest itself by the escape of dye.

Lewis & Grant (1924) inferred that the refractory state was not due to occlusion of the lumen of the vessels by viscous R.B.C. or other material, because it could be induced in a relatively bloodless arm, and because, even though whealing was inhibited, vascular reactions that indicated a fully patent vascular bed could still be elicited. There is no anatomically convenient site in the guinea-pig for occlusion tests as applied by Lewis & Grant, and the dermis is too dense for direct observation of vascular changes. The vessels were proved patent by direct test. Injections of 5 or 10 μg histamine were made at intervals, in the skin over the right scapular region of normal guinea-pigs under urethane anaesthesia. At the end of the series of skin injections, 4 ml. indian ink was put into the right axillary artery. During the preparation of this artery, the blood supply to the skin was interrupted for the last 2–3 min of the experiment. The state of the vascular bed was deduced (*a*) by direct observation of the blackening of the skin; (*b*) from the pattern of the ink-filled vessels seen by low-power microscopy in excised skin areas fixed in 10% formalin, dehydrated and cleared with clove oil; and (*c*) from the distribution of ink in the lumen of capillaries seen in histological section of individual blebs. In tests by these methods on eight animals, there was no evidence of blocked vessels in histamine lesions from 5 to 30 min old.

Skin reactions to 48/80

Intradermal injection in the trunk. In the skin of blued animals, 48/80, like histamine, induces a round area of blueing whose diameter is linearly related to the log. dose injected in a constant volume of 0·1 ml. (Text-Fig. 5*b*). The slope of the dose-response line is much less than that of histamine, varying between 1·5 and 2·0. The blueing develops in 3–5 min and is more intense than that produced by histamine; the minimal effective dose distinguishable from traumatic blueing by the injection needle is about 0·2 μg. With doses of 2–4 μg the blue first appears at the periphery of the bleb and invades the centre. In cross-section of 48/80 lesions the blue area is confined to the two upper layers of the skin (Text-fig. 1*e*). The blueing is not uniform as it is with histamine; it is more intense in the region of the two plexuses of blood vessels, and especially that immediately over the muscle layer. The 48/80 does not spread downwards to the subcutaneous tissues. Even with doses of 30 μg in 0·1 ml., only the upper part of the muscle layer is blued, and there is no blueing of subcutaneous tissues or underlying muscle. This is not due to absence of histamine available for liberation in the muscle or subcutaneous tissues, because direct injection of 48/80 into these sites induced intense localized blueing. This difference in the spread of 48/80 and histamine from the injection site is not likely to be due to the difference in molecular weights, which are of the order of 540 for the trimer of 48/80 and 310 for the acid phosphate of histamine; the difference confirms the conclusions about the greater adsorption of 48/80 derived from the results of the 'constant-amount' titrations (Text-fig. 3*c* and Table 2).

The centre of the 48/80 bleb remains unblued with doses over 5 μg; at most it becomes pink with a purplish tinge, and sometimes there are small patches of haemorrhage (Text-fig. 1*f*). The absence of response may in part be due to temporary vasoconstriction by 48/80 itself or by the histamine it liberates, followed by recovery of normal permeability before the vessels become patent. But it is to a large extent due to a more permanent damage, presumably thrombotic, to the blood vessels, because the pink area remains when the surrounding skin is blackened by intra-arterial injections of indian ink; and the blocking of the vessels is evident in stained sections of the ink-treated skin.

Intradermal injection in the ear. Concentrations of 10 and 100 μg/ml. caused an immediate flushing; with 10 μg/ml. blueing started in 7 min, and with 100 μg/ml. in 1½ min. Blueing in a histamine bleb develops uniformly over the treated area, whereas that due to 48/80 begins at the edge of the bleb and after 5 min, along the course of the large vessels, particularly the arteries, lying under it. These paravascular streaks then coalesce to form a uniformly blue lesion. Above 250 μg/ml. this blueing occurs only at the periphery of the bleb. There is a central area whose size varies with the concentration, in which within 1½ min, the small superficial vessels thrombose without prior constriction. The underlying larger vessels become invisible but refill with circulating blood after about 10 min. By this time there is very slight blueing in the central area, so that by naked eye the lesion is faint purplish pink in the centre surrounded by deep blue (Pl. 2A). This central thrombosis and inhibition persist for over 6 hr.

Though 48/80 spreads outwards to blue an area 50–200 times that of the initial bleb, the resulting lesion is always round. Unlike histamine, 48/80, even at 10,000 μg/ml. does not leak in the lymphatic plexus beyond the confines of the bleb to produce a wedge-shaped blue lesion. Even when 48/80 is injected directly into the plexus, the blue area is approximately round (Pl. 2B). After 15 min or more a blue prolongation towards the base of the ear may develop, but this is observably the coloration of the lymphatic ducts with blue exudate from the bleb; it is not due to an increased permeability of blood vessels adjacent to the channels, as with histamine. These observations confirm the conclusions from the behaviour of 48/80 lesions in the skin of the trunk, that it is fixed firmly and rapidly to the tissue, whereas histamine at first moves freely, and begins to be fixed only after 2–3 min; and that inhibition at the centre is due to thrombosis of the vessels. Moreover, since 48/80 lesions blue as fast as histamine lesions, but no faster, and since the duration of the 48/80 effect is almost identical with that of histamine (Text-fig. 7*a*), it is probable that 48/80 liberates histamine very quickly and its latent period is mainly that of the liberated histamine.

Factors that change the skin-reactivity to 48/80. Blueing by 48/80, like that by histamine, is partly inhibited during anaesthesia and shock, and by

warming the animals in an atmosphere at 37° C. Sickly animals respond poorly to 48/80; this is probably not due to a diminution in the skin of histamine available for liberation, because the same animals are proportionally insensitive to histamine itself. As with histamine, most of the states of apparent insensitivity to 48/80 appear to be due to a poor blood supply to the skin.

Neoantergan intravenously is much less effective with 48/80 than with histamine. The effect of 8 mg/kg body weight 30 min before the injection of 48/80 is shown in Text-fig. 5*b*. Three concentrations of 48/80 and a saline control were titrated in blued animals, the four doses being randomized in a Latin square over sixteen sites. Three animals were used in each group so that each point is the mean of twelve readings. There is a 3-fold decrease in the 48/80 effect. In other tests 6 mg neoantergan/kg decreased the effect 5-fold, and 2 mg decreased it 2·5-fold. In a few animals the neoantergan greatly diminished the intensity but not the area of blueing by 48/80. The relative inefficiency of circulating neoantergan in antagonizing 48/80 suggests that 48/80 may have a more direct action on capillary permeability; histamine activity was reduced 9-fold by 0·02 mg neoantergan/kg, whereas as much as 6 mg/kg was required for a 5-fold decrease of 48/80 activity. Moreover, doses up to 15 mg/kg, which is near the LD 50 of neoantergan, did not abolish the response to 48/80. It is possible that at the centre of the bleb, 48/80 itself is in a sufficiently high concentration to increase capillary permeability by direct action. Nevertheless, since the area of the 48/80 lesion is diminished by neoantergan, the drug in the concentrations obtaining at the edge of the lesion clearly act by liberating histamine. In the centre of the lesion, either the increase in permeability is not wholly due to histamine, or the available neoantergan is overwhelmed by histamine rapidly liberated by the high concentration of 48/80.

Fixation of 48/80. Superinjection of 0·2 ml. saline into lesions of various ages made by 2 μg of 48/80, failed to increase the lesion-diameter after 2–3 min. With larger doses, 48/80 remained free for a longer period, and superinjection increased the lesion-diameter after 10 min or more.

Duration. The increased permeability, as tested by late blueing, induced by a single dose, lasts for 7–10 min, after which it declines, so that after 20–25 min no blueing occurs. In the graph illustrating this recovery (Text-fig. 7*b*), the arrows indicate the period between 12 and 15 min, when the blueing ceases to be intense and becomes relatively feeble; the decrease in lesion-diameter does not fully indicate the recovery of the vessels, which is substantially complete at 12–15 min.

Immunity. The three experiments in Table 4 exemplify the immunity induced by 48/80 to test injection of the same substance. Thus in Expt. I, 2 μg partly, and 10 μg almost wholly, immunize to test doses of 10 μg. In Expt. II, 50 μg immunize to 50 μg to some extent after 30 min and well after 3 hr, whereas after 5 hr the immunity is wearing off. Immunity is maximum

between 1·5 and 3·5 hr (Expt. III). In the ear, a concentration of 20 μg/ml. immunizes well to test doses of the same concentration. Here also, the immunity is at a maximum between 1·5 and 3·5 hr, and then passes off gradually and is gone at 5–6 hr.

TABLE 4. Immunizing action of intradermal 48/80 and histamine. Means of four to six lesions

Expt.	Immunizing agent and dose (μg)		Age of primary lesion (hr)	Mean diameter (mm) and intensity* of reaction to test dose of: 48/80 (10 μg)	Histamine (2 μg)
I	48/80	0	2	8·5 + +	7·0 f.
		2	2	2·0 f.	2·0 f.
		10	2	8·0 f.	2·0 f.
II	48/80	50	0·5	9 +	8·5 +
			3	1·5 f.	2·0 f.
			5	4 +	8·0 f.
	Histamine	5	0·5	9 + +	2±
			3	9 + +	8 f.
			5	8·5 + +	9 +
	Saline		0·5	10 + +	10 + +
			3	10·5 +	10·5 +
			5	10·5 +	10·5 +
III	48/80	30	0·5	5·2±	7·3 +
			1·5	1·8 +	2·0 f.
			2·5	0·0	0·7±
			3·5	3·8±	5·5 +
			4·5	3·3 +	6·5 +
			5·5	7·2 +	6·7 +
			6·5	6·2 +	6·7 +
	Saline		2·5	8·7 + +	6·7 +

* Intensity: symbols as Table 3.

The site of action of 48/80. The accumulation of blue at the level of the two plexuses of blood vessels in skin treated with 48/80, as compared with the more general blueing with histamine (Text-fig. 1) and the visible development of blueing along the larger vessels of similarly treated ears, suggests strongly that the main reservoirs of histamine available for liberation are closely associated with the smaller arteries and veins. The association of this histamine with blood vessels may also be inferred from the intensity of blueing induced in body skin by the two substances. The amount of histamine that can be extracted from the skin of the trunk is about 3 μg/g (Feldberg & Miles, unpublished work); and a 10 mm lesion occupies about 150 mg skin. The intensity of blueing induced by enough 48/80 to give a lesion-diameter of 10 mm is far greater than that produced by injecting 0·5 μg histamine (the amount that 48/80 presumably liberates) in about 0·35 ml. saline, a volume that will give a 10 mm lesion (see Text-fig. 3*b*). We conclude that after injection a great deal of this dose of histamine is ineffective because it is distributed through relatively non-vascular tissue, and that the endogenous histamine which can be liberated by 48/80 must be concentrated near or in the blood vessels.

Cross-immunity with histamine. As already noted, the lesions developing

after the injection of a substance into an already-injected site are variable and not easily measured. Cross-immunity is nevertheless obvious by reason of a decline in intensity or size or both, of the blued area. Tables 3 and 4 exemplify the results usually obtained. Histamine and 48/80 each induces a good immunity to itself; histamine induces only a fair immunity to 48/80, but 48/80 induces a good immunity to histamine. In the ear, concentrations of 20 μg/ml. of both induced a good though not marked cross-immunity between 48/80 and histamine. It appears, therefore, that 48/80 immunizes both by liberating histamine, which in turn immunizes the susceptible vessels, and either by exhausting the histamine which can be liberated in the skin or by interfering with the mechanism of release of the bound histamine not liberated by the immunizing dose.

Skin reactions to leukotaxine

In most respects, the reaction of the blood vessels to leukotaxine were remarkably similar to those produced by 48/80. The chief difference lay in the very high concentration of leukotaxine required to induce thrombosis of the vessels.

Intradermal injection in the trunk and the ear. Like 48/80, leukotaxine induces in 3–5 min a round area of intense blueing, the diameter of which is in proportion to the log. dose in the constant-volume titration (Text-fig. 3*d*) and to the log. volume in the constant-amount titration (Text-fig. 5*c*). Leukotaxine appears to be strongly adsorbed to the skin tissues (Table 2); minimal blueing dose in 0·1 ml. is about 1 μg; inhibition of blueing at the centre of the bleb may occur with amounts from 10 μg upwards, absent in some animals until 100–500 μg is reached. This inhibition is not permanent; the area blues within 15–25 min. With large doses, 2 mg or more, small areas of permanent inhibition are produced. On cross-section, the blue leukotaxine blebs are like those of 48/80 (Text-fig. 1*e*).

In the ear, concentrations of 200 μg/ml. and over induce a general dilatation of the small vessels within 30 sec, and a rapid blueing that starts first along the small vessels, then the larger veins and finally along the larger arteries within the bleb. When concentrations of 300 μg or more per ml. are placed near one of the larger veins or arteries, a narrow band of vasoconstriction may appear within 1 min, disappearing after 1–2 min. With concentrations above 2·5 mg/ml., thrombosis of the vessels may occur at the centre of the bleb, though it is less severe and less regular in occurrence than that induced by 48/80. The blued areas are always approximately round (cf. Pl. 2B) whether made by intradermal or intralymphatic injection.

Effect of anaesthesia and neoantergan. Blueing is diminished in area and intensity during bromethol and urethane anaesthesia. Intravenous neoantergan, 3–8 mg/kg body weight, diminishes the leukotaxine effect from 1·5- to

3-fold (Text-fig. 5*c*); the degree of neutralization is of the same order as that of 48/80, but rather less marked.

Time-course of the leukotaxine effect. By the superinjection method, putting 0·2 ml. saline into 5 μg lesions, all the leukotaxine appeared to be fixed in 16 min and most of it in the first 5–8 min. The capillaries of the skin treated with 10 μg wholly regained their normal impermeability within 25 min; most of them had recovered after 10 min (Text-fig. 7*a*, *b*).

Immunity. Table 5 typifies the results of several tests of leukotaxine immunity. Three immunizing doses of each substance, histamine, 48/80 and leukotaxine were used, and immunity tested after 2 hr by superinjecting the lowest dose again. As is common in all such tests, immunity is best when the

TABLE 5. Immunity and cross-immunity induced in the skin by leukotaxine. Primary lesions 2 hr old. Means of four lesions

Immunizing agent and dose (μg)		Mean diameter (mm) and intensity* of reaction to test dose of: Histamine (2 μg)	48/80 (5 μg)	Leukotaxine (17 μg)
Histamine	8	7·4±	N.T.	7·0++
	4	7·0±	N.T.	6·2++
	2	7·1±	7·0±	7·4++
	0	10·0+	7·8++	7·6++
48/80	20	N.T.	3·4±	2·0+
	10	N.T.	3·5±	2·2+
	5	8·0±	4·6±	5·0+
	0	9·0+	7·2++	8·0++
Leukotaxine	67	N.T.	N.T.	4·0±
	33	N.T.	N.T.	3·4±
	17	9·0±	7·1+	4·8±
	0	9·5+	7·8++	7·4++

* Intensity: symbols as in Table 3.
N.T. = No test.

immunizing dose is 4–10 times greater than the test dose. In this example immunity must be judged as much by decrease in intensity as by decrease in diameter of the blueing. Leukotaxine immunizes well to itself but only moderately to histamine and 48/80. Histamine immunizes moderately to itself and 48/80, and slightly to leukotaxine; whereas 48/80, though immunizing only moderately to histamine, immunizes well to itself and leukotaxine. Leukotaxine immunity is maximal in 1–2 hr, and has passed off by the 4th hr.

DISCUSSION

The study of increased permeability in the skin of the trunk of blued guinea-pigs has shown that after a latent period of 1½–3 min, injected histamine induces a gross increase in capillary permeability, which is maximum within the next 5 min. In concentrations of from 1 to 100 μg/ml. it can be characterized by the response of blued animal to varied doses in constant injection-volume

and to constant doses in varied injection-volume. By the superinjection of saline into histamine lesions of varying ages, and by the intravenous injection of dye at varying periods after the intradermal histamine, it can be shown that from 3 to 10 min free histamine is disappearing from the lesion, and the capillaries are recovering their normal low permeability. By the superinjection of histamine into recovered histamine-treated areas, it can be shown that as they recover, the vessels become partly immune to histamine; the immunity increases up to 2 hr, and declines after 4 hr. This immunity is not due to any interruption of the blood supply to the immunized tissues. With concentrations above 100 μg/ml. no blueing due to increased permeability is detectable.

In the ear, the outstanding peculiarity is the high concentration required to produce even a 15 min inhibition of blueing at the centre of the lesion, and the transience of the effect with lower concentrations (p. 237 and Table 6).

TABLE 6. Comparison of the approximate minimal effective concentrations of histamine, 48/80 and leukotaxine in the skin of the trunk (10 mm blebs) and the ear (3 mm blebs)

Effect	Drug	Minimal effective concentration in μg/ml. Trunk	Ear
Blueing	Histamine	1–2	0·5
	48/80	2	5·0
	Leukotaxine	10	—
Temporary inhibition of blueing, and arterial constriction*	Histamine	10–30	50
	48/80	10–30	250
	Leukotaxine	100	300
Thrombosis of blood vessels	48/80	30–50	250
	Leukotaxine	2500	5000
Leak of free drug into surrounding lymphatic plexus	Histamine	—	1000
	48/80	—	>10000
	Leukotaxine	—	>10000

* Presumed in trunk, demonstrable in ear.

In the skin of the trunk inhibition lasts for hours. For this relative permanence we postulated an arterial vasoconstriction, and therefore an absence of exudation, for periods longer than the duration of increased permeability. This relation does not hold in the ear because vasoconstriction is visibly relaxed within 3–7 min, and increased permeability lasts up to 8 min and may persist, though feebly, for 12–15 min. The relative insensitivity of the ear to this inhibitory effect may be a reflexion of its greater vascularity; either the injected histamine is swept away by the blood and lymph streams more quickly or much of the injected histamine is taken up by receptors in the numerous small vessels, so that on reaching the underlying larger vessels its concentration is too low for vasoconstriction. As Table 6 shows, the ear displays the same relative insensitivity towards 48/80 and leukotaxine, and presumably for the same reasons.

The action of 48/80 in many ways resembles that of histamine. Both substances have a latent period of about 3 min. When blueing starts they become 'fixed' to the tissues; as blueing proceeds, the vessels recover their normal low permeability and are apparently normal at the end of 10–20 min. They are at this time partly immune to second doses of the same size, and the immunity increases for 2 hr; and after 4 hr it begins to decline. But there are marked differences, which raise the question how far the release of histamine accounts fully for the action of 48/80 on capillary permeability? First, the distribution of blueing by histamine is general, but that produced by 48/80 is concentrated in the region of the arteries and veins in both the skin of the trunk and the ear, suggesting that bound histamine is localized in or near these larger vessels. Secondly, whereas high concentrations of histamine inhibit blueing by altering the immunity of the vessels, high concentrations of 48/80 do so by damaging the vessels so that thrombosis occurs. Thirdly, the two substances differ in their susceptibility to circulating neoantergan; we have to assume either that 48/80 has a direct action on capillaries, or that the histamine it releases is less sensitive than injected histamine to neoantergan. Since 48/80 is well established as a potent histamine-liberator (Feldberg & Paton, 1951; Paton, 1951; Paton & Schachter, 1951) the second assumption is the more justified, particularly because insensitivity to antihistaminics is displayed by other known histamine-liberators (MacIntosh & Paton, 1949). Although in our experiments complete extinction of 48/80 blueing by neoantergan was impossible, partial neutralization was achieved by 200 times the dose required to neutralize histamine to the same extent. The parallelism of dosage-response in untreated and neoantergan-treated animals (Text-fig. 5*b*) is consistent with a true neutralization, and with the hypothesis that the unneutralized blueing is due to some 'pharmacological inaccessibility' of the liberated histamine rather than to a direct action of 48/80 on the capillaries. This inaccessibility may result from the ready adsorption of 48/80 to the tissues, and the concentration of the depots of available histamine near the blood vessels. This histamine is perhaps 'intrinsic' in Dale's (1948) sense that it is released from the cells on which it acts. Our observations are not exact enough to decide this point. But whatever reason for the relative inefficacy of neoantergan with 48/80, the fact indicates that we need not demand of a presumed histamine-liberator that its susceptibility to inhibition by neoantergan shall be of the same order as that of histamine.

Our observations on 48/80 provide a pattern of the intradermal behaviour of a histamine-liberator, for comparison with substances like leukotaxine, whose capacity to liberate histamine is in doubt. Menkin (1936, 1938*a*, *b*) isolated leukotaxine from sterile inflammatory exudates, and described its capacity to increase capillary permeability. Later work (Duthie & Chain, 1939; Spector, 1951) proved it to be a family of positively chemotactic

polypeptides, of which the larger members have this action on the capillaries. Rocha e Silva & Dragstedt (1941) postulated that leukotaxine acted via histamine, and Dekanski (1949) showed that on intradermal injection it increased the histamine equivalent in cat's skin, and that its 'blueing' action was neutralized by neoantergan. Menkin (1936, 1938*a*), and Cullumbine & Rydon (1946) deny the mediation of histamine because leukotaxine does not cause contraction in isolated smooth muscle, and the blueing is not antagonized by neoantergan (Cullumbine, 1947). We shall not, however, expect a histamine-liberator to cause isolated smooth muscle to contract. Menkin (1938*a*) showed that leukotaxine did not do so; but neither does 48/80 (Paton, 1951). Nor is Cullumbine's failure to detect a neoantergan effect by roughly quantitative tests unexpected. Leukotaxine in the skin can have a very shallow dosage-response slope, so that as much as a 10-fold drop in potency might diminish the diameter of the lesion by as little as 2 mm. Unless the neutralization test is made at several dose-levels in several animals, a genuine though small neutralization effect might well be missed. The relative insusceptibility of leukotaxine to neoantergan may well be determined by factors responsible for the similar behaviour of 48/80 and other liberators. Leukotaxine differs from histamine in spreading less readily through the tissues after local injection, as first recorded by Menkin, and in failing to induce inhibition at the centre of injection blebs, except in high concentrations. If it acts solely by histamine-liberation, inhibition of blueing may be impossible because there is not enough histamine to reach required concentration, even though it is localized round the vessels upon which it ultimately acts. An inhibiting dose of histamine, say 5 μg, produces a lesion about 8 mm in diameter, involving about 60 mg of skin. Ignoring spread to adjacent tissues, we arrive at an average inhibiting concentration of 83 μg histamine/g skin; whereas the normal content of extractable histamine in the skin of the trunk is of the order of 3 μg/g (W. Feldberg & A. A. Miles, unpublished work). This histamine is presumably all available for liberation. Leukotaxine, except for its inability to induce thrombosis in moderate concentrations, behaves in most respects like 48/80. Both these substances resemble histamine in several respects, notably the similarity of the time-course of blueing (the lag period and duration of permeability) and in the induction and duration of immunity.

We can attach weight to these likenesses as a proof that leukotaxine acts through histamine if the effects are peculiar to histamine. This may well be so, but it should be noted that the time-course of blueing, and duration of permeability is shared by a large number of blueing substances besides 48/80, e.g. neoarsphenamine, stilbamidine, acetylcholine, peptone and hypotonic saline (unpublished work); and though some of these substances are probably histamine-liberators, the time course of their effect may reflect very general properties of vascular endothelium. The evidence obtained from the cross-

immunization tests, which are summarized in Table 7, may be misleading for a similar reason, that in the endothelium there is only one kind of site which is affected, and made resistant, by a wide variety of substances. In addition, cross-immunity between substances other than histamine does not necessarily imply the same site of action. Cross-immunity between histamine and another substance may well be an expression of true histamine immunity; but cross-immunity between two presumed histamine-liberators may be due either to exhaustion of the available histamine, to the immunization of vessels by liberated histamine or to an inhibition of the mechanism of histamine release.

TABLE 7. Summary of tests of immunity and cross-immunity to increased capillary permeability in the skin

	Immunity		
	Tested with		
Induced by	Histamine	48/80	Leukotaxine
Histamine	+ +	+	+
48/80	+ + +	+ + +	+ + +
Leukotaxine	+ +	+	+ + +

+, + +, + + + = moderate, good and marked immunity.

It should be noted here that we have used the very general terms 'immunity' and 'immunization' in this connexion, rather than 'refractoriness' or 'refractory state' because we have applied them generally to drug-resistance induced in the skin by substances other than histamine. The terms can legitimately be extended to cover these phenomena; and though our histamine 'immunity' may be the same phenomenon as Lewis & Grant's (1924) refractoriness to histamine whealing in man, we are not in a position to equate them. The two phenomena have much in common; whealing was inhibited in man by occlusion of the blood supply for 10 min and our blueing is inhibited by withholding the circulating dye for 10 min. Both were relative, never demonstrably absolute. But whereas in man the refractory state lasts 5–10 min, the immune state lasts several hours in the guinea-pig. The immunity induced by histamine, leukotaxine and 48/80 in some respects resembles the immunity to whealing of nervous origin described by Grant, Pearson & Comeau (1935). In both cases it lasts for several hours, and both are explicable in terms of an exhaustion of a substance like histamine or H-substance increasing capillary permeability.

The cross-immunity we demonstrated was never very solid. This may have been due to deviations from the exact superposition of the test injection on the region of immunized tissue, and to the different degrees of tissue affinity of the three substances tested. It may, however, reflect a distinction between the modes of action; though as regards leukotaxine, it is probably significant that the histamine-liberator 48/80 immunized well against leukotaxine. Although cross-immunity should not be accepted as a sufficient single criterion

of similarity of action, in conjunction with other evidence it is strongly suggestive.

Our various tests of the increase of capillary permeability in the skin by the three substances, do not constitute a rigorous proof that the effect of 48/80 and leukotaxine on the capillary endothelium of the skin is mediated by histamine. Nevertheless, taking the evidence as a whole, our observations on these three substances are all consistent with the view that leukotaxine acts as a histamine-liberator in inflammatory lesions. The question whether histamine, through leukotaxine or some other endogenous liberator, is the *sole* mediator of the increased capillary permeability in inflammation, is less easy to answer. A given blood vessel made permeable by histamine or leukotaxine becomes substantially immune to the further action of the drugs within 20 min. The 'perpetuation of increased vessel permeability due to the gradual accumulation of peptides in the tissues' postulated by Spector (1951) in the natural course of inflammation must therefore be produced either by partial stimulation, and consequently only a partial immunization, of some parts of the vessel, leaving other parts for later stimulation; or, what is more consistent with the observed expansion of most progressive inflammatory lesions, by a gradual outward spread of leukotaxine to as yet unaffected vessels, leaving impermeable those already affected. But even with these refinements, the major role of leukotaxine is not necessarily established in inflammation, particularly infective inflammation. For example, the time-course of blueing by several clostridial exotoxins is quite different from that of leukotaxine (unpublished work); blueing may take an hour to develop and permeability persist for several hours; and cross-immunity with histamine and histamine-liberators is difficult to demonstrate. The behaviour of *Cl. oedematiens* exotoxin is singular, because the increased capillary permeability induced by a single dose persists for more than 30 hr. This observation probably has some bearing on the extensive oedema which accompanies infection by this microbe, but in this context it is chiefly interesting as indicating the existence of substances of pathological importance which appear to alter capillary permeability in a manner quite different from that of histamine or histamine-liberators.

SUMMARY

1. The increase in capillary permeability in the skin of the trunk, and ear of guinea-pigs was measured by the size and intensity of the blue lesion induced by the intradermal injection of histamine, the histamine-liberator 48/80, and leukotaxine, in animals with pontamine sky blue 6X in their circulation. In the trunk, the mean lesion-diameter from graded doses of these drugs in a constant volume is proportional to the log. dose; and for a constant dose in graded injection-volumes it is proportional to the log. volume.

2. The linear dosage-response to histamine, 48/80 and leukotaxine forms the basis of an assay method in the 'constant-volume' titration; 'constant-dose' measurements can be used to measure the affinity of skin tissue for the three substances.

3. Histamine has a relatively low, and 48/80 and leukotaxine a relatively high, affinity for skin tissue. Injected histamine spreads readily with the injection fluid to the subcutaneous tissues and lymphatic channels, whereas 48/80 and leukotaxine tend to remain in the skin.

4. All three substances increase capillary permeability within 3–5 min of injection, and their action is mostly finished in 10–15 min, and wholly so in 30 min. Between 15 and 30 min after the injection, the capillaries not only begin to recover their normal low permeability, but become immune to further doses of the drug. The immunity is greatest from 1–3 hr and declines after 4–5 hr.

5. Histamine blueing is general throughout the depth of the skin; 48/80 blueing is most intense in the region of arterioles, venules and the smaller arteries and veins, suggesting that the histamine available for liberation is located in these regions.

6. High local concentrations of histamine inhibit blueing by inducing severe local vasoconstriction during the period of increased permeability, so that by the time the constriction is relaxed the vessel walls have recovered their normal low permeability. High local concentrations of 48/80 inhibit blueing by thrombosing the blood vessels.

7. Anaesthesia, shock and chilling decrease blueing. The effect appears to be secondary to a decline in local intravascular pressures, and not to resistance of the capillaries to permeability-inducing substances.

8. Circulating neoantergan strongly antagonizes histamine blueing. It is over 200 times less effective with 48/80 and leukotaxine, but a definite antagonism to both substances is demonstrable.

9. The three substances induce a substantial cross-immunity to one another. The cross-immunity to histamine is presumably due to histamine liberated by 48/80 and leukotaxine. Cross-immunity between histamine-liberators may be due either to histamine immunization of the blood vessels, exhaustion of histamine or inhibition of the mechanism of histamine release.

10. The similarities in the time-course and duration of the permeability effect, and in the induction of cross-immunity, give strong support to the view that leukotaxine increases capillary permeability by liberating histamine. In all the tests made, the differences between leukotaxine and histamine were paralleled by differences between 48/80 and histamine, excepting the thrombosing effect of strong 48/80. Since 48/80 is an established histamine-liberator, these differences are not good evidence that substances displaying them do not liberate histamine.

We are greatly indebted to Dr E. J. de Beer at the Wellcome Research Laboratories, Tuckahoe, U.S.A., for a generous gift of the compound 48/80, and to our colleague Dr J. H. Humphrey for leukotaxine.

REFERENCES

BAIN, W. H. (1949). The quantitative comparison of histamine antagonists in man. *Proc. R. Soc. Med.* **42**, 615–623.

BALTZLY, R., BUCK, J. S., DE BEER, E. J. & WEBB, F. J. (1949). A family of long-acting depressors. *J. Amer. chem. Soc.* **71**, 1301–1305.

CULLUMBINE, H. (1947). Leukotaxine and histamine. *Nature, Lond.*, **159**, 841–842.

CULLUMBINE, H. & RYDON, H. N. (1946). A study of the formation, properties and partial purification of leukotaxine. *Brit. J. exp. Path.* **27**, 33–46.

DALE, H. H. (1948). Antihistamine substances. *Brit. med. J.* ii, 281–283.

DEKANSKI, J. (1949). The effect of protein hydrolysates (leukotaxine) on skin-histamine in cats. *J. Physiol.* **108**, 233–245.

DUTHIE, E. S. & CHAIN, E. (1939). A polypeptide responsible for some of the phenomena of acute inflammation. *Brit. J. exp. Path.* **20**, 417–429.

EVANS, D. G., MILES, A. A. & NIVEN, J. S. F. (1948). The enhancement of bacterial infections by adrenaline. *Brit. J. exp. Path.* **29**, 20–39.

FELDBERG, W. & PATON, W. D. M. (1951). Release of histamine from skin and muscle in the cat by opium alkaloids and other histamine liberators. *J. Physiol.* **114**, 490–509.

GRANT, R. T., PEARSON, R. S. B. & COMEAU, W. J. (1935). Observations on urticaria provoked by emotion, by exercise and by warming the body. *Clin. Sci.* **2**, 253–272.

HECHTER, O. (1946). Mechanism of hyaluronidase action in skin. *Science*, **104**, 409–411.

LEWIS, T. & GRANT, R. T. (1924). Vascular reactions of the skin to injury. II. The liberation of a histamine-like substance in injured skin; the underlying cause of factitious urticaria and of wheals produced by burning; and observations upon the nervous control of certain skin reactions. *Heart*, **11**, 209–265.

LONG, D. A. & MILES, A. A. (1950). Opposite actions of thyroid and adrenal hormones in allergic hypersensitivity. *Lancet*, i, 492–495.

MACINTOSH, F. C. & PATON, W. D. M. (1949). The liberation of histamine by certain organic bases. *J. Physiol.* **109**, 190–219.

MATOLSTY, A. G. & MATOLSTY, M. (1951). The action of histamine and anti-histaminic substances on the endothelial cells of the small capillaries in the skin. *J. Pharmacol.* **102**, 237–249.

MENKIN, V. (1936). Studies on inflammation. XII. Mechanism of increased capillary permeability. A critique of the histamine hypothesis. *J. exp. Med.* **64**, 485–502.

MENKIN, V. (1938*a*). Studies on inflammation. XIV. Isolation of the factor concerned with increased capillary permeability in injury. *J. exp. Med.* **67**, 129–144.

MENKIN, V. (1938*b*). Studies on inflammation. XV. Concerning the mechanism of cell migration. *J. exp. Med.* **67**, 145–152.

MILES, A. A. (1949). The fixation of diphtheria toxin to skin tissue with special reference to the action of circulating antitoxin. *Brit. J. exp. Path.* **30**, 319–344.

MILES, A. A. & NIVEN, J. S. F. (1950). The enhancement of infection during shock produced by bacterial toxins and other agents. *Brit. J. exp. Path.* **31**, 73–95.

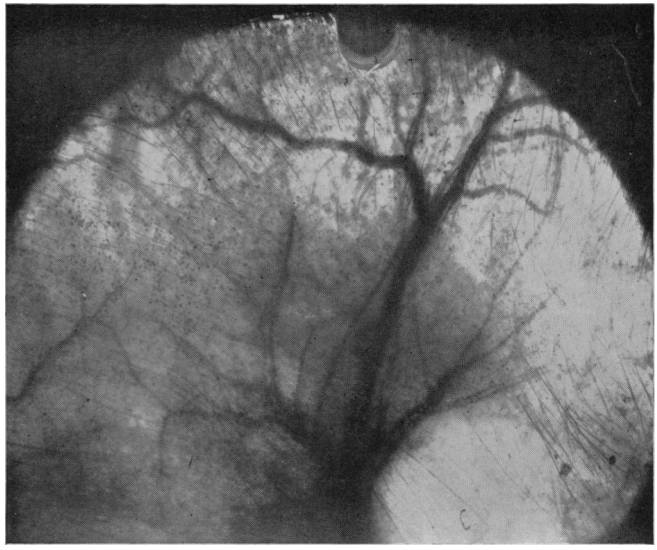
PATON, W. D. M. (1951). Compound 48/80: a potent histamine liberator. *Brit. J. Pharmacol.* **6**, 499–508.

PATON, W. D. M. & SCHACHTER, M. (1951). The influence of an antihistamine drug on the release of histamine in the unanaesthetized dog. *Brit. J. Pharmacol.* **6**, 509–513.

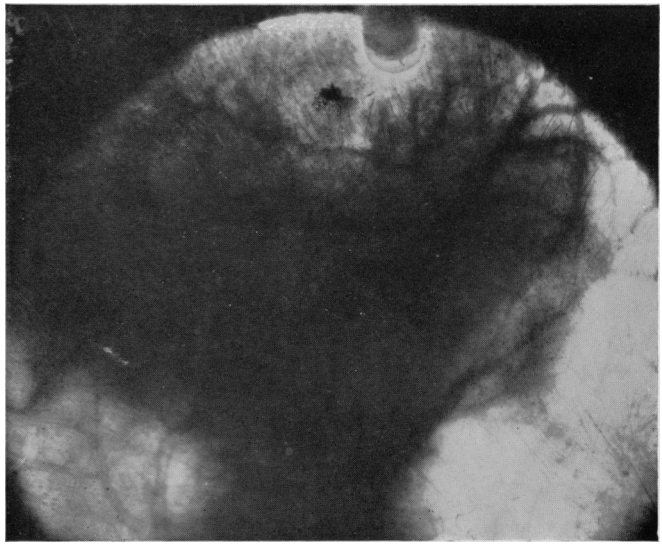
ROCHA E SILVA, M. & DRAGSTEDT, C. A. (1941). Observations on the trypan blue capillary permeability test in rabbits. *J. Pharmacol.* **73**, 405–411.

SPECTOR, W. G. (1951). The role of some higher peptides in inflammation. *J. Path. Bact.* **63**, 93–110.

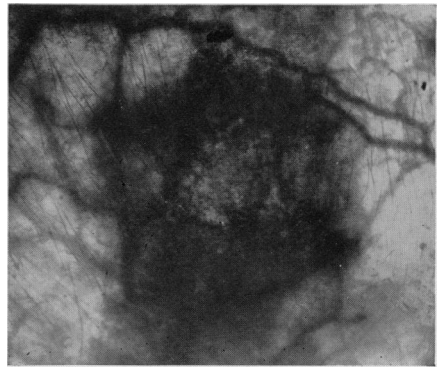
WADLEY, F. M. (1949). The use of biometric methods in comparisons of acid-fast allergens. *Amer. Rev. Tuberc.* **60**, 131–139.



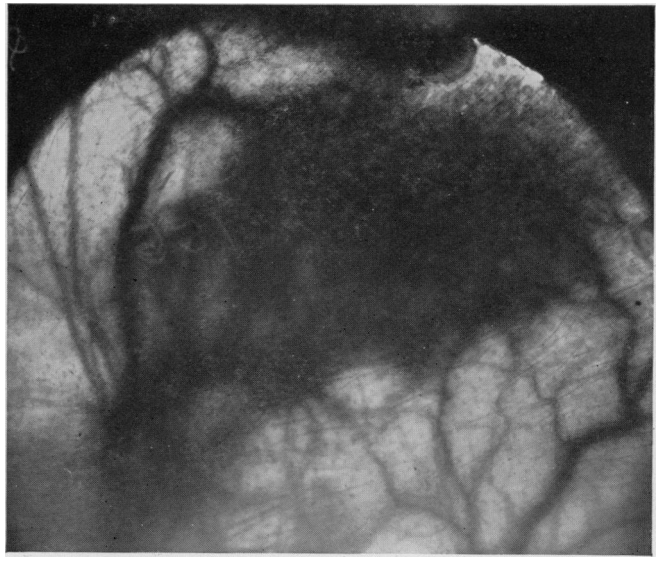
A



B



A



B

EXPLANATION OF PLATES

PLATE 1

A. Untreated ear of blued guinea-pig, showing the degree of initial opacity near the main vessels, due to the relative thickness of the ear in that region. ×5.

B. The same ear as in A after injection of strong histamine (1000 μg/ml.) directly into the lymphatic plexus. The indian-ink stain on the skin at the top marks the injection site. The area of intense blueing is approximately wedge shaped, with the apex at the base of the ear. ×5.

PLATE 2

A. Ear of a blued guinea-pig injected with strong 48/80 (500 μg/ml.). There is inhibition of blueing at the centre of the lesion, with the thrombosed superficial vessels in sharp focus. ×5.

B. Ear of a blued guinea-pig after injection of strong 48/80 (150 μg/ml.) directly into the lymphatic plexus. The opacity round the main vessels on the left is due to the thickness of the ear. The area of intense blueing is approximately circular. ×5.