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CUTANEOUS ANTIGEN-ANTIBODY REACTIONS IN THE RAT

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In an earlier paper (Brocklehurst, Humphrey & Perry, 1955) we showed that when rabbit antibody was injected into the skin of rats, and antigen administered intravenously, the local rapid increase in capillary permeability (passive cutaneous anaphylaxis, PCA) and the inflammatory changes of the Arthus reaction proceeded normally even in skin depleted of up to 90 % of its histamine content. Furthermore, in normal rats or in rats with greatly reduced skin histamine, antihistamine compounds failed to modify Arthus and PCA reactions unless these drugs were given in such large doses as to produce signs of collapse in the rats. In histamine-depleted animals, treated with an antihistamine drug, the PCA reaction remained unchanged although the amount of histamine required to produce a similar reaction by injection was about 1000 times as great as was present in the corresponding area of skin. We concluded therefore that histamine release, as a consequence of the antigen-antibody reaction, could not be the cause of the observed phenomena.

Inderbitzin & Craps (1957) have confirmed these findings in the rat, and Alberty & Takkunen (1957) found mepyramine to be relatively ineffective against cutaneous anaphylaxis in actively sensitized guinea-pigs. Halpern, Liacopoulos & Briot (1956) have reported, however, that the threshold dose of antigen required to produce a detectable increase in capillary permeability in the skin of actively sensitized rats was increased some 500 times by promethazine (2·5 mg/kg).

Another possible mediator in anaphylactic reactions, besides histamine, is 5-hydroxytryptamine (5-HT), which has been shown to be released by antigen-antibody reactions *in vitro* from blood platelets (Humphrey & Jaques, 1955), and *in vivo* in the rabbit (Waalkes, Weissbach, Bozicevich & Udenfriend, 1957). Parratt & West (1957*b*) showed that rat skin contained 5-HT, but concluded that most of this substance was located elsewhere than in the mast cells, which contained some 90 % of the skin histamine.

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They showed that, although compound 48/80 released both histamine and 5-HT from rat skin, reserpine could greatly diminish the 5-HT while hardly affecting the histamine. Inderbitzin & Craps (1957) reported that bromolysergic acid diethylamide (BOL), a potent antagonist of 5-HT, did not modify the PCA response in rats. Our previous studies had presumably excluded participation of 5-HT in PCA reactions, since the animals were thoroughly treated with 48/80, but this point had not been studied specifically. We have now extended our earlier work by investigating both reversed and direct cutaneous anaphylactic reactions, with particular reference to the effect of antihistamine drugs and BOL, in rats whose skin content of both histamine and 5-HT had been greatly reduced. The results indicate that neither histamine nor 5-HT are primarily involved in these skin reactions.

METHODS

Animals. The rats used in these experiments were drawn from the same colony as were those used in our previous studies (Brocklehurst *et al.* 1955).

Depletion and estimation of skin histamine. The procedures for depleting rat skin of histamine by compound 48/80 and for determining the skin histamine content were those described previously (Brocklehurst *et al.* 1955).

Depletion and estimation of skin 5-HT. The use of 48/80 to deplete the skin of histamine also causes a marked reduction in 5-HT (Parratt & West, 1957*b*). We have also used reserpine as an independent means of reducing the 5-HT content of the skin. Large doses of reserpine produce very severe systemic effects including dehydration, which make the animals wholly unsuitable for investigations of skin reactions. We used doses of reserpine of from 0·5 to 2·0 mg/kg, which did not induce prolonged systemic effects, and, in combination with the treatment with compound 48/80, reduced the skin 5-HT to some 10% of normal. A typical course of treatment is shown in Table 1.

TABLE 1. Typical course of treatment for depletion of skin histamine and 5-HT

	Dose (mg/kg)	
Day	Compound 48/80	Reserpine
1	0·8	—
2	1·2 and 1·4	—
3	1·8 and 2·2	—
4	3·0	—
5	3·8	—
6	5·0	—
7	—	1·0
8	—	1·5
10	—	2·0
14	Animals used for experiment	

The method of estimating the amount of 5-HT in the skin was based on that of Parratt & West (1957*a*). 150–200 mg of shaved skin was taken from areas of the thorax and abdomen, adjacent to the reaction site, and freed from subcutaneous fat. This was dropped into 5 ml. of acetone at 0° C and, after being chopped fine with scissors, was kept at 4° C for 36 hr. The supernatant fluid was then collected, and 2 ml. acetone was added to the tissue, which stood for a further 2 hr. The acetone solutions were mixed, and evaporated to dryness at below 10° C; 0·5 ml. water was then added and the sample was kept at −15° C. The 5-HT

content was estimated on the rat uterus, at 30° C, in Tyrode's solution modified according to Schwartz, Masson & Page (1955) and aerated with 95 % oxygen + 5 % CO_2.

Production of reversed PCA reactions. Reversed PCA reactions were produced as before (Brocklehurst *et al.* 1955). The antigens used were (1) crystallized Bovine Serum Albumin (BSA, Armour Laboratories), (2) Human Serum Albumin (HSA. kindly supplied by Dr W. D'A. Maycock, M.R.C. Blood Products Unit, Lister Institute, Elstree) and (3) hen ovalbumin (EA), recrystallized three times. Rabbit antisera were prepared by repeated intravenous injection of antigen adsorbed on alum. The antibody content, measured by quantitative precipitation at optimal proportions, was 5–10 mg/ml. Rat antibodies were prepared as described below under 'Active sensitization'.

Production of direct PCA reactions. The same antigen–antibody systems were used as for the reversed PCA reaction. In preliminary experiments antibody prepared in rabbits or rats was administered intraperitoneally or intravenously to normal rats in doses of 2–12 mg/100 g body weight. Skin reactions to various amounts of intracutaneous antigen were tested 24 or 48 hr later. We found that when antiserum was administered intraperitoneally, whatever the quantity of antiserum used, there was very little response to intracutaneous antigen. When the antiserum was given intravenously, a graded cutaneous anaphylactic response was obtained over a suitable range of test doses of antigen.

Reproducible graded skin responses of convenient size, and with satisfactory dose–response slope, were obtained when 6 mg antibody/100 g was administered intravenously, and the skin test doses of antigen were 0·5–3 μg (in 0·05 ml.). The slope of the dose–response curve was less steep than with PCA reactions, and became very flat at higher doses of antigen.

Active sensitization. Rats are not readily sensitized by the usual techniques, but a method based on that of Lipton, Stone & Freund (1956) was successful. BSA, HSA or EA was incorporated at a concentration of 10 mg/ml. in an oil-in-water emulsion containing killed tubercle bacilli. Two doses of 0·5 ml. were injected intramuscularly one week apart. Five weeks after the second injection 1 mg antigen adsorbed on aluminium hydroxide was administered intravenously, and a further similar injection was given a week later. The second intravenous injections caused temporary prostration (anaphylatic shock), and the precipitating antibody levels subsequently rose to 1·5–7 mg/ml. Ten to twelve days after the last injection the rats were used for tests of active cutaneous anaphylaxis, or were bled in order to provide rat antibodies for use in PCA tests using homologous serum.

Design of experiments. The experimental designs, as before (Brocklehurst *et al.* 1955), included randomization of injections and blind reading of results. Histamine acid phosphate and 5-HT creatinine sulphate were made up as aqueous solutions containing 1 % base, and the pH was adjusted to 7·5 with N/3-NaOH. Subsequent dilutions for intradermal injection were made with Tyrode's solution.

RESULTS

Effect of compound 48/80 *and reserpine on content of* 5-*HT in rat skin*

After the combined course of treatment with compound 48/80 and reserpine described above, the content of 5-HT in the rat skin was found to be reduced by about 90 % at the time when the cutaneous anaphylactic reactions were produced. The results of one experiment showing the extent of variation from animal to animal are given in Table 2.

In most experiments the skin samples (from various groups) were pooled before assay. The mean values were similar. The skin histamine content of these animals at this time of performing the anaphylactic reactions was not more than 15 % of that in control rats. These results agree with the figures

given by Parratt & West (1957*a*) and by Bhattacharya & Lewis (1956). The animals remained in good condition at the end of the course of treatment.

TABLE 2. The effect of treatment with compound 48/80 and reserpine on the 5-HT content of rat abdominal skin. (For dose schedule see Table 1.) The values are μg base/g skin

Control animals	0·66, 0·79, 1·4, 1·56	Mean 1·10
Treated animals	0·1, 0·1, 0·12, 0·15 0·1, 0·1, 0·16, 0·12	Mean 0·12

The effect of antihistamine drugs and BOL on reversed PCA reactions

Both in rats with low skin histamine and 5-HT and in control rats the responses to intracutaneous injections of histamine and 5-HT were used as an index of the effectiveness as inhibitors of mepyramine and BOL. The results are shown in Fig. 1, in which three experiments are combined. It will be seen that BOL (2 mg/kg), freshly prepared and injected intravenously as a 0·2 % aqueous solution about 30 min before the tests were made, reduced the skin response to injected 5-HT somewhat more than fiftyfold. In the skins of rats depleted of 5-HT by combined treatment with compound 48/80 and reserpine, the responses to intracutaneous 5-HT (but not to histamine) were somewhat larger, and the effect of BOL was to reduce these responses even more than in control rats. This may be related to the fact that the skin of rats treated with 48/80 is very thin, and the distribution of an injected dose will consequently be modified. BOL did not diminish the size of the responses to injected histamine, but they were distinctly paler than in control rats. Mepyramine (50 mg/kg intraperitoneally 45 min before testing) reduced the response to histamine from sixty to two hundred times in this and other experiments, without significantly affecting the response to 5-HT.

Reversed PCA reactions were carried out in control rats and in rats pretreated with compound 48/80 and with reserpine, with and without mepyramine and BOL treatment. The results are shown in Fig. 2. The mean diameters of the PCA lesions were very similar in all groups, the greatest variations observed corresponding to less than a twofold increase of dose. It would seem therefore that neither histamine nor 5-HT, separately or together, determine the size of the lesion which results from reversed passive anaphylactic reactions in the rat skin. There were differences, however, in the intensity of the reactions. Rats treated with BOL gave PCA reactions notably paler than rats not so treated. This was observed in rats with normal or greatly diminished skin histamine and 5-HT.

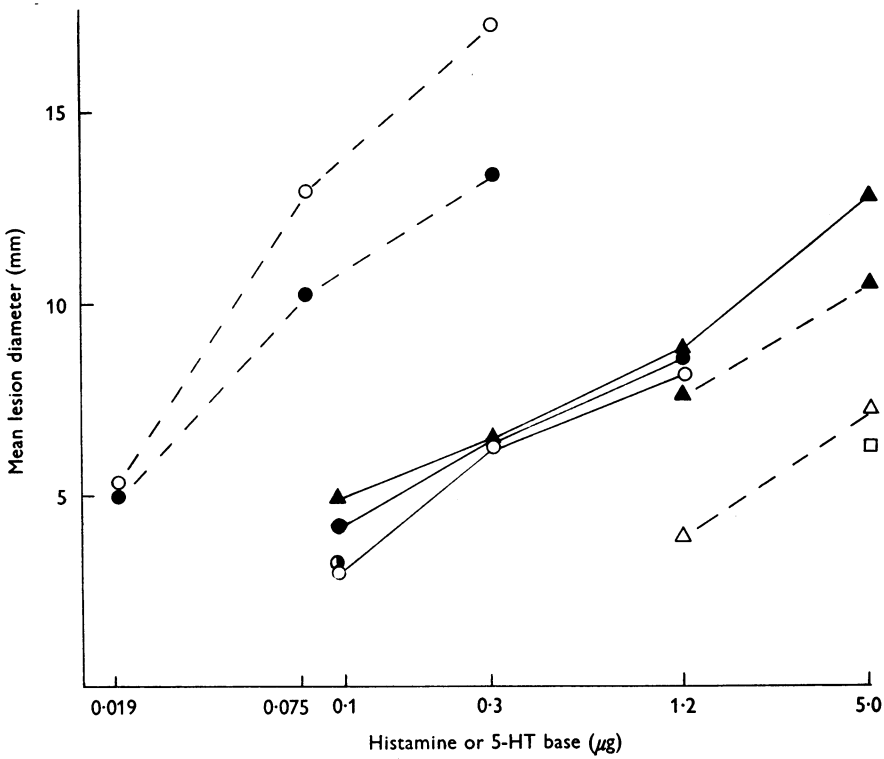

Fig. 1. Diameter of blue areas produced by intracutaneous injection of histamine and 5-HT in normal and treated rats, in the presence and absence of BOL. Filled symbols denote normal rats, open symbols denote rats treated with 48/80 and reserpine: ◑, treated with reserpine only; ●○, no antagonist; △▲, BOL; □, BOL + mepyramine; —— = histamine response; - - - = 5-HT response. Semi-log. scale. In this and subsequent figures each point represents the mean response in 4–10 animals.

Direct cutaneous reactions in actively and passively sensitized rats

When direct PCA reactions were elicited by injection of antigen into the skin of rats passively sensitized with rabbit or rat antibody, the area of the response was not diminished significantly by either mepyramine or BOL, but the intensity of blueing was reduced by BOL. This was particularly evident in the smaller lesions, which were sometimes too pale to be measured. The results of one experiment are given in Table 3.

The combined results of two experiments with rats which had been actively sensitized are shown in Fig. 3. The doses of the inhibitors tested were sufficient to depress the responses to intracutaneous histamine and 5-HT by a factor of well over 100, yet the response to antigen was reduced only twofold by mepyramine, and BOL had no effect on the area and a

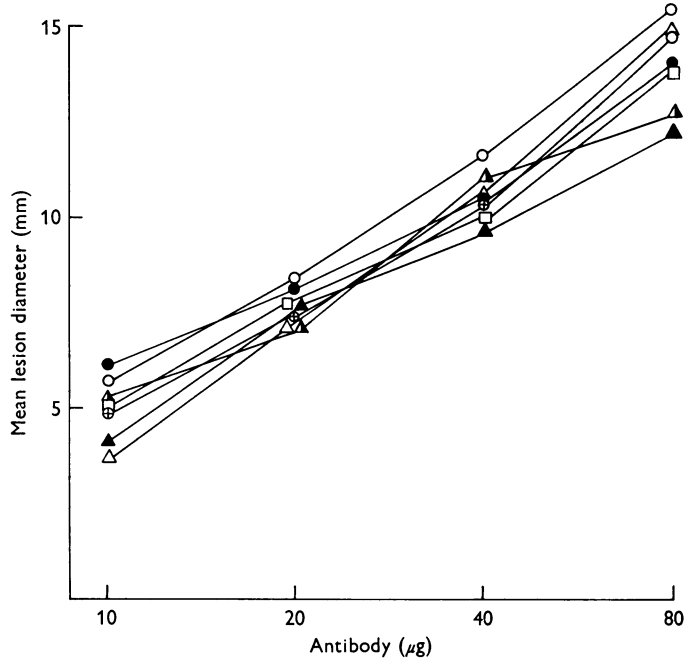

Fig. 2. Diameter of PCA reactions in normal and treated rats in the presence and absence of BOL and/or mepyramine. Filled symbols denote normal rats, open symbols denote rats treated with 48/80 and reserpine: ●○, no antagonist; △▲, BOL; □, BOL + mepyramine; ⊕, treated + mepyramine; ▲, reserpine + BOL. Semi-log. scale.

TABLE 3. Direct cutaneous anaphylaxis in passively sensitized rats. Mean lesion diameter (mm). Intensity of blue indicated on a scale of + to + + + + +

Antagonist	Antigen (μg) 1/8	1/2	2	8
None	4·6 ++	6·8 +++	9·8 +++	11·6 ++++
Mepyramine	3·2 ++	6·6 ++	9·1 +++	11·0 ++++
BOL	5·0 +	6·0 +	8·5 ++	10·4 +++
BOL + mepyramine	Unreadable ±	6·0 +	9·1 ++	12·0 +++

Antigen given intradermally in 0·05 ml. Tyrode solution 44 hr after antibody given I.V. The antagonists reduced the response to 5-HT and histamine by a factor of at least 100.

very slight effect on the intensity of the lesions. Promethazine (20 mg/kg) reduced the size of the lesions produced by antigen, in that, after promethazine, 5–10 times more antigen was required to elicit responses similar to those in untreated rats.

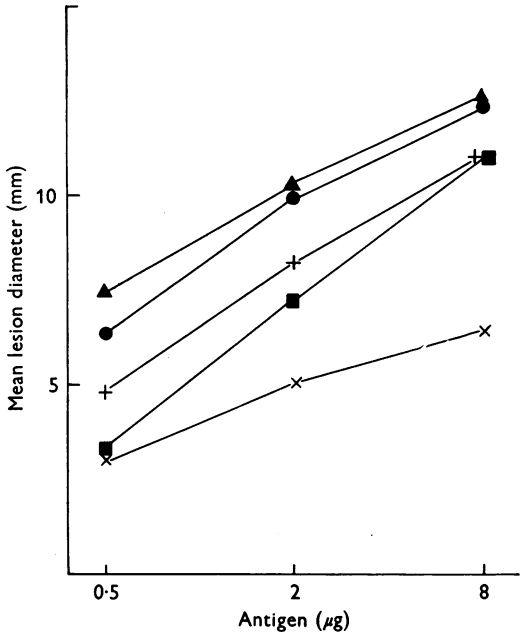

Fig. 3. Diameter of direct cutaneous anaphylactic reactions in actively sensitized rats. The rats were actively sensitized with HSA or EA, and had not been treated with 48/80 and reserpine. Both antigen–antibody systems gave comparable results. ●, no antagonist; ▲, BOL; +, mepyramine; ■, BOL+mepyramine; ×, promethazine. Semi-log. scale.

The effect of promethazine on other cutaneous reactions

Promethazine was the only antihistamine drug significantly to affect cutaneous anaphylactic reactions in this and in our previous series of experiments. Benacerraf & Fischel (1949) found that in the rabbit the Arthus reaction and the effect of an erythrogenic toxin from streptococci were markedly inhibited by comparable doses of promethazine, and concluded that this drug acts directly on capillaries, reducing their susceptibility to damage. Furthermore, promethazine (20 mg/kg), unlike mepyramine, diminished the skin response of rats to 5-HT by about eightfold, in addition to more than a thousandfold reduction of the skin response to histamine.

It was therefore interesting to examine the effect of promethazine on the action of the permeability factor of guinea-pig serum described by Wilhelm,

Miles & Mackay (1955). This factor is activated by dilution or ether fractionation of serum, and is concentrated in the G 2α globulin, a sample of which was kindly provided by Professor A. A. Miles. The factor has been shown by Wilhelm and his colleagues to act independently of histamine, and not to be a histamine liberator in the rat (Wilhelm, Mill, Sparrow, Mackay & Miles, 1958). The effect of promethazine (20 mg/kg) and mepyramine (50 mg/kg) on the areas of increased capillary permeability due to graded doses of the G 2α fraction of guinea-pig serum is shown in Fig. 4.

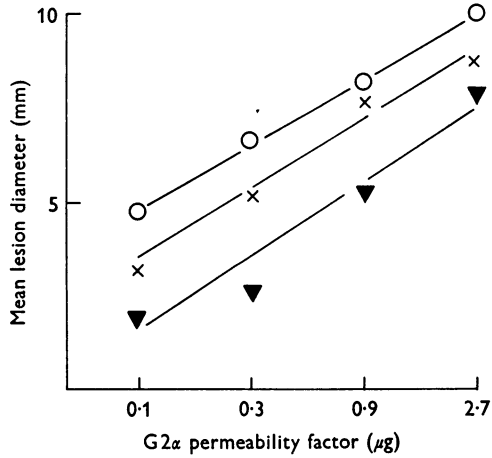

Fig. 4. Diameter of skin reactions to guinea-pig G 2α permeability factor. The material was injected in 0·05 ml. Readings were made on the under side of the skin 25 min after injection. ○, no antagonist; ×, mepyramine 50 mg/kg; ▼, promethazine 20 mg/kg. Semi-log. scale.

It is interesting to note that mepyramine diminished the response about twofold, since Wilhelm *et al.* found that rat permeability factor, tested in the rat, was also weakly inhibited by mepyramine. Promethazine, however, diminished the response some sixfold and affected not only the area but the intensity of the weaker responses, which were relatively pale and faded at the edges.

DISCUSSION

We have extended our earlier observations on reversed PCA reactions to include the use of both heterologous and homologous antibodies and also experiments on direct cutaneous anaphylaxis in actively and passively sensitized rats. In no case have we been able to show that depletion of the skin 5-HT and histamine affects the size of the cutaneous anaphylactic reaction, even when the cutaneous reaction to injected 5-HT and histamine is inhibited with BOL and mepyramine. Two points require some amplification, however. First, BOL definitely reduced the intensity of blueing in

the area of the lesions produced both by intradermal injections of histamine and by cutaneous anaphylactic reactions. Furthermore, in skin depleted of both histamine and 5-HT, mepyramine also decreased the intensity of the anaphylactic reactions. It is known that histamine is released in these reactions and although there is no direct evidence for a release of 5-HT, the effect of BOL suggests that this also occurs. Both histamine and 5-HT therefore may play some part, almost certainly secondary, in increasing the intensity of anaphylactic reactions in rat skin. The underlying mechanism, however, appears not to be dependent on them, a conclusion already reached by Inderbitzin & Craps (1957) and by Sanyal & West (1958). The only tissue reaction which appears to be mediated principally by 5-HT is the anaphylactic contraction of the mouse uterus (Fink & Rothlauf, 1955); this must not be taken to apply generally, since one of us (Brocklehurst, 1958) has shown that the anaphylactic contraction of the rat uterus cannot be suppressed by antagonists of 5-HT.

Secondly, attention is drawn to the action of promethazine. This is the only antihistamine drug which we have found to affect significantly the size of the cutaneous anaphylactic reaction, and Halpern *et al.* (1956) found it even more potent in raising the threshold of this response. This action of promethazine seems not to depend on its antihistamine properties but upon some other non-specific effect on capillaries which is not shared by mepyramine. Inhibition by promethazine is much less obvious in tests with reversed PCA than in direct cutaneous anaphylactic reactions. Halpern *et al.* came to similar conclusions, and have doubted whether reversed PCA reactions should be classed as true anaphylactic reactions. In an appendix to this paper the mechanisms of both reactions are analysed, in order to explain why apparently contradictory results may be obtained with inhibitors, and why reversed PCA reactions are more suitable than direct reactions for the study of the underlying processes of anaphylaxis.

One other point requires discussion, namely the difference in the relation between dose and diameter of skin reactions to injected histamine and to 5-HT. These amines are similar in molecular size; neither binds very markedly to tissues; and the responses of both were measured after 20 min. The slope of the line relating log. dose to response might therefore be expected to be similar also. In fact the slope with 5-HT was the steeper, which would be explained if, in the skin, histamine were inactivated enzymically more rapidly than 5-HT; this explanation receives some support from the observations of Vane (1959) that 5-HT appears to be resistant to the amine oxidases of intact rat stomach preparations whereas amines lacking the –OH group are not.

SUMMARY

1. Reversed passive cutaneous anaphylactic reactions, and direct cutaneous anaphylactic reactions have been studied in passively and actively sensitized rats. The effect upon these reactions of depleting the skin of histamine and 5-HT by compound 48/80 and/or reserpine, and of treatment with mepyramine and/or BOL, has been examined.

2. No evidence was found for significant effects of any of the treatments, alone or in combination, on the size of the anaphylactic responses, although in rats treated with BOL they were paler.

3. Promethazine reduced the anaphylactic responses, but reasons are given for considering this effect to be due to some property other than its antihistamine action.

4. It is concluded that histamine and 5-HT are not primary mediators of the anaphylactic reaction in the rat skin, but play a secondary part.

5. The differences between reversed and direct cutaneous anaphylactic reactions are considered to be due to the mechanical factors determining the spread of antigen which reacts with antibody, and reasons are given for preferring the former for study of antigen–antibody reactions of this kind.

We wish to thank Professor A. A. Miles for the sample of G 2α guinea-pig permeability factor; Messrs Ciba for reserpine; Messrs Burroughs Wellcome for 48/80; Abbott Laboratories for 5-HT; Sandoz for Brom LSD; Specia for promethazine; and Messrs May and Baker for mepyramine.

APPENDIX

Comparison of direct (active and passive) and reversed passive cutaneous anaphylactic reactions

When any locally active agent is injected into, or produced in, the skin, the size of the response will depend on the area over which a threshold concentration is reached and maintained long enough for the response to appear. The mechanical factors involved in intracutaneous injections in the guinea-pig have been discussed by Miles & Miles (1952); in the rat skin, which is thinner, the process is probably similar, but the fluid mass will be retained at the injection site for a shorter time. Spread of injected material probably occurs by a combination of mass movement of fluid in the connective tissue, and, to a lesser extent, by diffusion. It will be limited by any process which inactivates the material, and by adsorption on to tissues. The areas over which a response is produced by two different concentrations of the agent have been found experimentally to be proportional to the log. dose. Rate of spread is therefore proportional to the amount present, resembling in this respect processes controlled mainly by diffusion. A mathematical treatment of such processes has been given by Humphrey & Lightbown (1952), which predicts that the slope will be increased the longer the interval between injection of the test material and the reading of the reaction.

In the case of reversed PCA the injected antibody spreads unimpeded in the skin, except for slight adsorption, during 3–4 hr before antigen and indicator dye are administered intravenously. There is evidence that antigen–antibody combination is most damaging when antigen is present in moderate excess (Rosenberg, Chandler & Fischel 1958; Trapani, Garvey & Campbell, 1958). Since, in PCA, excess antigen is injected, this condition is

reached rapidly and simultaneously over the whole area and produces a reaction wherever antibody is present at a supraliminal concentration. A steep slope therefore results. In direct anaphylactic reactions, on the other hand, when graded doses of antigen are given in a fixed volume intracutaneously, they reach only a limited area of skin and there the reaction begins at once. The skin contains a uniform concentration of antibody, and, depending on this concentration and the amounts of antigen used, much of the antigen will combine with the antibody at the injection site, and will not be free to spread over a wider area. Only where there is excess of antigen will the chain of events leading to increased capillary permeability be started, but a large excess of antigen will not necessarily be more effective than a moderate excess. Furthermore, the reaction is read after 25 min, which is a very short time for any antigen remaining uncombined to spread over a wider area and enlarge the lesion. These are conditions which will produce a small slope.

In studying passive direct cutaneous anaphylactic reactions we found that suitable doses of antigen were related to the amount of antibody given, and that the range was rather critical. In the lower dose ranges the slopes of the dose–response curves were sometimes very small, while at higher dose levels the curves showed a plateau. With still higher doses the responses consisted only of a blanched central area with a surrounding indefinite ring of blue. Furthermore, antibody levels vary quite widely in a group of actively sensitized animals; the same dose of antigen may therefore not be optimal for all the animals in the group. For this reason passive sensitization is to be preferred for quantitative work, although it perhaps resembles natural sensitization less closely than does active sensitization. The use of a threshold reaction (in which the end point is the smallest dose of antigen able to produce a detectable lesion) has severe limitations because it gives no indication of the relation between dose and response. An agent such as promethazine, which in our experiments reduced the vascular response to various different agents several fold, apparently non-specifically, could raise the concentration of antigen needed to produce a threshold reaction by a large factor, as indeed was found by Halpern *et al.* (1956). However, there need be no quantitative relation between this increased threshold and, on the one hand, the specific inhibition of the anaphylactic reaction, or, on the other, the antagonism of substances acting directly, such as histamine and 5-HT. For example, if the level of sensitization were such that over a wide range of antigen test doses the effect of the antigen–antibody reaction on capillary permeability exceeded the threshold by a relatively small amount, a non-specific agent capable of raising the threshold by this amount would completely prevent the skin from showing a response, irrespective of the dose of antigen.

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