Immunology, 1959, **2**, 403.

The Mechanism of Action of Anaphylatoxin. Its Effect on Guinea Pig Mast Cells

I. MOTA

Laboratory for Cell Physiology, Department of Histology, Faculty of Medicine, Univ. of S. Paolo, Brazil

Summary. In the guinea pig anaphylatoxin produces mast cell damage that is similar to that produced by antigen-antibody reaction but is different from that produced by chemical histamine liberators. This damage is inhibited by iodoacetate, *p*-chloromercuribenzoate, phenol and cold, but is not inhibited by calcium lack or previous heating of the tissue to 45° C. Mepyramine reduces but does not abolish the contraction of the ileum produced by anaphylatoxin. Previous heating of a sensitized ileum to 45° C., although completely abolishing the anaphylactic response, does not interfere with the contraction induced by anaphylatoxin. Furthermore desensitization to anaphylatoxin does not modify the anaphylactic contraction. The implication of anaphylatoxin with the anaphylactic reaction is discussed. It is concluded: (*a*) that anaphylatoxin shares with the anaphylactic reaction part of the pathway leading to histamine release; (*b*) that the mechanism of action of anaphylatoxin is quite different from that of the chemical histamine liberators.

INTRODUCTION

WE recently reported that mast cell alterations promoted by antigen in sensitized guinea pig tissues occur simultaneously with histamine release, and that both processes were inhibited alike by several agents. It was also shown that mast cell alterations produced by compound 48–80 and octylamine are morphologically different from those induced by antigen and that they can also be distinguished by the effect of several inhibitors of anaphylaxis (Mota, 1958).

It is well known that anaphylatoxin releases histamine from guinea pig tissues (Rocha e Silva, Bier and Aronson, 1951), but whether anaphylatoxin acts by a mechanism similar to that of antigen or to that of chemical histamine liberators is not yet known. Anaphylatoxin has been suggested as a mediator in the mechanism of anaphylaxis (Rocha e Silva, 1954; Hahn, 1957) and it seemed interesting to find out whether anaphylatoxin causes mast cell damage, whether such damage is similar to that induced by antigen and how far the inhibitors of anaphylaxis would also inhibit anaphylatoxin. The present paper is an account of our results.

MATERIALS AND METHODS

Guinea pigs of either sex weighing 250–350 g. were used. Mesentery from sensitized guinea pigs, which releases histamine in contact with antigen or histamine liberators (Mota, 1959) and is a suitable tissue in which to observe mast cell damage, was used throughout in this paper. The mesentery was dissected away from the small intestine and cut into several small pieces which were dropped into cold Tyrode solution. Three or four of these pieces were used as a sample. The samples were incubated for 15 min. with 0·5 ml. of Tyrode solution or Tyrode solution containing the required concentration of

inhibitor. 0·5 ml. of anaphylatoxin was then added and the tissue was incubated for a further period of 15 min. For the microscopical observation of mast cells the mesentery was fixed with a lead subacetate solution in acidified alcohol as previously described (Mota and Vugman, 1956), stained with 0·5 per cent toluidine blue in aqueous solution and examined as a whole-mount preparation. The mast cells in thirty microscopical fields were counted with the aid of a square-ruled ocular micrometer, magnification ×80.

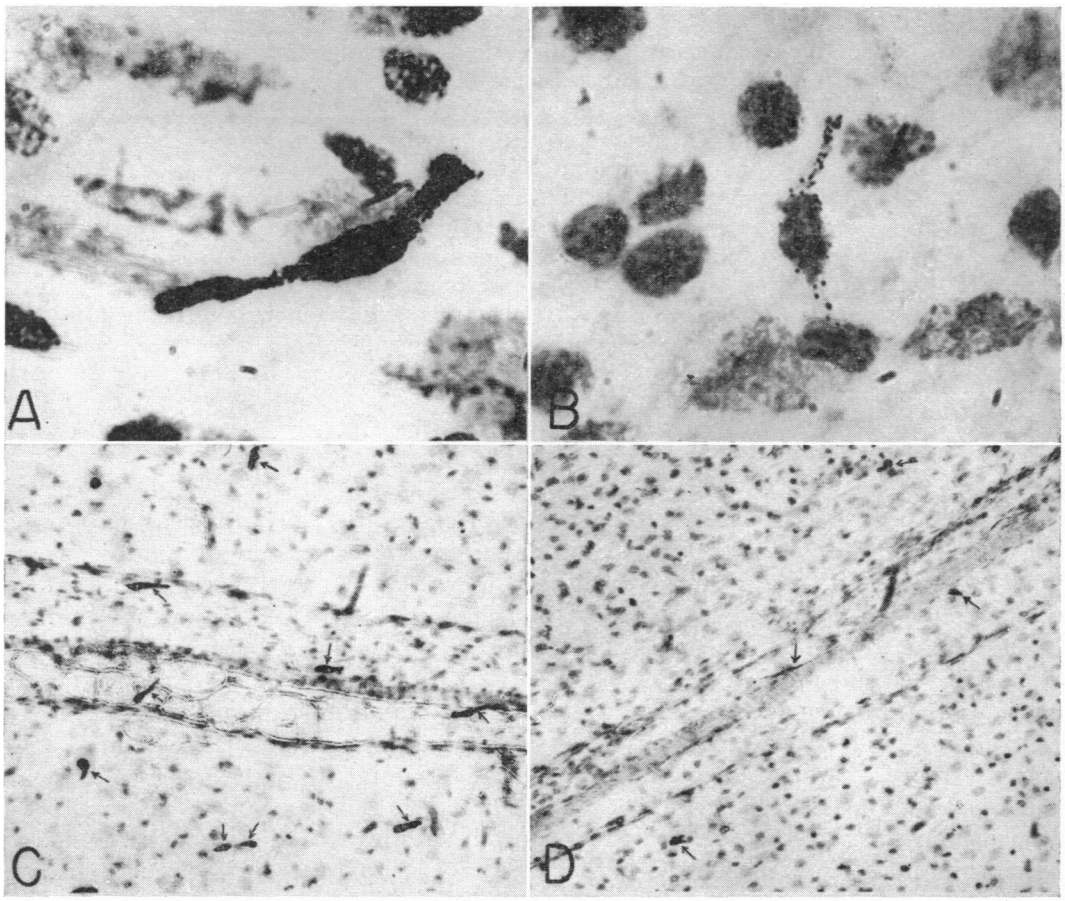

FIG. 1. Photomicrographs of whole-mount guinea pig mesentery after fixation in lead subacetate-ethanol-acetic acid and staining in toluidine blue. (A) Mast cell in guinea pig mesentery incubated in Tyrode solution. Observe cell crowded with granules. × 1000. (B) Mast cell in guinea pig mesentery incubated with anaphylatoxin. Observe that the cell has lost most of its granules. × 1000. (C) Mast cells in mesentery incubated in Tyrode. solution. Observe several mast cells (arrows), some of them following the vessels. × 250. (D) Mast cells in guinea pig mesentery incubated with anaphylatoxin. Observe reduction in mast cell number. × 250.

Anaphylatoxin was prepared by incubating rat serum or heparinized plasma with agar (1 mg./ml.) at 37° for 30 min. Agar was removed by centrifuging and the supernatant was dialysed against cold saline for 24 hours. The activity was tested on the isolated guinea-pig ileum and the activated serum or plasma kept at 4° C. until use.

Guinea pigs were sensitized by injecting a 5 per cent solution of ovalbumin or bovine

γ-globulin (1 ml. subcutaneously and 1 ml. intraperitoneally) and were used three weeks later.

The experiments with the isolated ileum were performed in a Dale's apparatus with 7 ml. capacity.

RESULTS

EFFECT OF ANAPHYLATOXIN ON MAST CELLS

When pieces of mesentery were incubated with anaphylatoxin mast cell damage resulted, characterized by partial or complete disappearance of their granules (Fig. 1) and consequent reduction in the number of cells containing metachromatic granules (Table 1). This reduction is variable and similar to that induced by antigen in sensitized tissues (Mota, 1959). Under similar conditions mesenteries incubated with non-activated rat serum or plasma showed no mast cell alterations. Moreover when the serum was heated at 56° for half an hour before incubation with agar, to abolish anaphylatoxin formation, subsequent contact of the mesentery with this preparation caused no mast cell damage.

TABLE 1

MAST CELL COUNTS* IN GUINEA PIG MESENTERY INCUBATED WITH TYRODE SOLUTION OR ANAPHYLATOXIN

Guinea Pig	*Mast Cell in*	
	Tyrode	*Anaphylatoxin*
1	28	20
2	31	16
3	30	14
4	25	21
5	20	13
6	23	13
7	29	17
8	20	13

* Means of 30 microscopical fields at a magnification of ×80.

EFFECT OF INHIBITORS OF ANAPHYLAXIS ON MAST CELL DAMAGE INDUCED BY ANAPHYLATOXIN

Since mast cell damage by anaphylatoxin resembled closely that produced by antigen in sensitized tissue, several agents known to inhibit anaphylaxis (Mongar and Schild 1957a, b; 1958) were tested for their effect on mast cell damage induced by anaphylatoxin. The results of these experiments are summarized in Fig. 2.

Iodoacetate, p-chloromercuribenzoate and Phenol

When iodoacetate (0·001 M), *p*-chloromercuribenzoate (0·001 M) or phenol (0·01 M) were left in contact with pieces of mesentery at 37° for 15 minutes before the addition of anaphylatoxin the destruction of mast cells by anaphylatoxin was greatly reduced.

Effect of Calcium Lack

Pieces of mesentery were incubated for 5 min. in calcium-free Tyrode containing 0·01 per cent "Versene" and then transferred to anaphylatoxin containing 0·1 per cent of "Versene" at 37° for 15 min. Mast cell counts showed that calcium lack did not inhibit mast cell damage by anaphylatoxin.

Effect of Previous Heating of the Tissue to 45°

Pieces of mesentery were kept at 45° for 5 min. and restored to 37°. When the mesentery was subsequently incubated with anaphylatoxin it was found that this treatment had produced no inhibition of mast cell damage by anaphylatoxin.

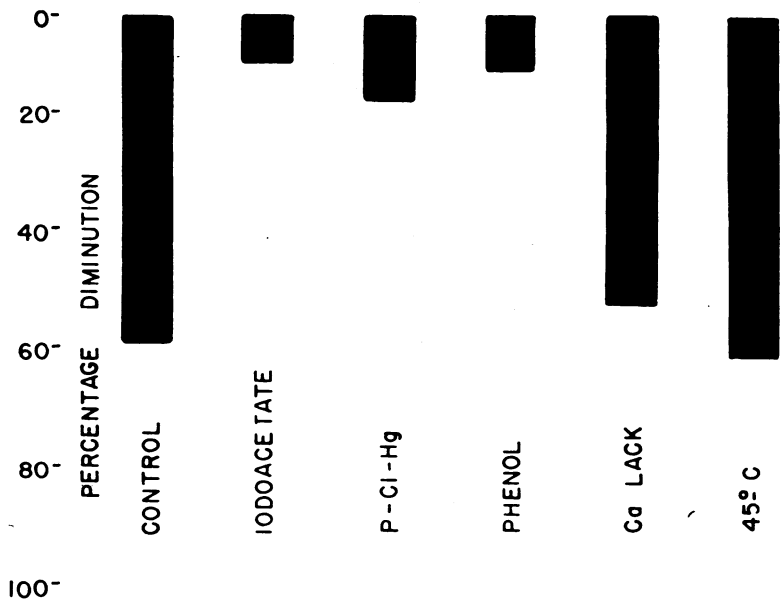

FIG. 2. Effect of various inhibitors of anaphylaxis on mast cell reduction induced by anaphylatoxin. Mast cell diminution is expressed as a percentage of mast cell number in the non-treated mesentery sample. Control represents the reduction induced by anaphylatoxin without inhibitor.

Effect of Cold

In order to test whether low temperature would inhibit mast cell damage by anaphylatoxin, pieces of mesentery were kept in Tyrode solution or in anaphylatoxin at 15° for 15 min. and then transferred to anaphylatoxin at 37° for a further period of 15 min. and fixed. Some of the pieces incubated with anaphylatoxin in the cold were transferred directly to fixative. These experiments showed that cold inhibits mast cell damage by anaphylatoxin, and that previous contact with anaphylatoxin in cold almost completely abolishes the mast cell damage induced by later contact with anaphylatoxin at 37° (see Fig. 3).

Desensitization to Anaphylatoxin in the Presence of Phenol

Phenol is known to inhibit mast cell degranulation induced by antigen without inhibiting desensitization (Mota, 1959). Experiments were done to test whether phenol would also

inhibit mast cell degranulation by anaphylatoxin without preventing desensitization to a further dose of anaphylatoxin. Pieces of mesentery were incubated for 15 min. with phenol (0·01 M) in Tyrode solution. Phenol was then removed, the mesentery washed in Tyrode solution and transferred to anaphylatoxin. In other experiments anaphylatoxin was added to mesentery in the presence of phenol; after 15 min. the mesentery was washed in phenol solution to remove anaphylatoxin, then in Tyrode solution to remove phenol, and finally a second dose of anaphylatoxin was added.

The results were as follows: (*a*) Prior contact of mesentery with phenol did not prevent mast cell degranulation on subsequent incubation with anaphylatoxin; (*b*) when a first dose of anaphylatoxin was added in the presence of phenol and a second dose in the absence of phenol there was no reduction in mast cell number. This implies that contact of the tissue with anaphylatoxin in the presence of phenol inhibited mast cell degranulation without inhibiting desensitization.

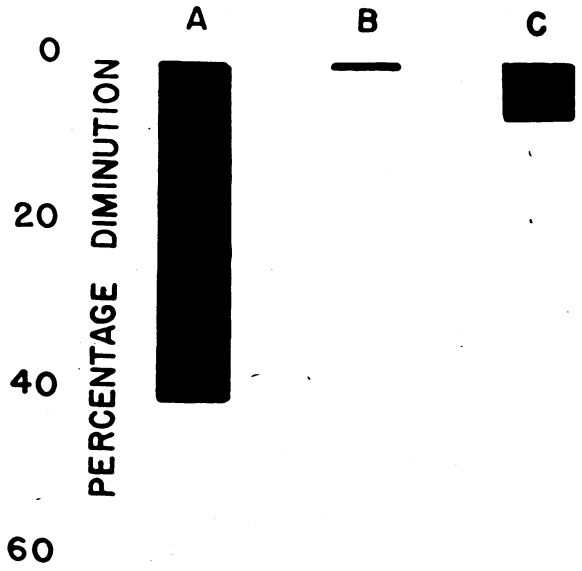

FIG. 3. Effect of cold on mast cell reduction induced by anaphylatoxin. In *A* the mesentery was kept in Tyrode solution at 15° and then transferred to anaphylatoxin at 37°. In *B* the mesentery was incubated with anaphylatoxin at 15° and then fixed. In *C* the mesentery was kept in anaphylatoxin at 15° and then transferred to anaphylatoxin at 37°.

EXPERIMENTS WITH THE ISOLATED GUINEA PIG ILEUM

Anaphylatoxin causes a strong contraction of the isolated guinea pig ileum, followed by desensitization (Rothschild and Rocha e Silva, 1954). A typical response to anaphylatoxin is shown in Fig. 4. There is a rapid and transient contraction followed by a prolonged contraction at a lower level. Such a response is very similar to that given by a sensitized ileum on contact with antigen. It is known that antihistamines do not completely antagonize the anaphylactic response of the ileum (Hawkins and Rosa, 1956), that phenol prevents the response of this organ to antigen without preventing desensitization, and that previous heating of the ileum to 45° completely abolishes its anaphylactic response (Mongar and Schild, 1957b). Thus the effect of these agents on the response of the ileum to anaphylatoxin was studied.

Effect of Antihistamines

The effect of mepyramine on the contraction of guinea pig ileum produced by anaphylatoxin is shown in Fig. 4. Whereas a control piece of ileum gave a typical biphasic response

on addition of 0·2 ml. of anaphylatoxin to the bath, when the same dose of anaphylatoxin was added to another piece of the same ileum in presence of mepyramine 10^{-6} mg./ml. only a slow contraction was obtained. This result was obtained consistently, although more obvious with some ilea than with others. Higher doses of mepyramine (10^{-4} mg./ml.), however, completely inhibited the response of the ileum to anaphylatoxin.

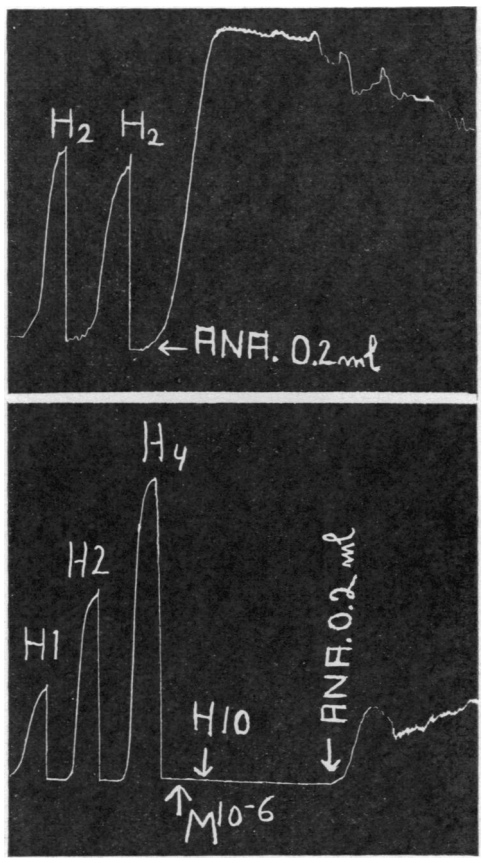

FIG. 4. Effect of mepyramine on the contraction of the ileum induced by anaphylatoxin. A control piece in Tyrode solution (above) gave a typical and maximal contraction with 0·2 ml. of anaphylatoxin, while a piece of the same ileum in presence of mepyramine (M) 10^{-6} mg./ml. responded with very small contraction to the same dose of anaphylatoxin. H, histamine 10^{-7} mg./ml.

Experiments with phenol

The effect of phenol (0·01 M) on the response of the ileum to anaphylatoxin is shown in Fig. 5. While the addition of 0·1 ml. of anaphylatoxin to a control piece of ileum produced a maximal response, another piece of the same ileum gave no response to 0·5 ml. of anaphylatoxin when this was added in presence of phenol and left in contact with the tissues 15 min. Furthermore when phenol was washed out, restoring the sensitivity to histamine, 0·5 ml. of anaphylatoxin added to the bath produced only a small contraction. The tissue was partially desensitized to anaphylatoxin, but complete desensitization was never obtained.

Effect of Previous Heating to 45°

Ileum from a sensitized guinea pig was divided into two pieces. One was kept at 37° as a control and the other held at 45° for 15 min. The pieces were then suspended in

oxygenated Tyrode solution at 37° and the effect of anaphylatoxin and antigen tested. A control piece gave a maximal contraction to both antigen and anaphylatoxin. The piece heated to 45° gave no response to antigen but gave a maximal response to anaphylatoxin, showing that previous heating to 45°, although completely abolishing the anaphylactic response, does not interfere with the contraction induced by anaphylatoxin (see Fig. 6).

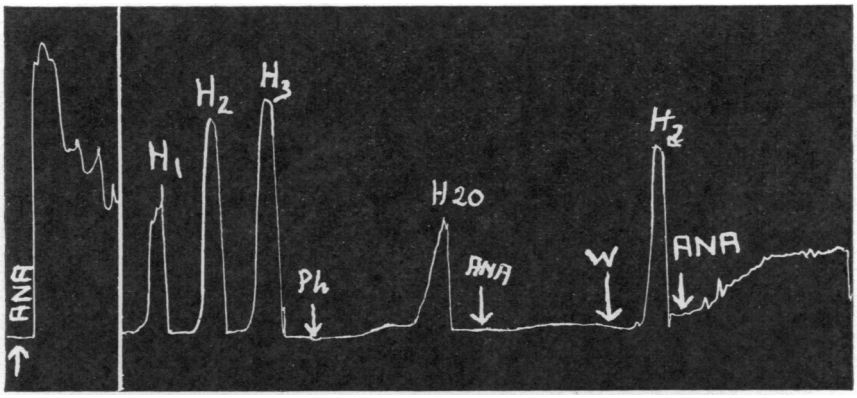

FIG. 5. Effect of phenol on the contraction of the ileum induced by anaphylatoxin. In the left side of the figure a control piece responded to 0·1 ml. of anaphylatoxin (ANA) with a maximal contraction. After the addition of phenol (Ph) the response to histamine was reduced and 0·5 ml. of anaphylatoxin produced no contraction. After washing (W) sensitiveness to histamine was restored and 0·5 ml. of anaphylatoxin produced a very small response. H, histamine 3×10^{-8} mg./ml.

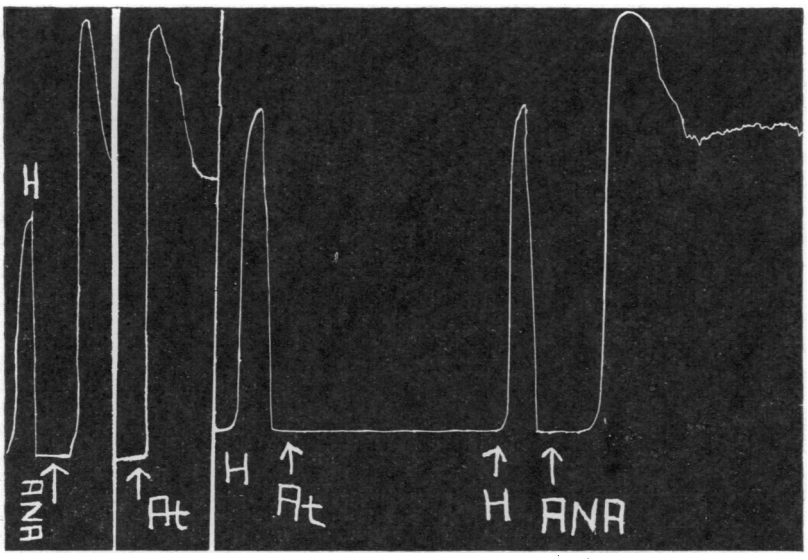

FIG. 6. Effect of previous heating of the tissue to 45° on the contraction induced by anaphylatoxin or antigen. On the left side of the picture 0·1 ml. of anaphylatoxin or 0·1 mg. of antigen (ovalbumin) produced a maximal contraction in a control non-heated piece of sensitized ileum. In another piece of the same ileum previously heated to 45° antigen (At) produced no response but anaphylatoxin (ANA) produced a maximal contraction. H, histamine 5×10^{-8} mg./ml.

Effect of Previous Desensitization to Anaphylatoxin on the Anaphylactic Response

It is known that desensitization (tachyphylaxis) follows a number of successive additions of a same dose of anaphylatoxin and some experiments were performed to find out whether desensitization to anaphylatoxin would abolish or modify the anaphylactic contraction. Successive increasing doses of anaphylatoxin were added to a piece of sensitized ileum until 1 ml. of anaphylatoxin produced no response. Antigen (ovalbumin or bovine γ-globulin) was then added. In all experiments the addition of antigen induced a typical and maximal anaphylactic contraction. Furthermore even when a piece of ileum was immersed in undiluted anaphylatoxin, which produced a maximal and sustained contraction, and was left so for 45 min., the subsequent addition of antigen produced the usual anaphylactic response. It was observed, however, that in a piece of ileum that had been completely desensitized to anaphylatoxin the induction of a anaphylactic contraction restored the sensitivity to anaphylatoxin (Fig. 7). This seemed to be a specific effect of the anaphylactic contraction, since neither a strong contraction induced by histamine nor the addition of antigen (ovalbumin or bovine γ-globulin) to a non-sensitized piece of ileum restored the sensitivity of the tissue to anaphylatoxin.

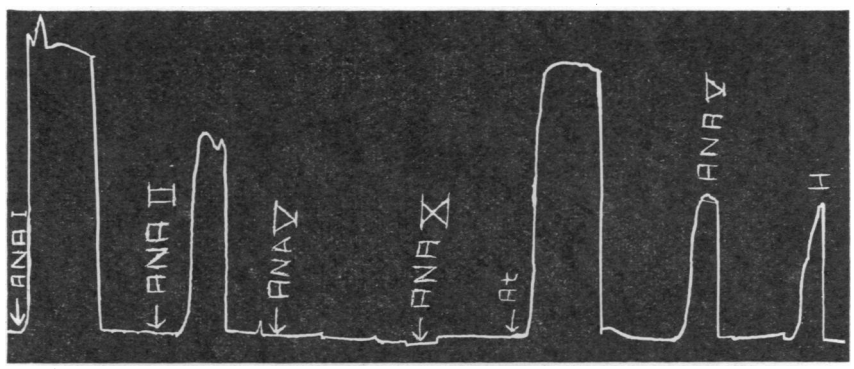

FIG. 7. Effect of desensitization to anaphylatoxin on the anaphylactic contraction. Each dose of anaphylatoxin (ANA) was added at five minutes interval during which anaphylatoxin was left in contact with the tissue. After desensitization to 1 ml. of anaphylatoxin 0·1 mg. of antigen (At = bovine γ-globulin) still produced a maximal contraction. After this contraction anaphylatoxin in an amount that had previously produced no contraction was again able to contract the ileum. ANA I = 0·1 ml., ANA II = 0·2 ml., ANA V = 0·5 ml. and ANA X = 1 ml. of anaphylatoxin. H, histamine 2×10^{-8} mg./ml.

DISCUSSION

It is an old observation that incubation of guinea pig serum and antigen-antibody complex activates some toxic principle called anaphylatoxin, so that reinjection of the activated serum into a normal guinea pig produces a shock syndrome very similar to anaphylactic shock. The same result is obtained by incubating rat or guinea pig serum or plasma with agar and several other substances, and rat anaphylatoxin is stronger than guinea pig anaphylatoxin. More recently it was showed that antihistamines protect guinea pig from fatal anaphylatoxin shock (Hahn and Oberdoff, 1950) and that anaphylatoxin releases histamine from perfused guinea pig lungs (Rocha e Silva, Bier and Aronson, 1951).

We have shown not only that anaphylatoxin damages the same cell as the antigen-

antibody reaction, but that this damage is like that caused by antigen and quite unlike that caused by compound 48–80 or octylamine.

When the effect of the inhibitors of anaphylaxis on anaphylatoxin is analysed there are points of similarity and discrepancy as can be seen in Table 2. The discrepancies, however, are more apparent than real. The failure of calcium lack to prevent mast cell damage by anaphylatoxin agrees with previous experiments by Rocha e Silva (1954) showing that calcium, although necessary for anaphylatoxin formation, is not necessary for anaphylatoxin action. Anaphylatoxin formation could be secondary to antigen-antibody combination and a factor in the production of the anaphylactic reaction, and yet absence of calcium could inhibit histamine release and mast cell destruction by antigen. As regards the effect of heating to 45°, it seems reasonable to suppose that such treatment might not prevent the action of preformed anaphylatoxin, and yet destroy some factor necessary for the sequence of events leading to the formation of anaphylatoxin in anaphylaxis.

TABLE 2

EFFECT OF VARIOUS TREATMENTS ON MAST CELL DAMAGE INDUCED BY ANAPHYLATOXIN, ANTIGEN OR OCTYLAMINE

Treatment	*Inhibition of Mast Cell damage induced by*		
	Anaphylatoxin	*Antigen**	*Octylamine**
Iodoacetate	+P	+P	—
P-Cl-Hg benzoate	+P	+P	—
Phenol	+P	+P	—
Cold	+T	+T	+T
Ca++lack	—	+T	—
Heating to 45° C.	—	+T	—

+P Partial inhibition
+T Total inhibition
— No inhibition
* Data from Mota, 1959.

The fact that iodoacetate and *p*-chloromercuribenzoate, which inhibit histamine release and mast cell damage by antigen (Mongar and Schild, 1957a; Mota, 1959), also inhibit mast cell destruction by anaphylatoxin agrees with results of Rothschild and Rocha e Silva (1956) showing that these substances inhibit histamine release by anaphylatoxin, and with results of Moussatche and Danon (1956) who showed that sulphydryl blocking agents inhibit the response of the guinea-pig ileum to anaphylaxis and anaphylatoxin.

The results of the experiments with phenol are interesting, since they suggest that anaphylatoxin acts indirectly by activating some transient intermediate substance, perhaps an enzyme. Furthermore the fact that desensitization to anaphylatoxin occurred in the cold suggest that anaphylatoxin may activate this substance on the mast cell surface, since anaphylatoxin would not diffuse into a living cell.

The experiments with the isolated ileum show that rat anaphylatoxin is able to produce a strong contraction of this organ, as already described by Rothschild and Rocha e Silva (1954), and that this contraction is very similar to that induced by anaphylaxis and is partially abolished by low concentrations, and totally abolished by higher concentrations of mepyramine.

Anaphylatoxin could be introduced in the scheme for the anaphylactic reaction proposed by Mongar and Schild (1957b) by assigning to it the role of their active enzyme, or of a factor activated by this enzyme. If so part of the histamine releasing mechanism of the antigen-antibody reaction would be common to that of anaphylatoxin, as follows:

Antigen + Antibody + Enzyme precursor I (inactivated at 45°)
↓
Active enzyme I + Anaphylatoxin precursor
↓ Ca++
Anaphylatoxin + Enzyme precursor II
↓
Active enzyme II + Bound histamine (cold, phenol, iodoacetate would act here)
↓
Free histamine

However, the fact that a piece of ileum completely desensitized to anaphylatoxin still gives a maximal anaphylactic response is hard to reconcile with the hypothesis that anaphylatoxin is a mediator in the chain reaction of anaphylaxis. This agrees with observations by Dale and Kellaway (1922) showing that sensitized guinea pigs' tissues rendered refractory to anaphylatoxin are still able to present anaphylactic shock when challenged with the specific antigen and with more recent observations by Greisman (1958). Nevertheless if anaphylatoxin were formed in the tissues during anaphylaxis it might well be more active than anaphylatoxin added from the outside. If this were so, our observation that the anaphylactic contraction restores the sensitivity of desensitized tissue to anaphylatoxin might make it not so difficult to accept anaphylatoxin as a mediator in anaphylaxis, since the antigen-antibody reaction might rapidly restore the sensitivity of the tissue to anaphylatoxin. Furthermore the possibility remains that the anaphylatoxin-like substance that might be formed into guinea pig tissues during anaphylaxis is not quite identical with rat blood anaphylatoxin. Thus until direct experimental proof is obtained, the implication of blood anaphylatoxin in anaphylaxis remains uncertain. However, our present findings do suggest that anaphylatoxin shares with the anaphylactic reaction part of the pathway leading to histamine release. They also suggest that the mechanism of action of anaphylatoxin is quite different from that of the chemical histamine liberators.

ACKNOWLEDGMENT

The author is much indebted to Dr. J. H. Humphrey, National Institute for Medical Research, London, for his valuable help and advice.

REFERENCES

DALE, H. H. and KELLAWAY, C. H. (1922). 'Anaphylaxis and anaphylatoxins.' *Phil. Trans. B.*, **211**, 273–315.

GREISMAN, S. E. (1958). 'The role of anaphylatoxin during *in vitro* anaphylaxis.' *J. Immunol.*, **81**, 214–19.

HAHN, F. (1957). 'On anaphylatoxin.' *Int. Arch. Allergy*, **11**, 119–28.

HAHN, F. and OBERDOFF, A. (1950). 'Antihistaminica und Anaphylaktoide Reaktionen.' *Z. Immun.-Forsch.*, **107**, 528–38.

HAWKINS, D. F. and ROSA, L. M. (1956). 'Some discrepancies in the histamine theory of anaphylaxis in smooth muscle.' Ciba Foundation Symposium on Histamine, p. 180. London, Churchill.

MONGAR, J. L. and SCHILD, H. O. (1957a). 'Inhibition of the anaphylactic reaction.' *J. Physiol.*, **135**, 301–19.

MONGAR, J. L. and SCHILD, H. O. (1957b). 'Effect of temperature on the anaphylactic reaction.' *J. Physiol.*, **135**, 320–38.

MONGAR, J. L. and SCHILD, H. O. (1958). 'The effect of calcium and *p*H on the anaphylactic reaction.' *J. Physiol.*, **140**, 272–84.

MOTA, I. (1958). 'Mast cells and anaphylaxis.' *J. Physiol.*, **140**, 6P.

MOTA, I. (1959). 'Effect of the antigen-antibody reaction on mast cells and histamine content of

REFERENCES (*continued*)

guinea-pig tissues. Comparison with octylamine action.' *J. Physiol.* (In the press.)

MOTA, I. and VUGMAN, I. (1956). 'Effects of anaphylactic shock and compound 48/80 on the mast cells of the guinea-pig lung.' *Nature, Lond.*, **177**, 427–29.

MOUSSATCHE, H. and DANON, A. P. (1956). 'Inhibition by sulphydril blocking agents of the guinea-pig gut reaction to anaphylaxis and to anaphylatoxin.' *Naturwissenschaften*, **10**, 227.

ROCHA E SILVA, M. (1954). 'Anaphylatoxin and histamine release.' *Quart. Rev. Allergy*, **8**, 220–38.

ROCHA E SILVA, M., BIER, O. and ARONSON, M. (1951). 'Histamine release by anaphylatoxin.' *Nature, Lond.*, **168**, 465.

ROTHSCHILD, A. M. and ROCHA E SILVA, M. (1954). 'Activation of a histamine-releasing agent (anaphylatoxin) in normal rat plasma.' *Brit. J. exp. Path.*, **35**, 507–18.

ROTHSCHILD, A. M. and ROCHA E SILVA, M. (1956). 'Mecanismo da liberação de histamina por anafilatoxina, composto 48–80 e soluçoes hipotônicas.' *Cienc. e Cult.*, **8**, 180.