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52. MECHANISM OF HISTAMINE RELEASE BY ANIMAL VENOMS

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The ability to release histamine from tissues is a pharmacodynamic property of many animal venoms. Although not generally considered as a major cause of the lethal actions of such venoms, the hypotension and increased peripheral vascular permeability which are produced by histamine, can enhance the toxic actions of other venom components, as well as of endogenous factors released by the venom in the bitten subject. In this presentation I shall limit myself to a discussion of the biochemical aspects of the release of histamine induced by two venoms: that of the Brazilian rattlesnake *Crotalus durissus terrificus* and that of the honey bee *Apis mellifera*. The results to be presented are mostly those of our own studies, performed in collaboration with Dr. Mercedes P. de Oliveira, A. Castania, Vera Portieri and Suzana Ribeiro da Costa.

It has been known since 1956 that crotamine, a basic protein found in the venom of CROTALIDAE of southern Brazil (1), is capable of releasing histamine from rat tissues. This result was first obtained by Moura Gonçalves and Rocha e Silva (2) using the perfused hind leg preparation of the rat; the authors concluded that crotamine is the cause of the well-known histamine releasing ability of this crotalic venom. There are however, certain varieties of Brazilian rattlesnakes, more abundant in the northern and central regions of the country, which do not contain crotamine in their venoms. In a study of the pharmacodynamic properties of such venoms, we noted that they were highly active histamine releasing agents, having a potency equal or higher than that of crotamine itself. Fig. 1 shows the release of histamine from isolated rat mast cells by crotamine and crotamine-free whole rattlesnake venom. In this, as well as in most subsequent studies, histamine release was assayed on the washed mast cells isolated from the peritoneal cavity of the rat. Such cells are good representatives of the tissue-bound forms of these cells which, as it is well-known, are histamine storage sites in many species. It is by the stimulation of their granular secretion or by unspecific cytolytic damage, that histamine release is brought about *in vivo*.

It is known that the so-called hemolytic snake venoms are often powerful histamine releasing agents *in vivo* or in perfused tissues. Trethewie in Australia, has actually suggested (3) that these two activities e.g. hemolysis and amine releasing action are due to the same chemical entity. We have tried to verify whether in crotamine-free rattlesnake venom, histamine-releasing activity was associated with hemolytic or rather, to use a more precise term, phospholipase A

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activity. To this end, we made a chromatographic analysis of crotamine-free rattlesnake venom on a Amberlite Irc-50 ion-exchange column according to the technique described by Habermann (4). Fig. 2 shows the results. It can be

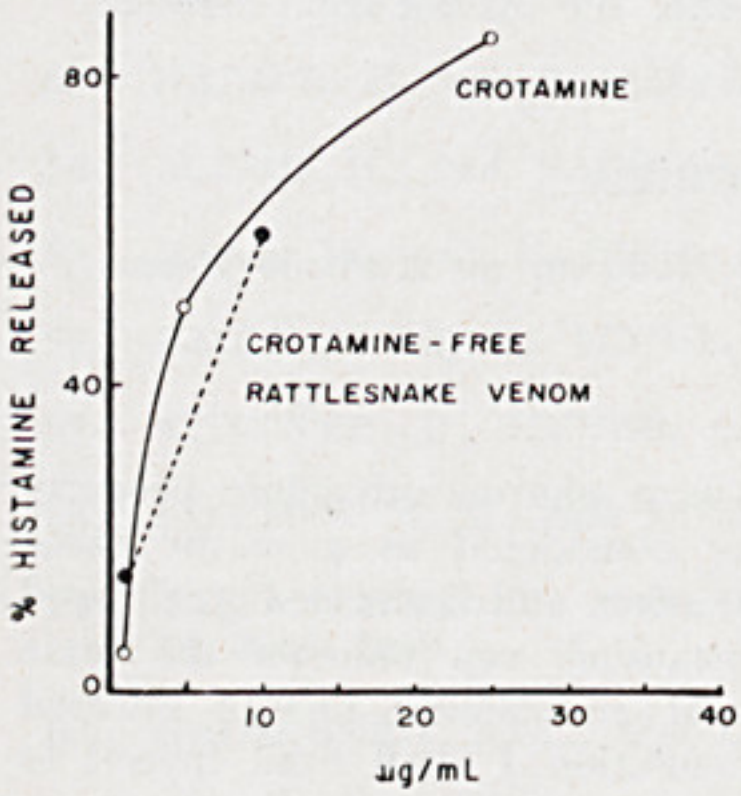

Fig. 1 — Histamine release from isolated rat mast cells by crotamine and crotamine-free whole rattlesnake venom.

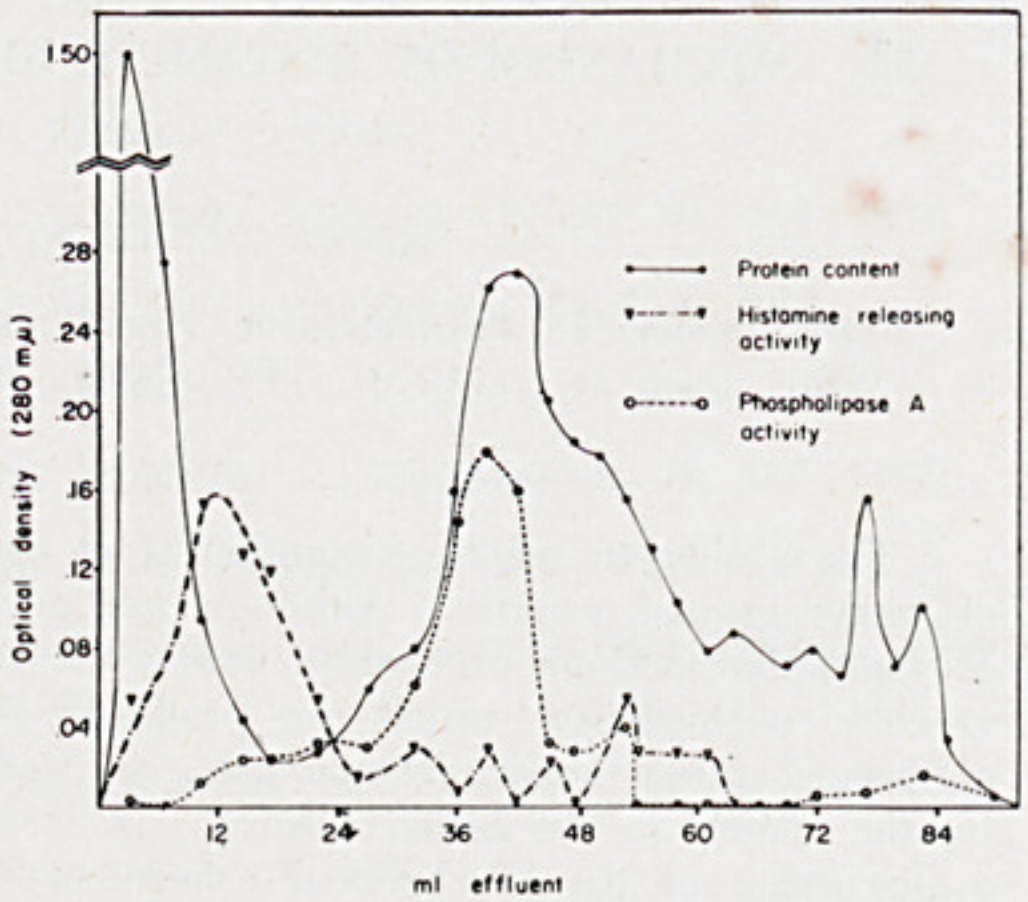

Fig. 2 — Chromatographic analysis of crotamine-free crotalic venom on Amberlite XE-64, CG-50 ionic resin.

seen that hemolytic activity, measured as phospholipase A by the egg-yolk coagulation test, could be clearly differentiated from histamine releasing activity as measured by the isolated mast cell technique. This activity was eluted shortly after, but definitely not together with crotactin (tall peak), the major protein of this rattlesnake venom, which also seems to be the major factor responsible for the lethality of such venom (4). It thus becomes clear that the histamine releasing factor of crotalic venom, which we have tentatively called enzyme fraction I, is neither crotactin nor phospholipase A. By using the combined fractions showing highest histamine releasing activity, we proceeded with an analysis of its properties. These are shown in Table I, which also compares them with

TABLE I — HISTAMINE RELEASING ACTIVITY OF CROTAMINE AND OF ENZYME FACTOR I FROM RATTLESNAKE VENOM

Treatment	Histamine releasing activity *	
	Enzyme factor I	Crotamine
Heat (5', 100°C)	Lost	Retained
Dialysis	Retained	Lost
Digestion by trypsin	Lost	?
Electrophoretic mobility (pH 7.7)	Slow, anionic	Fast, cationic

* Assayed on rat isolated peritoneal fluid mast cells.

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those of crotamine. It can be seen that we are most probably dealing with an enzyme as the thermal instability, protein nature and high molecular weight data indicate. We suspect that enzyme factor I is a proteolytic enzyme having chymotrypsin-like properties and base this assumption on three facts: a) chymotryptic activity has been demonstrated in crotalic venom by Deutsch & Diniz (5) in 1955; b) chymotrypsin is a histamine-releasing agent (6, 7) in contrast to trypsin which will not act on the isolated mast cell; c) our product was able to destroy bradykinin, a property of chymotrypsin, and which is an extremely sensitive, although not specific test, for this enzyme.

The absence of a direct histamine releasing effect of crotalic phospholipase A was a somewhat unexpected finding, even more so because of reports in the literature (Uvnäs and collaborators (8)) showing that snake or bee venom phospholipase A was indeed a potent histamine releasing agent on isolated mast cells of the rat. It seemed possible to us that the preparations used by Uvnäs could have been contaminated with other venom factors capable of releasing histamine. In order to investigate this problem, we decided to study the histamine releasing properties of a highly purified and very active preparation of phospholipase A from bee venom, obtained through the courtesy of Prof. Habermann (9) of Germany. It turned out, that this enzyme was only slightly active on the isolated mast cell, even when assayed in relatively high doses as shown in Table II. Not

TABLE II — EFFECT OF PHOSPHOLIPASE A TREATED EGG YOLK ON THE RELEASE OF HISTAMINE FROM ISOLATED RAT MAST CELLS

Releasing agent *	Percent histamine released
Phospholipase A, 20 μg/ml	7.3
Egg yolk (4%) pretreated with phospholipase A (37°, 40')	91.0
Egg yolk (4%)	0.0

* Allowed to act for 20 min, 37°C on the mast cell suspension.

unexpectedly, phospholipase A was also inactive as a hemolytic agent, an obvious conclusion from the known fact that the red cell's membrane phospholipids are shielded from attack by this enzyme (10). They are however highly sensitive to the action of the product of this enzyme's action on a susceptible substrate like egg yolk's fresh lecithin. The data on Table II indicate that rat mast cells readily loose their histamine when treated with the products of the action of phospholipase A on egg yolk, which we know to contain lysolecithin. Thus, *in vitro* release of histamine by phospholipase A can only take place in an indirect way.

The question which now arose in our minds was: would the enzyme behave in the same manner in an *in vivo* test for histamine-releasing activity? Would therefore the numerous results about the histamine releasing activity of hemolytic venoms *in vivo* or in perfused tissues demonstrated by other authors, have to be ascribed entirely to the presence of non-phospholipase A components? In order to answer these questions, we performed a very simple test for the detection of histamine releasing activity *in vivo*. This is the well-known Trypan blue skin

capillary permeability test, which consists of the following: a rat or a guinea-pig is intravenously injected with a dye like Trypan or Evans blue, which normally does not leave the circulation to enter tissue fluid spaces because of its inability to cross capillary wall permeability barriers. If such barriers are however altered by an experimental procedure such as the local release of histamine, a substance highly active on capillary permeability, diffusion of the dye across the vascular wall can take place and a blue spot will appear at the site of histamine release. Fig. 3 shows the results of intradermal injection of bee venom phospholipase A

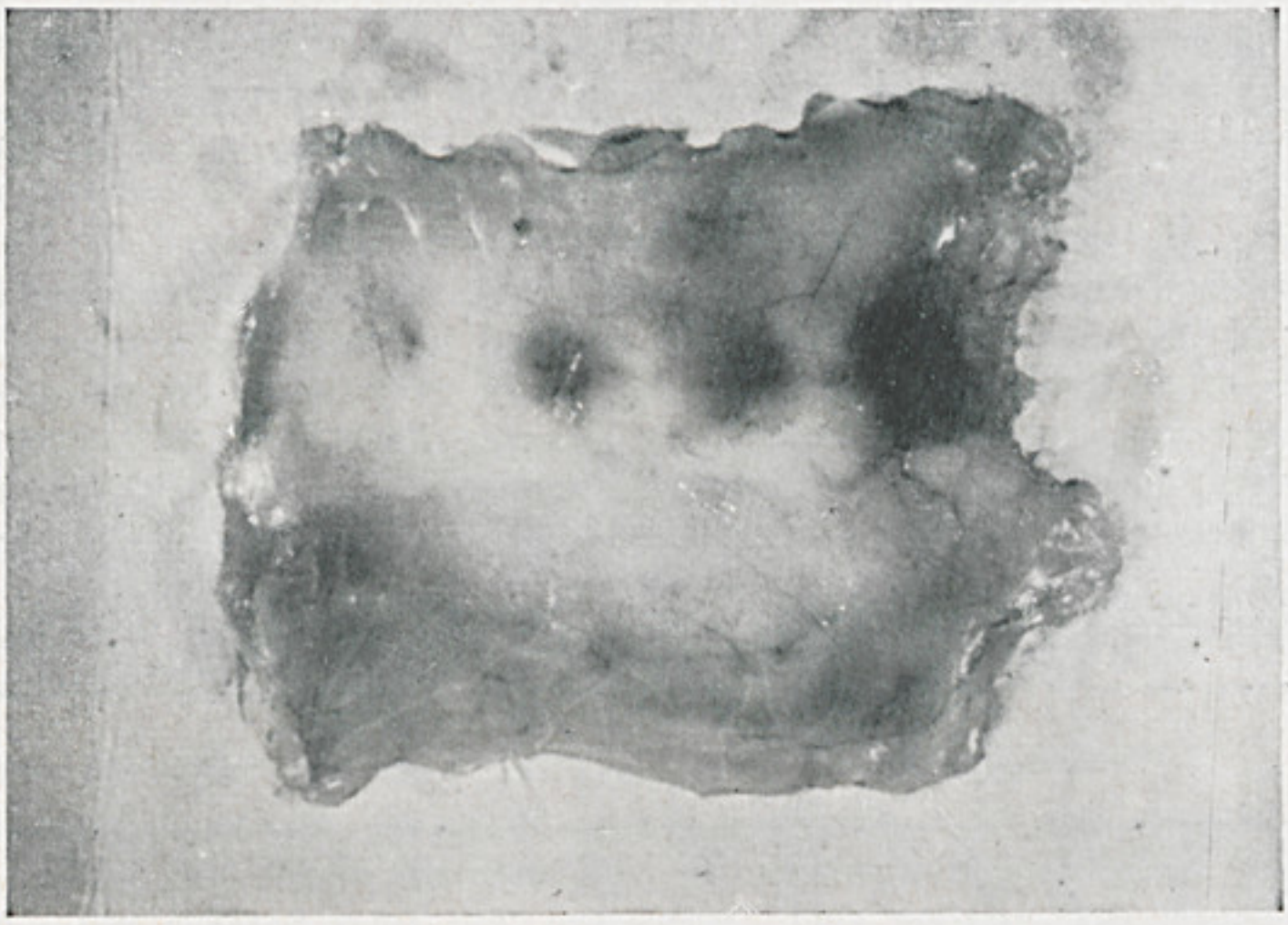

Fig. 3 — Effect of bee venom phospholipase A on capillary permeability in rat skin. Dark areas correspond to the injection of respectively 0.01, 0.1, 1.0 and 5.0 μg of enzyme. Contra-lateral injections of saline failed to produce comparable responses.

into rat skin in doses ranging from 10 μg to 5 μg. It can be seen that the enzyme was highly effective in lowering capillary diffusion barriers and that this effect bore relation to the dose used. This effect was not a direct one; it is probably mediated by the release of skin histamine and serotonin since it could be shown (13) that the permeability effects of phospholipase A could be completely suppressed in animals previously treated with a mixture of an inhibitor of the vascular effects of histamine (diphenhydramine) and an inhibitor of the vascular effects of serotonin (BOL-148, bromlysergic acid diethylamide). This latter had to be used because as it is well-known, whenever mast cell damage occurs in the rat, not only histamine but serotonin as well, is released. Serotonin is even more potent on rat capillary permeability than histamine. The obvious conclusion from these results was then, that phospholipase A was able to release mast cell amines *in vivo*, even though it proved incapable of doing the same on the isolated mast cell *in vitro*.

Table II had shown that phospholipase A can induce copious histamine release from isolated mast cells *in vitro* but only in an indirect way, namely, through the formation of lysolecithin. As it is well-known, this substance, a highly cytolytic agent, is the result of the splitting of an acid radical from lecithin by

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phospholipase A. Could this reasoning explain the potent effects of the enzyme on rat skin *in vivo*? We believe this to be so. Firstly, because tissue fluids, specially lymph, are known to contain phospholipids which could function as substrates for phospholipase A, yielding cytolytic phosphatides in the *in vivo* situation; secondly, because we have actually been able (13) to show a significant, even though small, histamine releasing action of the bee venom enzyme on rat skin *in vitro*. To our understanding, these results indicate that histamine release by phospholipase A is always an indirect process. It can take place in rat skin because this tissue, even *in vitro*, possesses enough free phospholipid to serve as a source for lysolecithin; in contrast, free, washed mast cells have no other phospholipid besides that contained in their cell membranes or cytoplasma. This phospholipid material must be shielded from the direct attack by even as potent a phospholipase A as is the bee venom enzyme.

Before finishing this presentation, I would like to show a few photomicrographs illustrating the effects of histamine-releasing compounds on rat mast cell morphology. Fig. 4a shows the typical, toluidine blue stained mast cell of rat mesentery spreads, with its metachromatically stained, heavy population of granules which, besides heparin, contain all of the mast cell's histamine. Fig. 4b shows the unchanged appearance of the mast cell, following incubation with 25 μg/ml of phospholipase A. When phospholipase A-treated egg yolk was added to the mesentery fragment however, distinct signs of cell degranulation could be observed (Fig. 4c). This phenomenon, of mast cell degranulation, occurs whenever histamine release takes place. It probably represents the primary response of mast cells to

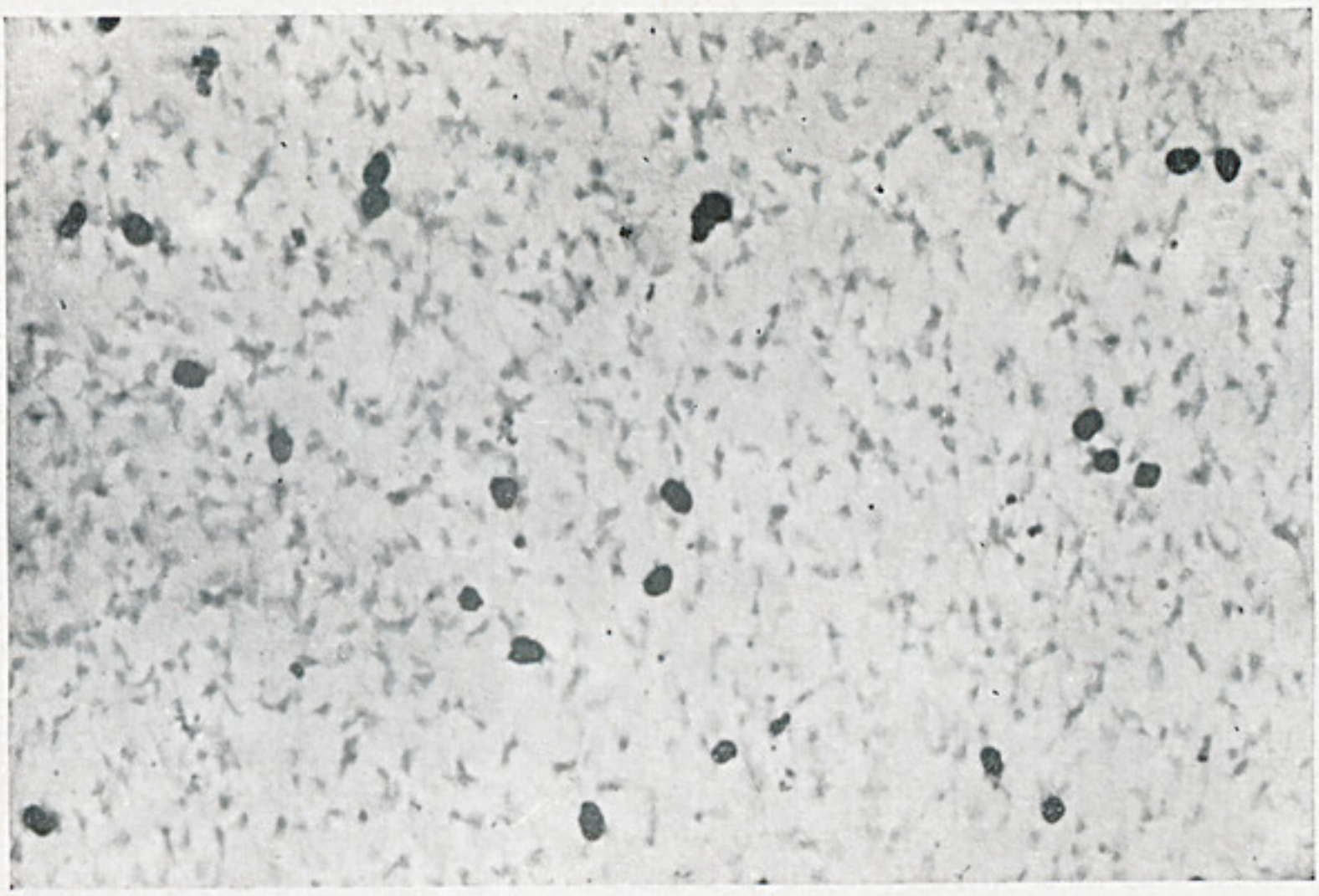

Fig. 4a

Fig. 4 — *In vitro* morphological alterations of rat mesentery mast cells by bee venom phospholipase A, mellitin and compound 48/80. a) Untreated controls; b) incubated for 25 min at 37° C with 25 μg/ml of phospholipase A; c) incubated for 25 min with the product of the action of phospholipase A (25 μg/ml) on 4% egg yolk; d) incubated for 10 min with cp. 48/80 (5 μg/ml); e) incubated for 25 min with 25 μg/ml of mellitin.

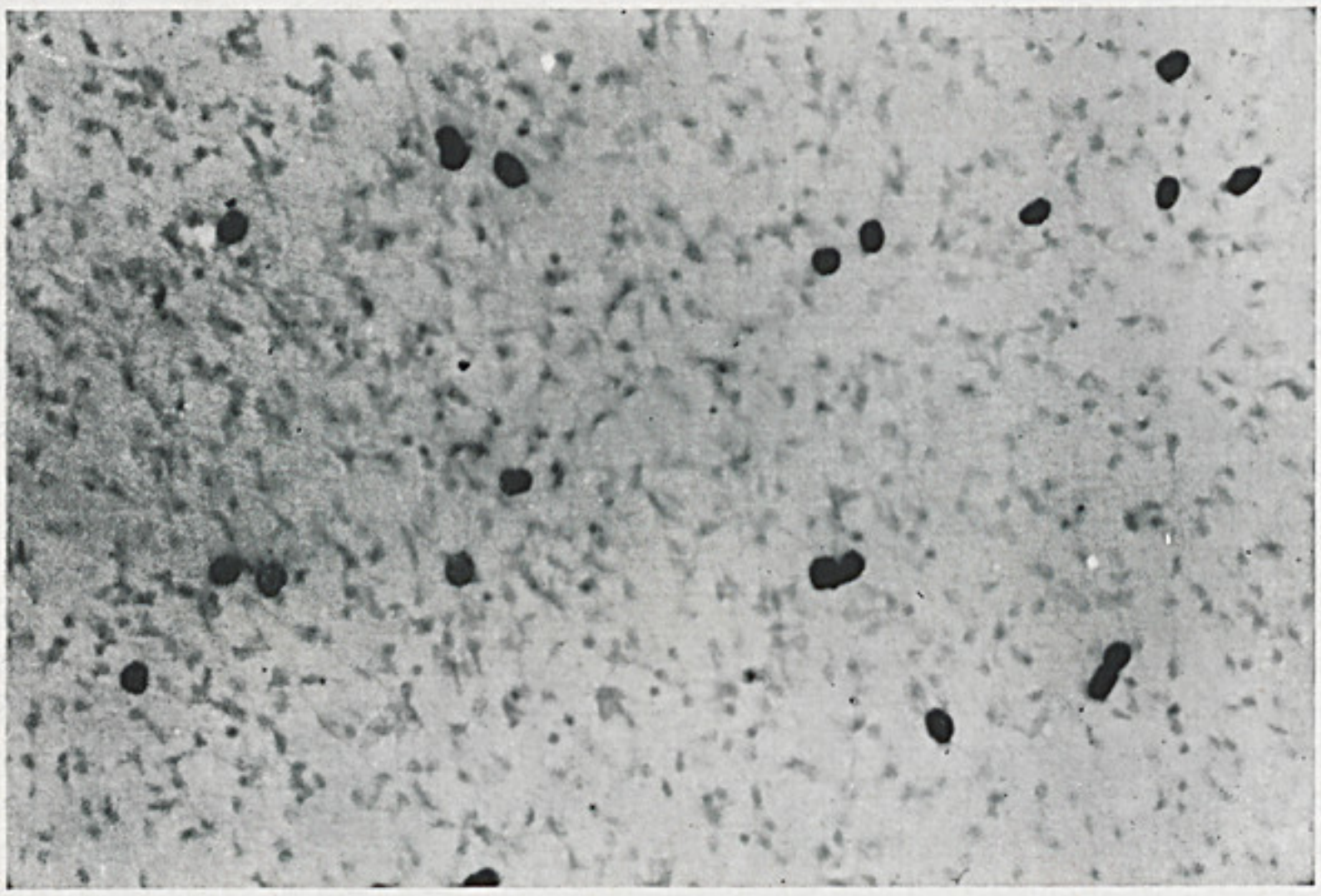

Fig. 4b

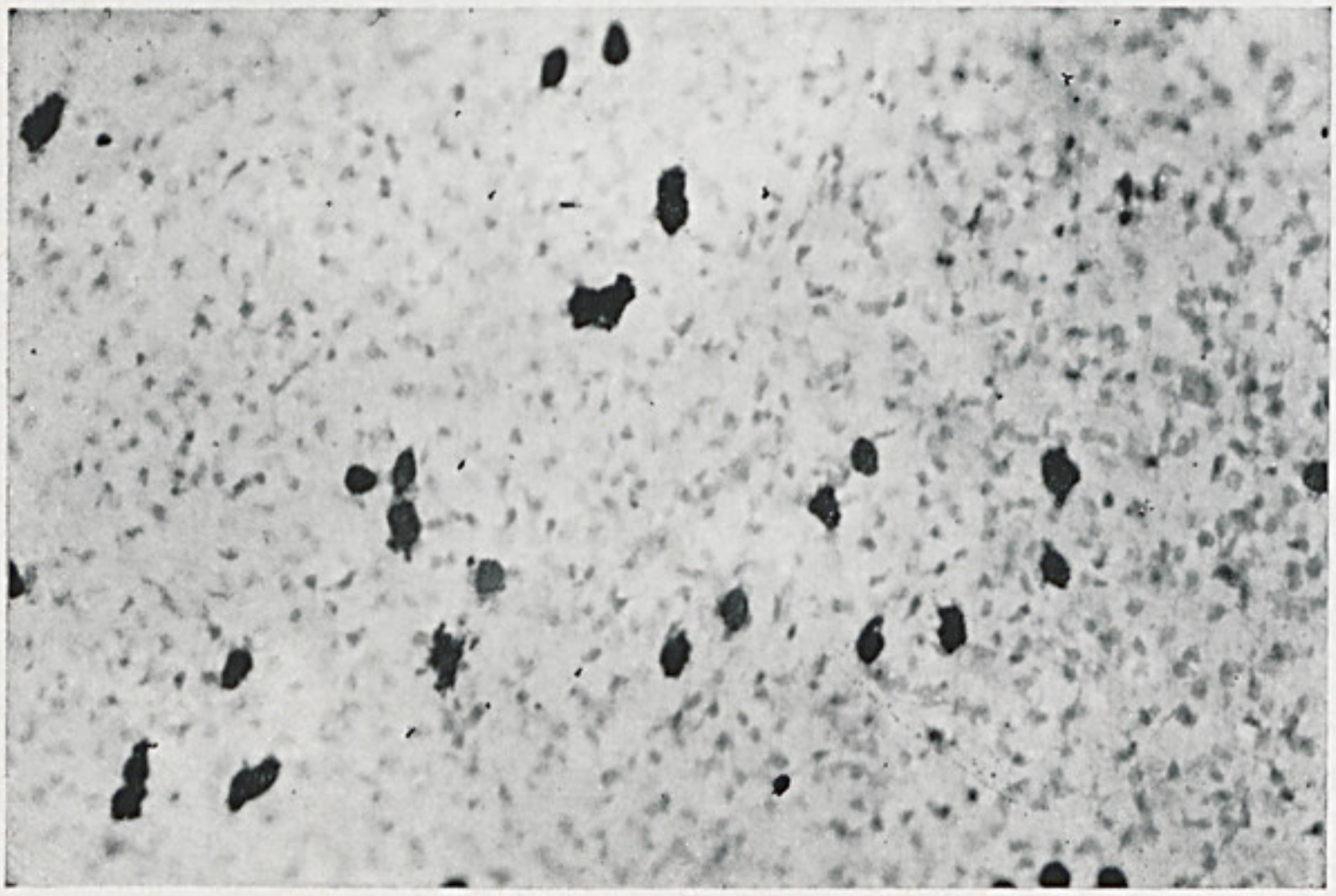

Fig. 4c

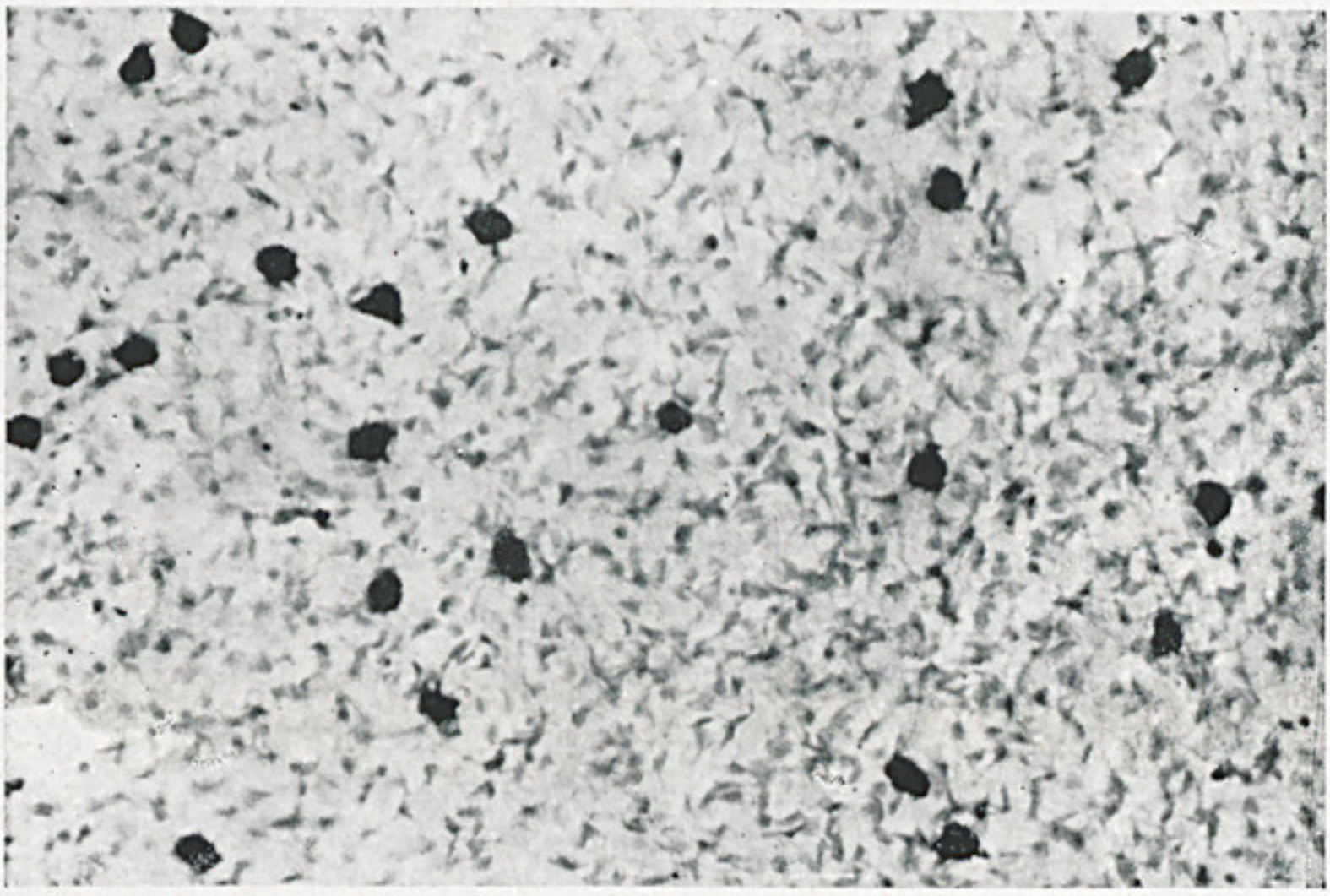

Fig. 4d

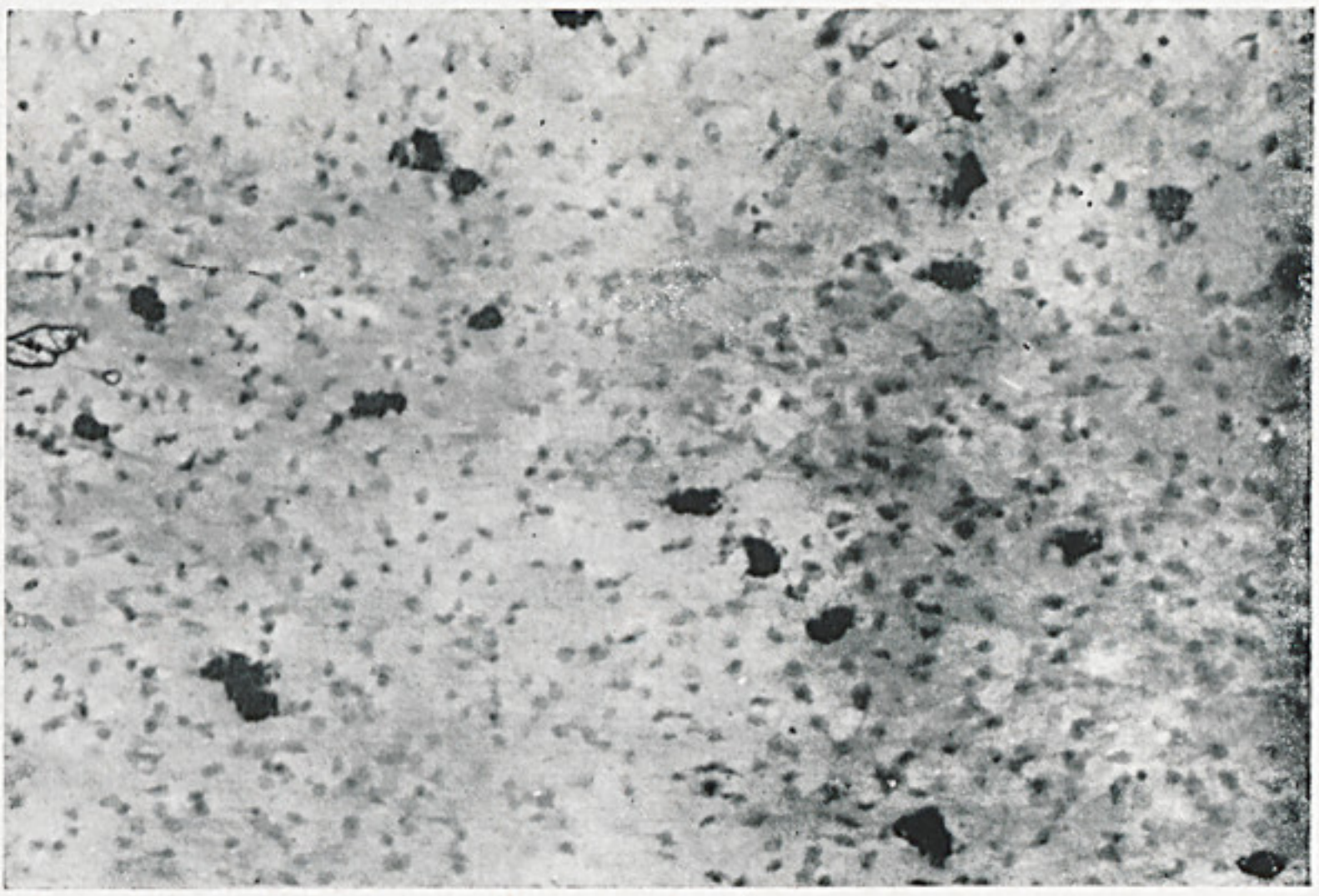

Fig. 4e

histamine releasing stimuli and it is likely, that it is only in the shed granules that histamine breaks loose from its intracellular ties, becoming free to exert pharmacological actions in the body. Fig. 4d shows, for the sake of comparison, the effect of compound 48/80, the most potent chemical histamine releaser in the rat known, on the mast cells of another sample of mesentery. Fig. 4e shows

the effects of melittin; this is a polypeptide substance, isolated from bee venom by Habermann (14), which has an intense and apparently unspecific cytolytic effect. It induces 100 per cent release of histamine from isolated mast cells (13). As it can be seen, its effects are not so much a degranulation but rather a dissolution of the cell's boundaries. This aspect is typical of unspecific cell damage, and is, in its mechanism of action quite different from granule secretion as evoked by compound 48/80 or the antigen-antibody reaction. The action of melittin is however similar to that of lysolecithin. Table III shows that there is a means

TABLE III — MODES OF ACTION OF HISTAMINE RELEASING AGENTS

Group I:	Stimulants of mast cell secretory activity (effects inhibited by metabolic inhibitors (anoxia, DNP, NaCN)
	Antigen-antibody reactions
	Epinephrine
	Compound 48/80
	Curares
	Chymotrypsin
	Enzyme factor I from crotalic venom
	Crotamine
Group II:	Non-specific cytolytic agents (effects *not* inhibited by metabolic inhibitors)
	Surfactants (octylamine, Tween 20, etc.)
	Phospholipase A
	Lysolecithin (egg yolk)
	Melittin
	Crotamine

of distinguishing between the mechanism of action of histamine releasing agents. I consider those agents whose action is inhibited by metabolic inhibitors like oxygen lack, dinitrophenol or cyanide, to be true stimulants of the secretory machinery of the mast cell. In contrast, the group of histamine releasers whose action is not blocked by metabolic inhibitors, are to be considered unspecific cytolytic agents, capable of rupturing the mast cell in the same way as they would injure most any cell in the mammalian organism. It can be seen that among histamine releasing agents contained in animal venoms, phospholipase A, melittin and, to a partial extent, crotamine, are included in this group.

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Discussion

F. Kornalik: "How do you explain the fact, that ELAPIDAE venoms, especially the venom of *Naja naja* which are known to have big amounts of phospholipase A are not able to liberate any histamine from the tissue? Have you tested the phospholipase A from Prof. Habermann in another way than the skin test for the presence of spreading factor?"

A. M. Rothschild: "I have no personal experience with such venoms. However the statement that the venom of *Naja naja* does not release histamine from tissues contradicts observations reported by a considerable number of authors who have found this venom to be quite powerful as a histamine releasing agent. We have not done a characterization of this enzyme because Prof. Habermann (9) has presented convincing proof that it is essentially free of enzymatic contaminants including hyaluronidase. Furthermore, our skin tests definitely implicate histamine and serotonin as mediators; hyaluronidase is known not to release these amines."

J. L. Prado: "The bradykinin destroying effect of enzyme I fraction of snake venom seemed too slow to me; would it really be a chymotryptic enzyme?"

A. M. Rothschild: "There is, I believe, fair evidence that the histamine releasing activity of Fraction I is of an enzymic nature. Although our results do not conclusively characterize this activity as being chymotrypsin-like, they point in this direction. Slugishness of action does not necessarily rule out this possibility since even whole crotalic venom is slow in attacking a typical chymotryptic substrate like ATEE."

E. A. Zeller: "Have you tested the substrate pattern of your enzyme factor which you suggested appears to be a chymotrypsin-like proteinase?"

A. M. Rothschild: "No."