Br. J. Pharmac. (1970), **38**, 253–262.

Mechanisms of histamine release by compound 48/80

A. M. ROTHSCHILD

Department of Pharmacology, Faculty of Medicine of Ribeirão Prêto, University of São Paulo, Ribeirão Prêto, Brazil

Summary

1. Rat and guinea-pig lung tissues were incubated for 20 min at 37° C in Krebs-Ringer phosphate buffer at pH 7·4, or in Tyrode-Tris buffer at pH 8·2, and the release of histamine produced by adding different concentrations of compound 48/80 to the incubation medium was determined.

2. At pH 7·4, increasing concentrations of 48/80 increased the release of histamine from the rat lung, with a tendency towards a maximum. No release of histamine from guinea-pig lung was observed at this pH. At pH 8·2, histamine release occurred both from rat and guinea-pig lung, and was proportional to the logarithm of the concentration of compound 48/80.

3. Histamine release from rat lung by 20 μg/ml. of 48/80 decreased when the pH was raised from 7·4 to 8·2 ; but the release caused by 1 mg/ml. of 48/80 increased both in rat and guinea-pig lung as the pH was raised.

4. 2-4-Dinitrophenol (DNP) inhibited the release of histamine from rat lung by a concentration of 20 μg/ml. of 48/80 ; the inhibition was prevented by glucose. DNP did not affect histamine release from rat or guinea-pig lung by a concentration of 1 mg/ml. of 48/80 and enhanced the release when the pH was raised from 7·4 to 8·2.

5. 1 mg/ml. of 48/80 did not inhibit the enhanced oxygen consumption produced by DNP in the isolated rat diaphragm.

6. Iodoacetic acid (IAA) or a Ca/Mg-free medium inhibited the release of histamine by 20 μg/ml. of 48/80 from rat lung but not the release produced by 1 mg/ml. in either rat or guinea-pig lung.

7. The degranulation of rat mesentery mast cells caused by 20 μg/ml. of compound 48/80 was inhibited by DNP. The degranulation evoked by 1 mg/ml. of 48/80 was also sensitive to this inhibitor ; in this instance, however, the metachromatic staining reaction of the mesentery mast cells was greatly diminished.

8. It is concluded that two processes of histamine release by compound 48/80 occur in rat lung. One, dependent on cell metabolism, involves mast cell granule secretion. The other, independent of cell metabolism, seems to consist of a simple exchange reaction between histamine and compound 48/80, and this is the only one occurring in guinea-pig lung.

Introduction

Moussatché & Danon (1958), Rothschild (1961), Diamant & Uvnäs (1961), and Saeki (1964) have shown that metabolic energy is required for histamine release from sensitized tissues exposed to antigen. A similar requirement for the release of histamine by compound 48/80, a powerful "chemical" releaser of histamine, has been questioned. Van Ardsel & Bray (1961) showed that supression of its action on rat tissue by metabolic inhibitors like 2-4-dinitrophenol (DNP) or cyanide was overcome by increasing the concentration of the releaser, while Moussatché & Danon (1957) found that the release of histamine from guinea-pig lung *in vitro* was unaffected by metabolic intermediates like succinate or ketoglutarate, which nevertheless enhanced oxygen consumption by the tissue. Recent experiments (Rothschild, 1965, 1966; Yamasaki & Endo, 1967) suggest that compound 48/80 releases histamine from tissues by a metabolically dependent as well as by a metabolically independent pathway, and an attempt is made in the present experiments to provide some insight into the nature of these processes.

Methods

Albino guinea-pigs weighing from 300 to 400 g and Wistar rats weighing from 200 to 250 g, of either sex, were used. They were stunned and decapitated.

Histamine release in lungs

The lungs were excised and washed with cold saline solution. The guinea-pig lungs were sliced with a razor blade and the rat lungs were fragmented with sharp scissors into pieces weighing approximately 20 mg each. Tissue samples of 100–200 mg were placed in 10 ml. beakers containing 2 ml. of the incubation medium, incubated at 37° C, gassed with air in a Dubnoff metabolic incubator and shaken at 300 strokes/min. The incubation media employed were either Krebs-Ringer phosphate buffer, pH 7·4, containing one half of the originally recommended concentration of $CaCl_2$ (Umbreit, Burris & Stauffer, 1957), or a Tyrode solution stabilized at pH 8·2 by 0·01 M Tris buffer. The samples were pre-incubated for 20 min at 37° C in these solutions either with or without the inhibitor, and after adding compound 48/80, incubation was continued for 25 min. All experiments, except those using dinitrophenol, were performed in the presence of 0·1% glucose.

Following incubation, the tissue samples were placed in 0·1 N HCl and the residual histamine was extracted as described by Feldberg & Talesnik (1953). The incubation media containing the released histamine were acidified to pH 1–2 and neutralized before assay.

Histamine was assayed on the atropinized ileum of the guinea-pig; comparisons were made with standard solutions of histamine hydrochloride. At high concentration, compound 48/80 decreased the response of the ileum to histamine. This interference was overcome by adding an equivalent concentration of the compound to the standard employed for comparison. The results are expressed as percentages of the total tissue histamine released during incubation.

Oxygen consumption by excised rat diaphragm

Approximately 100 mg of rat diaphragm were placed in the main compartment of the flask of the Warburg apparatus and Krebs-Ringer buffer containing 1 mg/ml.

48/80 was added. The centre well contained 20% KOH. After a 10 min equilibration period, the experiment was started by adding the solution of 2-4-dinitrophenol from the side arm.

Mast cell examination in rat mesentery

Spreads of rat mesentery were stained with toluidine blue and prepared for microscopic examination as described by Mota & Ishii (1960).

Compound 48/80 was generously supplied by the Wellcome Research Laboratories, Tuckahoe, N.Y.

Results

Figure 1 shows that the histamine release differed according to whether the lung tissue was incubated in Krebs-Ringer solution buffered at pH 7·4 or in a solution of Tyrode-Tris buffer at pH 8·2. On incubation in the Krebs-Ringer solution, compound 48/80 released histamine from the rat only, not from the guinea-pig lung, and the release from the rat tissue appeared to be exponentially related to the concentration of compound 48/80. On incubation in the Tyrode-Tris buffer solution, histamine was released from both tissues, but the guinea-pig lung was approximately 100 times less sensitive than the rat lung. In both tissues the release was linearly related to the concentration of compound 48/80.

The difference in effectiveness of 48/80 in the two media seemed to be due to the difference in pH. Figure 2 gives the results obtained with both tissues incubated at varying pH in a simple saline phosphate buffer solution consisting of 0·9% NaCl, 10 mM NaH_2PO_4, 4·5 mM glucose. On incubation of guinea-pig lung with

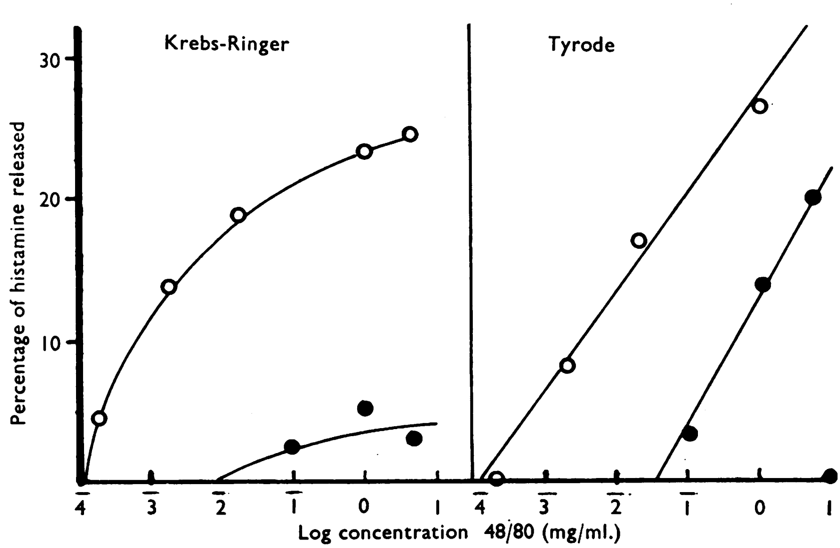

FIG. 1. Release of histamine by compound 48/80 from rat (○——○) and guinea-pig (●——●) lung incubated in Krebs-Ringer buffered at pH 7·4 and in Tyrode at pH 8·2. Each point is the mean from three experiments and is corrected for blank values; that is, the histamine released on incubation without compound 48/80 has been deducted. Abscissa, 48/80 log concentration mg/ml.; ordinate, percentage of histamine released.

1 mg/ml. of compound 48/80 there was a gradual increase of histamine release on raising the pH of the medium from 7·4 to 8·2 (line A). The results obtained with rat lung differed according to the concentration of 48/80 used. Raising the pH from 7·4 to 8·2 caused a lowering of the histamine release when the concentration of compound 48/80 was 20 μg/ml. (line B) but an augmentation when the concentration was 1 mg/ml. (line C).

These results suggested that more than one mechanism was responsible for the release of histamine from rat lung. This possibility was investigated by examining the effects of metabolic inhibitors on the release. The first inhibitor used was 2-4-dinitrophenol (DNP), which had been shown to inhibit histamine release by compound 48/80 (Diamant & Uvnäs, 1961 ; Rothschild, Vugman & Rocha e Silva, 1961). In the present experiments it was found that the release of histamine by 20 μg/ml. of compound 48/80 from rat lung incubated in a glucose-free solution of Krebs-Ringer phosphate buffer was strongly inhibited by DNP, but that this inhibition did not occur when glucose was present in the incubation medium. This finding contradicts the suggestion of Van Ardsel & Bray (1961), that DNP, an acidic substance, exerts its inhibitory effect by combining with the basic amino groups of compound 48/80.

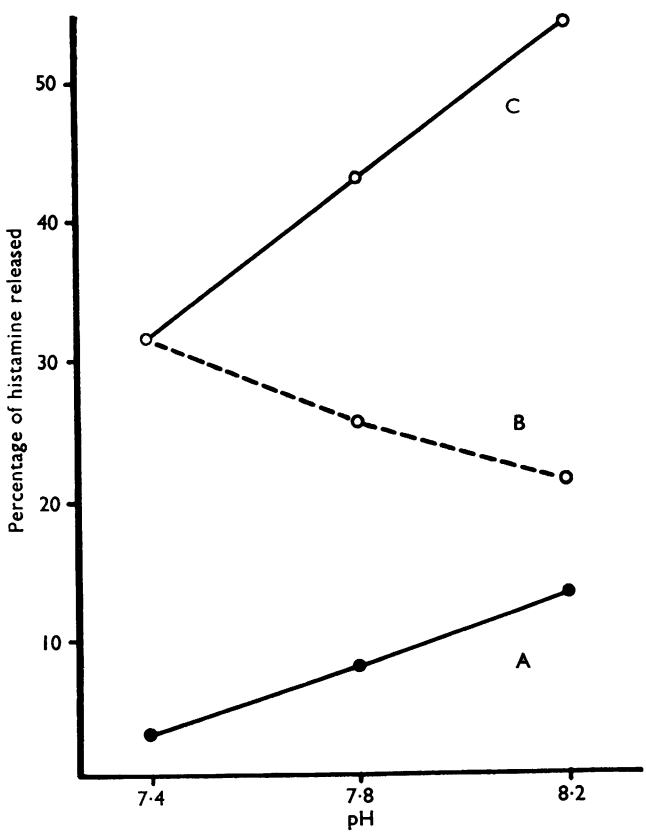

FIG. 2. Release of histamine by compound 48/80 from rat and guinea-pig lung incubated in saline-phosphate buffer (see text) at different pH. Guinea-pig lung incubated with 1 mg/ml. 48/80 (A) ; rat lung incubated with 20 μg/ml. 48/80 (B) and with 1 mg/ml. 48/80 (C). Each point is the mean of three experiments, corrected for blank values. Abscissa, pH ; ordinate, percentage of histamine released.

DNP, however, did not inhibit the release of histamine produced by 1 mg/ml. compound 48/80 even when the lung tissue was incubated in a glucose-free medium. As shown in Fig. 3, this result was obtained for rats as well as for guinea-pig lung incubated in a glucose-free solution of Tyrode-Tris buffer at pH 8·2.

The failure of DNP to inhibit the release of histamine by 1 mg/ml. of 48/80 does not result from disappearance of free DNP from the medium due to acid-base-binding with the excess of 48/80. This is evident from the results given in Table 1. They show that the characteristic enhancement of oxygen uptake brought about by DNP in the respiring rat diaphragm was not abolished by the presence in the medium of compound 48/80 in a concentration of 1 mg/ml. If the 48/80 had removed the DNP from the medium, the enhancement would not have occurred.

The effect of pH on the histamine release produced in DNP treated rat lung by different concentrations of 48/80 is shown in Fig. 4. At pH 7·4, histamine release

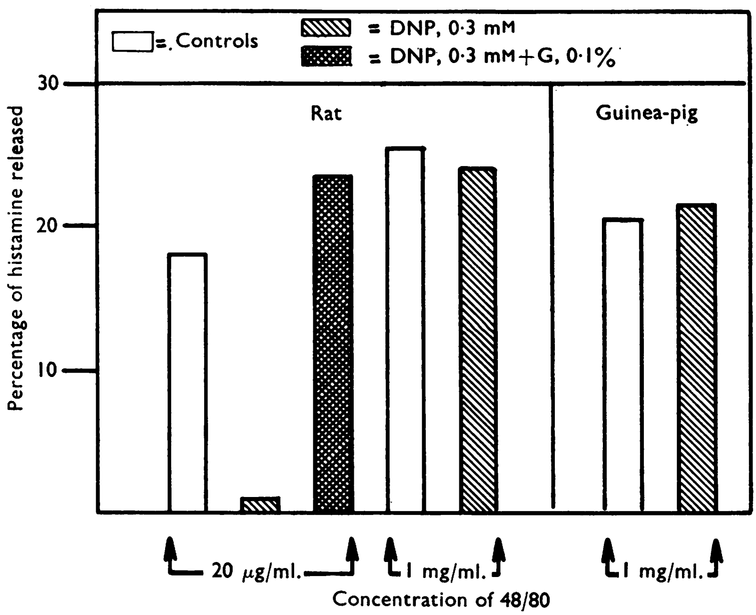

FIG. 3. Release of histamine by compound 48/80 from rat and guinea-pig lung incubated with or without 0·3 mM DNP. The results obtained with 0·20 μg/ml. 48/80 were obtained on incubation in Krebs-Ringer solution buffered at pH 7·4 without and with glucose (G) as indicated; the results obtained with 1 mg/ml. 48/80 were obtained on incubation in a glucose free solution of Tyrode-Tris buffer at pH 8·2. Each column gives the mean of three experiments corrected for blank values. Abscissa: concentration of 48/80 (mg/ml.). Ordinate, percentage of histamine released.

TABLE 1. *Influence of compound* 48/80 *on oxygen consumption of rat diaphragm treated with* 2-4-*dinitrophenol* (*DNP*)

Additions to incubation medium	Oxygen consumption (μl./min per g) 0–10 min	Effect of DNP	10–20 min	Effect of DNP
—	26·0±3·1	+17·9	21·3±3·3	+22·8
DNP, 0·03 mM	43·9±2·3		44·1±3·2	
48/80, 1 mg/ml.	23·1±2·3	+15·7	20·8±3·3	+19·0
48/80 plus DNP	38·8±1·4		39·8±3·1	

Figures represent the mean of three experiments. The changes produced by DNP were statistically significant ($P<0·05$) (Student's t test).

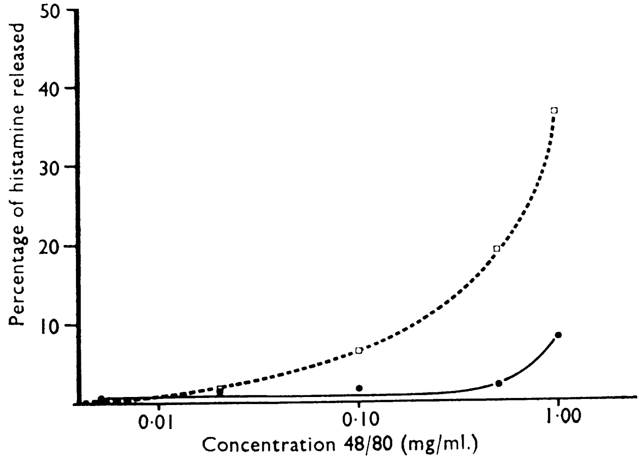

FIG. 4. Release of histamine by compound 48/80 from rat lung incubated at different pH with 0·3 mM DNP. At pH 7·4 (●——●) the incubation medium was Krebs-Ringer solution without glucose; at pH 8·2 (□- - -□) it was Tyrode-Tris buffer without glucose. Each point is the mean from three experiments corrected for blank values. Abscissa, concentration of 48/80 (mg/ml.); ordinate, percentage of histamine released.

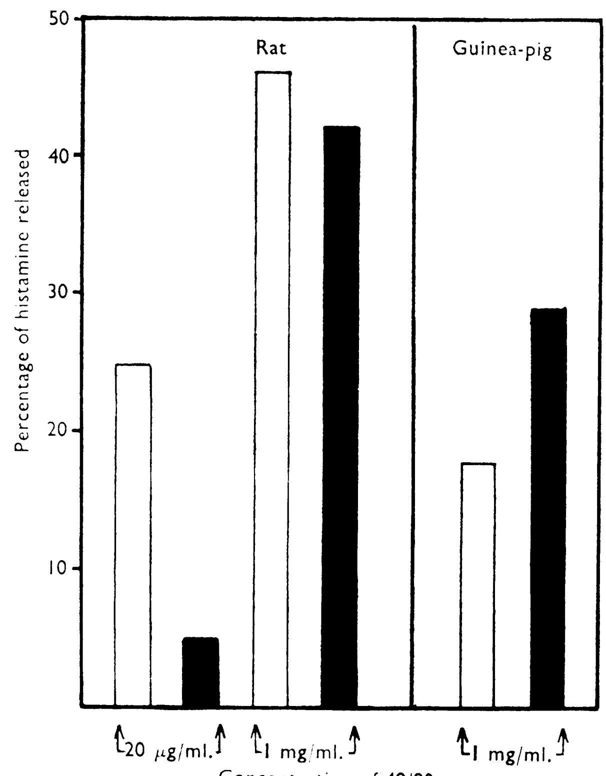

FIG. 5. Release of histamine by compound 48/80 from rat and guinea-pig lung incubated without (□) or with (■) 1 mM IAA. Results with 0·02 mg/ml. 48/80 obtained on incubation in Krebs-Ringer solution buffered at pH 7·4; results with 1 mg/ml. 48/80 obtained on incubation in Tyrode-Tris buffer solution at pH 8·2. Each column gives the mean from three experiments corrected for blank values. Abscissa: Concentration of 48/80 (mg/ml.). Ordinate, percentage of histamine released.

became noticeable only when the concentration was at least 1 mg/ml. By contrast, at pH 8·2 compound 48/80 was approximately 10 times more effective.

Aerobic glycolysis probably plays an important role in rat mast cell energy metabolism (Chakravarty, 1965). Figure 5 shows that 1 mM iodoacetate (IAA), an inhibitor of this process, blocked the release of histamine from rat lung produced by 20 μg/ml. of compound 48/80, but did not diminish the release produced by 1 mg/ml. from rat or guinea-pig lung incubated in the Tyrode-Tris medium; the release from the guinea-pig lung was in fact enhanced. This effect had been noted first by Mongar & Schild (1957) and is probably due to inhibition by IAA of the enzymatic histamine destruction, a process reported to be sensitive to SH group reagents (Buffoni, 1966).

Mota & Ishii (1960) have demonstrated a dependence on Ca^{++} for the histamine release from rat skin by 48/80. Results obtained on lung tissue incubated in Ca^{++} and Mg^{++}-free buffered solution are illustrated in Fig. 6. At pH 7·4 in Krebs-Ringer solution minimal amounts of histamine were released from rat lung by 20 μg/ml. of compound 48/80, but at pH 8·2 in Tyrode-Tris buffer solution the amounts released by 1 mg/ml. from rat as well as from guinea-pig lung were of the same magnitude as in the controls incubated with Ca^{++} and Mg^{++}.

It has been shown that rat mesentery mast cells exposed to 20 μg/ml. of compound 48/80 show degranulation and scattering of metachromatic granular material (Mota, Beraldo, Ferri & Junqueira, 1953), but that these effects are inhibited if the

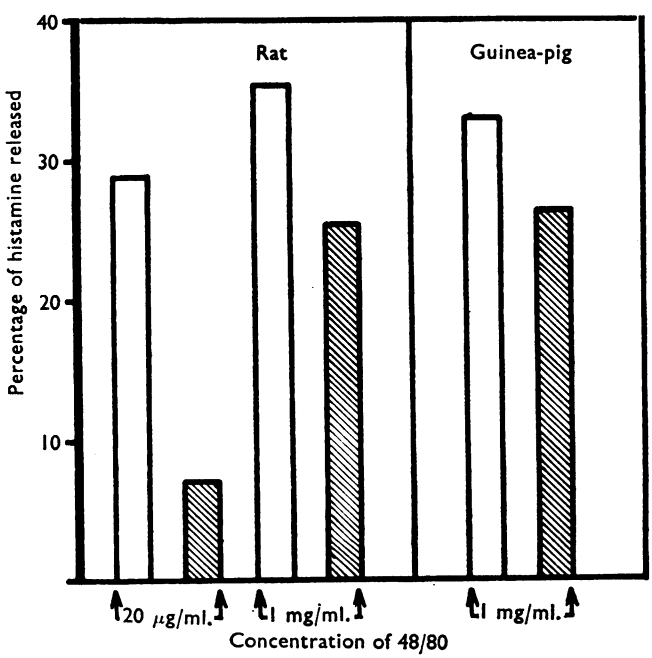

FIG. 6. Release of histamine by compound 48/80 from rat and guinea-pig lung incubated in the presence of Ca^{++} and Mg^{++} (□); or, in their absence, when 0·2% edetic acid (disodium ethylenodiamine tetra-acetate) was added to the medium (▨). Results with 20 μg/ml. 48/80 were obtained on incubation in Krebs-Ringer solution buffered at pH 7·4; results with 1 mg/ml. 48/80 on incubation in Tyrode-Tris buffer solution at pH 8·2. Each column gives the mean from three experiments corrected for blank values. Abscissa: concentration of 48/80 (mg/ml.). Ordinate, percentage of histamine released.

mast cells had previously been exposed to DNP (Rothschild *et al.*, 1961). DNP treated mesenteric mast cells exposed to 1 mg/ml. of 48/80, however, responded in a different manner. The majority of the mast cells were found to be ortho-(blue) rather than metachromically stained by toluidine blue. There was, further, little evidence of granule extrusion and many cells exhibited an appreciably well preserved intracellular grain structure.

Discussion

The results presented in this paper suggest that on exposure to compound 48/80, rat lung releases histamine by two processes. One occurs with comparatively low concentrations of the compound. It is dependent on cell metabolism as revealed by its sensitivity to glucose, dinitrophenol, iodoacetate and lack of extracellular Ca^{++}, and it may be a manifestation of mast cell secretory activity. Mast cells have been considered to be mononuclear endocrine glands involved in microcirculatory control (Fulton, Riley & West, 1957).

The other pathway is probably non-enzymatic in nature. Characteristics for this release are enhancement by alkalinity, insensitivity to DNP, iodoacetate and lack of Ca^{++}, as well as a requirement for a relatively high concentration of releaser for threshold response from metabolically inhibited tissue. Although other schemes can be suggested, these properties fit the amine-exchange mechanism of histamine release described by Jaques & Küttner (1961). Using a basic dye of strong affinity for mast cells, these authors showed that compound 48/80 and other bases having histamine releasing activity displaced the dye from its attachment to anionic, model sulpho-polysaccharides. This effect was roughly correlated to the histamine releasing capacity of the basic compounds. Compound 48/80 is a polymeric base possessing secondary amino-groups whose pK_A lies close to 9·0 (unpublished results). The ratio undissociated base/free-cation for such a compound increases approximately 6-fold when the pH of the medium is changed from 7·4 to 8·2. Histamine release by the amine-exchange mechanism would require access of the releaser to intracellular binding sites of histamine. Alkalinity should enhance this process, for, by repressing the ionization of 48/80, it would favour the passage of the compound across lipoid barriers on the mast cell membrane. The favourable effect of high pH is clearly shown in experiments using DNP-treated lung submitted to 1 mg/ml. of compound 48/80: approximately 4·5 times more histamine was released at pH 8·2 than at 7·4 in these conditions.

The concentration-activity relationship describing the action of 48/80 on rat lung histamine changed according to the pH of the medium. At pH 7·4, at which a tendency towards a maximum of activity was noted, the metabolically dependent pathway of histamine release probably predominated. Mast cell degranulation and histamine release by ATP of exogenous and probably also of endogenous origin have been described (Diamant & Krüger, 1967; Oliveira Antonio & Rothschild, 1969); an analogy has been drawn between the release of histamine from mast cells and the membrane-ATP-ase activated catecholamine release from chromaffin cells (Poisner & Douglas, 1968). The metabolically dependent pathway of histamine release is probably a multiple-enzyme process initiated at 48/80-sensitive sites on the mast cell membrane. Such a process should tend towards a maximum resulting from saturation of one of its components by excess 48/80.

The results of the present experiments indicate that at pH 8·2 metabolically independent release of histamine by 48/80 takes place and may even predominate. A simple exchange reaction between the two amines, 48/80 and histamine, competing for the same intracellular binding site, would possibly have less tendency towards saturation than an enzyme-controlled, secretory process. This would explain the straight-line character of the relationship between concentration of 48/80 and release of histamine at pH 8·2 observed both in rat and guinea-pig lung.

Rat mast cells treated with 1 mg/ml. of compound 48/80 in the presence of DNP did not show the typical purple, metachromatic stain which untreated cells acquire after exposure to toluidine blue. In the normal mast cell, this colour is the consequence of the reaction between toluidine blue and intracellular sulphopolysaccharides (Lison, 1935), which also seem to be histamine binding sites. Toluidine blue is able to release histamine from rat mast cells (Smith, 1958); in the isolated mesentery this release is not inhibited by DNP in a glucose-free medium of pH 8·2 (unpublished observations); it is probably, like metabolically independent release by 48/80, due to amine exchange at histamine-binding sites. Mast cells in which such sites are occupied by tightly held 48/80 molecules (Jaques & Küttner, 1961) would be less prone to react with toluidine blue. This could explain the greatly diminished metachromatic reaction of mast cells treated with 1 mg/ml. of 48/80 in the presence of DNP.

Histamine release from guinea-pig lung by 48/80 did not occur in a medium of pH 7·4, and at pH 8·2 the release was resistant to metabolic inhibitors and lack of extracellular Ca^{++}. This indicates that the histamine release by 48/80 from guinea-pig lung, in contrast to rat lung, is brought about solely by a metabolically independent pathway. A similar conclusion does not apply to the histamine release from guinea-pig lung by other agents, for instance, antigen, since several authors (Mota & Ishii, 1960; Rothschild, 1961; Prouvost-Danon & Moussatché, 1961) have shown that the histamine release not only from the sensitized rat but also from the sensitized guinea-pig lung by antigen, is inhibited by the same metabolic inhibitors which block the release of the amine from normal rat mast cells by low concentrations of 48/80. It would seem that guinea-pig mast cells are devoid of the specific amine receptor site which in rat tissues are involved in the mast cell degranulation and histamine release by 48/80 through a mechanism having the characteristics of a metabolically stimulated, secretory process.

REFERENCES

BUFFONI, F. (1966). Histaminase and related amine oxidases. *Pharmac. Rev.*, **18**, 1163–1201.

CHAKRAVARTY, N. (1965). Glycolysis in rat peritoneal mast cells. *J. cell. Biol.*, **25**, 123–128.

DIAMANT, B. & UVNÄS, B. (1961). Evidence for energy-requiring processes in histamine release and mast cell degranulation in rat tissue induced by compound 48/80. *Acta physiol. scand.*, **53**, 315–329.

DIAMANT, B. & KRÜGER, P. G. (1967). Histamine release from isolated rat peritoneal mast cells induced by adenosine-5-triphosphate. *Acta physiol. scand.*, **71**, 291–302.

FELDBERG, W. & TALESNIK, J. (1953). Reduction of tissue histamine by 48/80. *J. Physiol., Lond.*, **120**, 550–568.

FULTON, G. P., RILEY, J. F. & WEST, G. B. (1957). Humoral aspects of tissue mast cells. *Physiol. Rev.*, **37**, 221–242.

JAQUES, R. & KÜTTNER, K. (1961). Selektive Farbstoffverdrängung an biogenen Polyelektrolyten als Modell für die Liberation biogener Amine. *Helv. physiol. pharmac. Acta*, **19**, 335–343.

LISON, L. (1935). Etudes sur la metachromasie. Colorants métachromatiques et substances chromotropes. *Arch. Biol.*, **46**, 599–610.

MONGAR, J. L. & SCHILD, H. O. (1957). Inhibition of the anaphylactic reaction. *J. Physiol., Lond.*, **135**, 310–319.

MOTA, I. & ISHII, T. (1960). Inhibition of mast cell disruption and histamine release in rat anaphylaxis *in vitro*. Comparison with compound 48/80. *Br. J. Pharmac. Chemother.*, **15**, 82–87.

MOTA, I., BERALDO, W. T., FERRI, A. G. & JUNQUEIRA, L. C. U. (1953). Protamine-like property of compound 48/80 and stilbamidine and their action on mast cells. *Proc. Soc. exp. Biol., N.Y.*, **83**, 455–457.

MOUSSATCHÉ, H. & DANON, A. P. (1957). The effect of succinate and malonate on the chemical release of histamine from guinea-pig lung slices. *Naturwissenschaften*, **44**, 637–638.

MOUSSATCHÉ, H. & DANON, A. P. (1958). Influence of oxidative phosphorylation inhibitors on the histamine release in the anaphylactic reaction *in vitro*. *Experientia*, **14**, 414.

POISNER, A. M. & DOUGLAS, W. W. (1968). A possible mechanism of release of posterior pituitary hormones involving adenosine triphosphate and an adenosine triphosphatase in the neurosecretary granules. *Mol. Pharmac.*, **4**, 531–540.

PROUVOST-DANON, A. & MOUSSATCHÉ, H. (1961). Influence of anaerobic glycolysis on the histamine release in the guinea-pig and rat anaphylactic reaction *in vitro*. *Nature, Lond.*, **192**, 361.

OLIVEIRA ANTONIO, M. P. & ROTHSCHILD, A. M. (1969). Histamine release and mast cell degranulation induced by 2-4 dinitrophenol in rat tissues. *Experientia*, **25**, 244.

ROTHSCHILD, A. M., VUGMAN, I. & ROCHA E SILVA, M. (1961). Metabolic studies on the release of histamine by compound 48/80 in the rat diaphragm. *Biochem. Pharmac.*, **7**, 248–255.

ROTHSCHILD, A. M. (1961). Effect of metabolic inhibitors on the release of histamine by anaphylatoxin and by antigen *in vitro*. *Experientia*, **17**, 555.

ROTHSCHILD, A. M. (1965). Mecanismos de Liberação de Histamina. Free-docent Dissertation: University of São Paulo, Ribeirão Prêto, Brazil.

ROTHSCHILD, A. M. (1966). Histamine release by basic compounds. *Hand. exp. Pharmac.*, XVIII, pp. 386–423. Heidelberg: Springer.

SAEKI, K. (1964). Effects of compound 48/80, chymotrypsin and antiserum on isolated mast cells under aerobic and anaerobic conditions. *Jap. J. Pharmac.*, **14**, 375–390.

SMITH, D. E. (1958). Nature of the secretory activity of the mast cells. *Am. J. Physiol.*, **193**, 573–575.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Technique and Tissue Metabolism*. Minneapolis: Burgess.

VAN ARDSEL, P. P. & BRAY, R. E. (1961). On the release of histamine from rat peritoneal mast cells by compound 48/80: effects of metabolic inhibitors. *J. Pharmac.*, **133**, 3–8.

YAMASAKI, H. & ENDO, K. (1967). Coexistence of energy dependent and nondependent processes in histamine release mechanism of compound 48/80 and sinomenine: experiments on chopped skin from various animal species. *Jap. J. Pharmac.*, **17**, 228–240.

(*Received July* 10, 1969)