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METABOLIC STUDIES ON THE RELEASE OF HISTAMINE BY COMPOUND 48/80 IN THE RAT DIAPHRAGM*

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Abstract—The inhibitory action of uncoupling agents like 2:4-dinitrophenol, salicylate, thiopental and sodium oleate on the release of histamine from isolated rat diaphragm by compound 48/80, is markedly decreased by glucose. Inhibitions due to sodium cyanide or anoxia are similarly affected. Rat mesentery mast cell damage by 48/80 *in vitro*, which is suppressed by dinitrophenol or sodium cyanide, will occur if the action of these inhibitors is tested in the presence of glucose. Sodium succinate cannot prevent the inhibition of histamine release by dinitrophenol, although oxygen consumption studies reveal that it is aerobically metabolized by dinitrophenol-treated tissue. The results obtained have been interpreted as an indication that metabolic intermediates necessary for the release of histamine from rat diaphragm can be generated by a mechanism, possibly glycolytic, functioning independently of the Krebs cycle.

INTRODUCTION

A CORRELATION between active metabolism and the release of histamine was first indicated by Parrot,[1] who demonstrated that the liberation of histamine induced by the antigen in sensitized guinea-pig tissue did not take place in the absence of oxygen. Efforts to clarify the mechanism of histamine release, by looking for a possible link between cell metabolism and the response of the tissue to antigen, have been made by several investigators. Working with minced guinea-pig lung preparations, Mongar and Schild[2] have shown that SH-group reagents, sodium cyanide, low temperature, low pH, and calcium lack, as well as pre-incubation at 45 °C, effectively prevent *in vitro*-release of histamine. Partial inhibitions were also obtained with antipyretics like salicylate, acetanilide, etc. Although some of these compounds can function as inhibitors of oxidative phosphorylation, Mongar and Schild noted that a potent uncoupling agent like 2:4-dinitrophenol (DNP), was ineffective in blocking histamine release by antigen, even when tested at levels (50 mM) which are 300 times higher than those required to uncouple oxidative phosphorylation in liver mitochondrial preparations.[3] These results were at variance with those reported by Moussatché and Danon[4]; using guinea-pig lung slices from sensitized animals, they reported that 0·1 mM DNP, as well as 0·01 mM pentachlorophenol, effectively blocked the release of histamine by antigen. The same authors[5] demonstrated that histamine release from guinea-pig lung could be appreciably increased by the addition of Krebs cycle intermediates to the Ringer–Barron fluid employed as incubation medium. They postulated that the

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release of histamine in guinea-pig anaphylaxis is dependent on energy-rich phosphorylated compounds supplied by aerobic oxidative metabolism.[4]

Following the discovery that several amines of relatively simple structure have histamine-releasing properties, use has been made of such compounds for the study of the histamine-releasing process. In 1949, Rocha e Silva and Schild,[6] using the isolated rat diaphragm, studied the histamine-releasing action of *d*-tubocurarine. Examining the influence of temperature on this process they found that, besides its dependence on physical factors like the diffusion of *d*-tubocurarine and histamine through the tissue, the action of the alkaloid seemed to have a chemically controlled phase. Using minced guinea-pig lung, Mongar and Schild[2] have studied the release of histamine by compound 48/80 and other amines, but their results have shown that in this tissue amines are unsuitable models for the study of histamine release in anaphylaxis.

The high potency of compound 48/80 in inducing the release of histamine from rat tissue[7, 8] as well as the sensitivity of the process to temperature[9] and metabolic inhibitors,[10] has been stressed by Rocha e Silva.[11] Using the isolated rat diaphragm technique, Rocha e Silva *et al.*[9] have shown that the histamine-releasing action of compound 48/80 is affected by inhibitors like salicylate, calcium lack, low pH, etc., in much the same manner as is the release of histamine in guinea-pig lung anaphylaxis. Similar results have been obtained more recently by Mota *et al.*,[12] who have shown that the release of histamine from rat tissue by *in vitro*-anaphylaxis or by compound 48/80 was sensitive to the same agents used by Mongar and Schild[2] to inhibit this release in guinea-pig lung anaphylaxis.

The conflicting results regarding the inhibitory effects of dinitrophenol on histamine release,[2, 4] could have been caused by differences in the incubation conditions employed. It was therefore considered of interest to reinvestigate this problem using the isolated rat diaphragm technique and 48/80 as the histamine-releasing agent.

MATERIALS AND METHODS

Compound 48/80, a condensation product of *p*-methoxiphenethyl methylamine and formaldehyde, was obtained from the Wellcome Research Laboratories, Tuckahoe, N.Y. Krebs–Ringer phosphate buffer, pH 7·3, was prepared using one-half of the originally recommended concentration of $CaCl_2$ suggested by Umbreit.[13]

Wistar rats of either sex, weighing from 150 to 200 g, were stunned and decapitated and their diaphragms excised. After removing fat and loose connective tissue, the diaphragms were cut into from 5 to 8 pieces of about 80 mg wet weight each. One piece was used in each incubation flask.

Incubations were performed at 37 °C in air, in 10 × 100 mm test tubes shaken by a back-and-forth movement of about 300 strokes/min. All samples were pre-incubated for 30 min at 37 °C in buffer or in the presence of inhibitor, or substrate, or both. After adding the releasing agent, incubations were continued for 25 min. Final vols. were 2·0 ml. Controls and blanks containing, respectively, either no inhibitor or no releasing agent, were always run in parallel with the experimental incubations.

In the experiments with sodium oleate, pre-incubations were performed in Krebs–Ringer buffer prepared without calcium and magnesium. Prior to the addition of the 48/80 the tissue samples were transferred to the complete Krebs–Ringer medium but containing no oleate. This procedure apparently did not interfere with the release of

histamine in the controls; it was employed to avoid interaction of oleate with calcium and magnesium ions during pre-incubation.

Following incubation, tissues were removed, gently blotted, weighed and placed in 0·1 N HCl. Residual histamine was extracted in the manner described by Feldberg and Talesnik.[8] Incubation media containing released histamine were kept frozen until the moment of assay.

Histamine was assayed on the atropinized ileum of the guinea-pig; the tissue was suspended in Tyrode's solution at 37 °C. Comparisons were made with standard solutions of histamine phosphate. Some of the inhibitors employed affected the response of the ileum to this standard. Prior to assay such compounds were removed by acidification and ether extraction (thiopental), or acidification and boiling (NaCN).

Oxygen consumption was measured in the Warburg apparatus. Approximately 100 mg of rat diaphragm were placed in the main compartment of the flasks, and buffer containing the substrate was added. The centre-well contained 20% KOH. After a 10-min equilibration period, the solution of DNP was added from the side-arm. Consumption of oxygen was measured during the 30- to 60-min interval which followed the addition of DNP. Of compound 48/80, 20 μg per ml, added from the second side-arm, were present during this period.

Experiments in anoxia were performed in Thunberg tubes. The nitrogen employed was freed from oxygen by passing it over incandescent copper filings, previously reduced by exposure to a stream of hydrogen. Oxygen in the incubation medium and in the tissue was removed by alternately evacuating and filling the tube with nitrogen.

TABLE 1. RELEASE OF HISTAMINE FROM ISOLATED RAT DIAPHRAGM BY COMPOUND 48/80
Influence of glucose and other metabolites on the inhibition induced by 2:4-dinitrophenol (DNP).

Addition to medium	Per cent histamine released	
	Total	Total − blank
—	43·6 ± 2·7* (6)	38·3
Glucose, 4·5 mM	51·3 ± 2·8 (6)	45·2
DNP, 0·03 mM	35·5 ± 3·8 (6)	31·0
DNP, 0·03 mM + glucose, 4·5 mM	43·5 ± 3·2 (6)	39·0
DNP, 0·3 mM	8·5 ± 2·9 (4)	4·8
DNP, 0·3 mM + glucose, 4·5 mM	48·2 ± 5·6 (4)	43·7
DNP, 0·3 mM + succinate, 5 mM	8·3 ± 1·1 (3)	3·8
DNP, 0·3 mM + fumarate, 5 mM	4·1 ± 2·2 (3)	2·1
DNP, 0·3 mM + acetate, 5 mM	4·6 ± 1·6 (2)	2·3
DNP, 0·3 mM + pyruvate, 5 mM	3·5 ± 2·8 (3)	1·5
DNP, 0·3 mM + D-xylose, 4·5 mM	8·7 ± 1·8 (3)	3·4

Figures within parentheses refer to the number of experiments performed.
* Standard error of the mean 48/80, 20 μg/ml.

RESULTS

Results are expressed as percentage of the total histamine of tissue released during incubation. The first column of figures of Table 1 shows that an average of 43·6 per cent of the histamine of the tissue sample is released when the tissue is incubated, in glucose-free buffer, with 20 μg of 48/80 per ml. A small but significant increase to 51·2 per cent ($P < 0·05$) was observed when 4·5 mM glucose was present during incubation. The second column of figures represents the net release of histamine. It was obtained by subtracting values for the spontaneous release of histamine (blanks)

from the data in the first column. In the experiments presented in this work, blank values ranged between 4 and 11 per cent of the total histamine present in the tissue.

Table 1 shows that 0·03 mM DNP added to glucose-free buffer induced a moderate but significant reduction ($P = 0{\cdot}05$) in the amounts of histamine released by 48/80. When assayed in the presence of 4·5 mM glucose, inhibition by DNP was no longer noticeable. The effect of glucose was shown to a much greater extent in experiments performed in the presence of 0·3 mM DNP. As indicated in Table 1, at this level DNP produced almost complete suppression of the histamine-releasing action of compound 48/80. In this case again, 4·5 mM glucose practically abolished the effect of DNP. Table 1 shows also that Krebs cycle metabolites like succinate, fumarate and pyruvate, as well as D-xylose, did not reverse the action of 0·3 mM DNP.

The relation between glucose concentration and the suppression of DNP-induced inhibition of the release of histamine is shown in Fig. 1. It can be seen that abolition

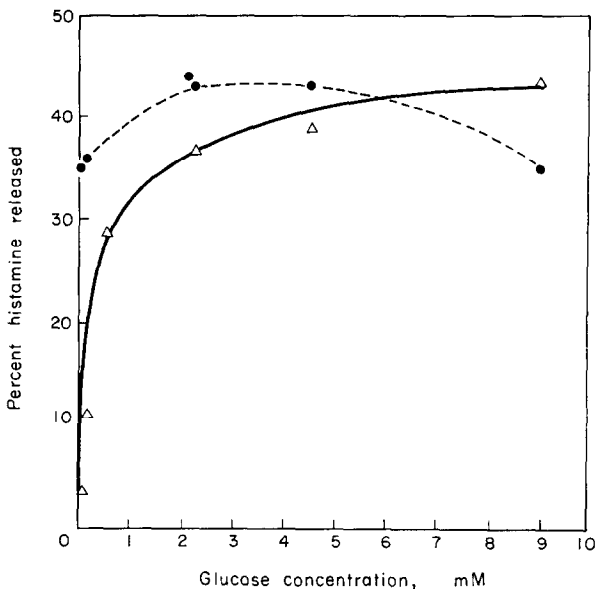

FIG. 1. Influence of glucose concentration on the DNP-induced inhibition of the release of histamine from rat diaphragm by compound 48/80. △——△: incubations performed in the presence of 0·3 mM DNP. ●——●: controls, incubated in the absence of DNP. Each point is the average of three experiments and is corrected for blank values.

of the action of DNP was obtained at glucose levels above 5 mM, but that lower concentrations of glucose were already effective. The second curve in this figure indicates that, even in the absence of DNP, glucose exerted a slight potentiation of the action of compound 48/80. This effect was no longer apparent at 10 mM glucose.

The uncoupling action of DNP in most tissues is usually associated with an increased oxygen consumption, which may be followed by a phase of decreased oxygen uptake. At higher concentrations of DNP, the latter effect often predominates and a depressed respiratory activity may be noted soon after the tissue came into contact with the inhibitor.[14] In order to investigate the effects of DNP on oxygen consumption of rat diaphragm, experiments were made in the Warburg apparatus. Table 2 shows that in an atmosphere of air, 0·03 mM DNP increased oxygen uptake by rat diaphragm both

in the presence and absence of glucose. At the 0·3 mM level, DNP induced a significant depression of oxygen consumption, the degree of which was slightly reduced by the presence in the medium of 4·5 mM glucose; this indicated that the tenfold increase in histamine release (shown in Table 1), was accompanied by a much smaller increase in overall oxygen consumption. The effect of succinate on oxygen consumption in the presence of 0·3 mM DNP was also studied. Table 2 shows that it increased oxygen uptake to levels obtained in the absence of DNP. When compared with the results obtained with succinate (Table 1), it becomes apparent that in this case there was again no correlation between oxygen consumption and histamine release by 48/80. The effects on oxygen consumption of 0·3 mM DNP, of glucose and of succinate were also examined in an atmosphere of pure oxygen. Table 2 shows that this environment did not change the relative values of oxygen uptake, although it increased these values in all samples.

TABLE 2. OXYGEN CONSUMPTION OF DNP-TREATED RAT DIAPHRAGM

Addition to medium	Oxygen consumption*	
	In air	In oxygen
—	11·4 ± 1·4† (5)	
Glucose, 4·5 mM	11·0 ± 0·8 (4)	17·7 ± 3·4 (3)
Succinate, 5 mM	11·3 ± 1·3 (5)	17·3 ± 3·3 (3)
DNP, 0·03 mM	16·2 ± 1·2 (3)	
DNP, 0·03 mM + glucose	18·0 ± 1·3 (3)	
DNP, 0·3 mM	4·2 ± 0·8 (5)	6·4 ± 0·6 (3)
DNP, 0·3 mM + glucose	6·2 ± 0·4 (4)	7·8 ± 0·7 (3)
DNP, 0·3 mM + succinate	12·8 ± 2·3 (5)	21·9 ± 6·1 (2)

Figures within parentheses indicate the number of experiments performed.
* μl/min per g wet weight.
† Standard error of the mean.

In a complex system such as the rat diaphragm, in which many different types of cells contribute to overall oxygen consumption, results like those presented on Table 2 give only incomplete evidence about the correlation between aerobic metabolism and the release of histamine by compound 48/80. It was felt that a clearer picture of the pathway by which glucose affected the histamine-releasing process would be obtained by examining its effects on tissue under anoxia or treated with sodium cyanide. These conditions can be expected to inhibit aerobic metabolism extensively in most mammalian cells. The results shown in Table 3 indicate that 1 mM cyanide inhibited the release of histamine by 48/80, as already shown by Mota.[12] Nitrogen anoxia was also inhibitory, although to a lesser extent. The presence of 4·5 mM glucose effectively reversed both inhibitions; however, a residual inhibition of about 20 per cent remained in both cases. In experiments in which from 2·25 mM to 9·0 mM glucose was used, this residual inhibition was not abolished.

Previous work in this laboratory [9] had indicated that salicylate inhibited the release of histamine from rat diaphragm incubated with compound 48/80 in buffered physiological saline. Salicylate also inhibits histamine release in antigen–antibody reactions,[2] and by anaphylatoxin;[15] its action as an uncoupling agent has been described.[16] Table 4 shows the effects of salicylate, in the presence and absence of glucose, on the release of histamine by 48/80; 25 mM salicylate was strongly inhibitory; in the presence

of glucose its action was depressed, a 30 per cent inhibition remaining. At the 10 mM level salicylate was also inhibitory; however, its action was almost completely reversed by the presence of glucose.

Sodium oleate and thiopental, although having no structural similarity to uncouplers like DNP or salicylate, have been described as having similar effects on mitochondria.[17, 3] The action of these two agents on the release of histamine by 48/80 is also shown in Table 4. The inhibition produced by thiopental was of the order of 70 per cent; it was markedly decreased by the presence of glucose. Sodium oleate, 1mM, inhibited the release of histamine to the same degree as thiopental; as with the barbiturate, the action of oleate was extensively reversed by glucose.

TABLE 3. RELEASE OF HISTAMINE FROM ISOLATED RAT DIAPHRAGM BY COMPOUND 48/80
Influence of glucose on the inhibition induced by anoxia and by sodium cyanide.

Addition to medium	Per cent histamine released	
	Total	Total − blank
—	45·2 ± 3·1* (6)	41·3
NaCN, 1 mM	5·9 ± 1·2 (6)	2·0
NaCN, 1 mM + glucose, 4·5 mM	36·7 ± 1·7 (6)	32·8
None (aerobic controls)	42·0 ± 2·8 (5)	36·7
Nitrogen atmosphere	25·1 ± 3·8 (5)	14·1
Nitrogen atmosphere + glucose, 4·5 mM	40·6 ± 2·1 (5)	29·6

* Standard error of the mean 48/80, 20 μg/ml.
Figures within parentheses indicate the number of experiments performed.

TABLE 4. RELEASE OF HISTAMINE FROM ISOLATED RAT DIAPHRAGM BY COMPOUND 48/80
Influence of glucose on the inhibition produced by sodium salicylate, sodium oleate and thiopental.

Addition to medium	Per cent histamine released	
	Total	Total − blank
—	50·0 ± 4·3* (5)	43·8
Salicylate, 25 mM	8·3 ± 1·8 (3)	2·0
Salicylate, 25 mM + glucose, 4·5 mM	36·5 ± 2·2 (4)	30·2
Salicylate, 10 mM	20·4 ± 1·6 (3)	14·5
Salicylate, 10 mM + glucose, 4·5 mM	43·0 ± 7·0 (2)	36·8
—	46·7 ± 4·5 (3)	38·2
Thiopental, 1 mM	21·8 ± 4·7 (3)	13·5
Thiopental, 1 mM + glucose, 4·5 mM	43·7 ± 2·9 (3)	35·2
—	52·0 ± 6·5 (4)	44·5
Oleate, 1 mM	20·7 ± 4·2 (4)	13·2
Oleate, 1 mM + glucose, 4.5 mM	55·6 ± 7·8 (4)	48·1

* Standard error of the mean 48/80, 20 μg/ml.
Figures within parentheses indicate the number of experiments performed.

Mast cells alterations

Typical morphological alterations of rat tissue mast cells are evoked by 48/80.[18] The effects of metabolic inhibitors on this action have been studied by Junqueira and Beiguelman[19] who, among other compounds, found DNP and NaCN to prevent the

extrusion of granules which is induced by 48/80. We have confirmed these results, but have found the inhibitory action of DNP to be evident to a marked extent only in the absence of glucose. Using pieces of rat mesentery, microscopic examination of the results of incubations with 20 μg of 48/80 per ml in Krebs–Ringer phosphate buffer revealed that: (a) in samples pre-incubated with 0·3 mM DNP, 6 per cent of the mast cells showed granule extrusion; (b) this value was increased to 70 per cent when 4·5 mM glucose plus 0·3 mM DNP were present; and (c) controls incubated in the absence of either compound presented granule extrusion in 99 per cent of the cells. Similar experiments using 1 mM sodium cyanide showed: (a) in controls, 95 per cent of the cells presented granule extrusion; (b) in the presence of NaCN this value was reduced to 8 per cent; and (c) NaCN plus 4·5 mM glucose revealed effects of 48/80 in 87 per cent of the cells. Each result refers to averages of two experiments, each performed in triplicate with samples of mesentery from one animal. A total of approximately 300 cells were counted in each sample.

DISCUSSION

The results presented in this paper indicate that the release of histamine from isolated rat diaphragm by compound 48/80 is effectively blocked by 2:4-dinitrophenol. The presence of glucose, but not of succinate, pyruvate, acetate or fumarate, reversed the inhibitory effect of DNP. The increased oxygen consumption of DNP-treated tissue, observed in the presence of succinate, indicated that this substrate was being metabolized, and yet was unable to affect the inhibition of the histamine-releasing system. Accordingly, it appeared that glucose was affecting the DNP-inhibition by a route other than the Krebs cycle and the cytochrome electron transport chain. The possiblity that glucose reversed the action of DNP by a direct chemical interaction was considered unlikely. D-Xylose, a compound having a similar chemical activity as glucose, but which is less readily metabolized by the mammalian cell,[20] was ineffective in relieving histamine release from inhibition by DNP.

A phenomenon similar to that encountered with DNP, was observed when glucose was used in the presence of two other inhibitors of the release of histamine by 48/80, namely, sodium cyanide and nitrogen anoxia. These inhibitors, although having another site of action than DNP, will ultimately affect aerobic cell metabolism in a similar way, e.g. by depressing the synthesis of high-energy phosphate. The ability of glucose to reverse the inhibition of histamine release by these agents, as well as by DNP and uncoupling agents like salicylate, thiopental or oleate, could be explained most logically by the suggestion that the sugar enables the cell to use other metabolic pathways to provide the histamine-releasing process with high-energy intermediates. Randle[21] has pointed out that, like insulin, inhibitors of oxidative phosphorylation can stimulate glucose uptake by rat muscle *in vitro* and probably have similar effects *in vivo*. A relationship between the release of histamine and the effects of insulin *in vivo* has been indicated by the results of Goth *et al.*;[22] these showed that in alloxan-diabetic rats the liberation of histamine by dextran or egg-white is severely depressed, but takes place normally following insulin treatment. It seems possible that in tissue treated with inhibitors of oxidative phosphorylation, the glycolytic breakdown of glucose supplies the metabolites required by the histamine-releasing process. A high glycolytic activity under aerobiosis has been noted in leucocytes.[23] In view of the similarity between tissues mast cells and the leucocytic basophils[24, 25] the search for

the relative importance of glycolysis in mast cell metabolism and especially in the amine-releasing mechanism are interesting possibilities for future work.

Results of studies[26] on the effects of DNP and glucose on histamine release in guinea-pig lung anaphylaxis have shown that the powerful inhibition of this process evoked by 0·3 mM DNP, although significantly sensitive to partial reversal by glucose, was less affected than DNP-inhibition of the release of histamine by compound 48/80 in rat diaphragm. In contrast, experiments on rat anaphylaxis have revealed that in the isolated sensitized diaphragm, the release of histamine by antigen, which is also abolished by 0·3 mM DNP, is not only reversed, but markedly potentiated when glucose plus DNP are present in the medium.

Thus, it becomes apparent that the study of the action of metabolic substrates like glucose or others on the effects of inhibitors of histamine release, may well provide a valuable tool for the clarification of the biochemical events which accompany the release of amines in animal tissues.

Note added in proof

Upon completion of this manuscript it was learned that B. Westerholm (*Acta Physiol. Scand.* **50**, 300, 1960) had shown that the *in vitro* release of histamine produced by 48/80 in cat skin is blocked by anoxia, and that glucose reverses this block. The author also refers to unpublished results by Diamant, in which effects of glucose and anoxia, similar to those described by us on Table 4, were obtained.

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