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INHIBITION OF HISTAMINE RELEASE BY SODIUM SALICYLATE AND OTHER COMPOUNDS

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There are few reports in the literature of drugs which protect against allergic reactions by mechanisms other than antagonism to released humoral agents. Substances which inhibited the chain of reactions leading to release of active substances such as histamine might be useful in the treatment of conditions, suspected to be allergic in origin, that do not respond to treatment with antihistaminic drugs. Knowledge of the mode of action of such inhibitors of the allergic reaction, even if weak, might be useful in suggesting possible structures for more effective agents.

Sodium oxalate, sodium citrate, and phenol, completely prevent *in vitro* anaphylactic histamine release in rabbit blood; heparin is partially effective (Dragstedt, Wells, and Rocha e Silva, 1942; McIntire, Roth, and Richards, 1949). There is little agreement, however, on the ability of heparin to protect against acute anaphylactic shock *in vivo*; sodium citrate does reduce the amount of histamine liberated during anaphylactic and anaphylactoid reactions in the dog (Rocha e Silva, Scroggie, Fidlar, and Jaques, 1947; Rocha e Silva, 1950). Sodium salicylate inhibits anaphylactic histamine release from guinea-pig lung *in vitro* (Trethewie, 1951; Ungar and Damgaard, 1955) and modifies the response of isolated guinea-pig ileum to the specific antigen (Gray, Pedrick, and Winne, 1951); both sodium salicylate and acetylsalicylic acid protect rabbits against acute anaphylactic shock (Lepper, Caldwell, Smith, and Miller, 1950) although the latter is not effective against histamine shock (Campbell, 1948). Sodium salicylate also gives some protection against the passive reversed Arthus reaction in rabbits and guinea-pigs (Smith and Humphrey, 1949) and against the Schwartzman reaction (Schwartzman, Schneierson and Soffer, 1950).

This paper is mainly concerned with a study of the effect of sodium salicylate on various reactions in which histamine is released, in an attempt to throw some light on its mode of action.

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METHODS

Sensitization of Rabbits.—Rabbits were sensitized by subcutaneous injections of a modified Freund type antigen (protein of Wellcome normal horse serum 5% w/v in 0.85% w/v NaCl, 5 g.; ointment of wool alcohols B.P., 5 g.; liquid paraffin containing heat-killed avian tubercle bacilli 2 mg./ml., 10 g.) (Freund, 1948). Each animal received 1 ml. at two different sites on the first day. This was repeated once between days 7 and 14 and then again 10 days later. Boosting doses (2 by 1 ml.) were given when necessary. Seven days were allowed to elapse after an injection before blood samples were withdrawn.

General Methods.—The collection of blood, the biological assay of histamine, and the polysaccharides used have been described previously (Haining, 1955). Plasma histamine was usually extracted by the method of McIntire, Roth, and Shaw (1947), but sometimes, including all experiments where estimates of cellular histamine were required, Code's (1937) modification of the method of Barsoum and Gaddum was employed. Antigen-antibody precipitate was obtained by incubating serum from a sensitized rabbit with a small quantity of horse-serum for 30 min. at 37.5° C. The precipitate was washed 3 times with, and suspended in, a volume of 0.85% NaCl equal to that of the original serum. When activated plasma was required the plasma of dilute heparinized blood (heparin 1.5 units/ml.) was incubated with antigen-antibody precipitate for 1 hr. at 37.5° C. and then centrifuged to remove the precipitate. Anaphylatoxins were prepared by the method of Bordet (1913), and were tested on isolated guinea-pig ileum. Concentrations of activators referred to in the text are those present in the final preparation.

Inhibition of Histamine Release in Rabbit Blood.—Samples (6 ml.) of dilute heparinized blood (heparin 1.5 units/ml.) were mixed with 1 ml. of 0.85% w/v NaCl with and without inhibitor. The samples were incubated at 37.5° C. for 15 min.; 0.5 ml. of 0.85% w/v NaCl or the same solution containing Wellcome No. 2 horse serum 6% v/v was then added and the incubation continued for a further 15 min. The samples were chilled in ice and centrifuged at 3,000 rev./min. for 20 min. at room temp. Aliquots of the supernatant plasma were extracted and assayed for histamine.

Results

When blood from a sensitized rabbit was incubated with the specific antigen there was almost always a marked increase in the concentration of plasma histamine. (In 21 experiments, the mean plasma histamine of control samples was 0.6 μg./ml. and after incubation with antigen 1.8 μg./ml.) When blood from normal rabbits was incubated with the same concentration of the antigen no release took place. (In 3 rabbits the mean level of plasma histamine in untreated blood was 0.45 μg./ml. and after treatment 0.49 μg./ml.) In sensitized blood treated with antigen, sodium salicylate caused a graded reduction in the level of plasma histamine depending upon the concentration employed (Fig. 1).

In early experiments the control levels of plasma histamine were high, presumably because of a non-specific release, which also appeared to be reduced by the drug. Both sodium chloride and potassium chloride were effective inhibitors, but only at much higher molar concentrations than were needed with sodium salicylate. Since the regression lines for sodium chloride and potassium chloride were so close together it seemed likely that the inhibitory activity could be attributed to the anion. The inhibitory effect of sodium benzoate was found to equal that of sodium salicylate.

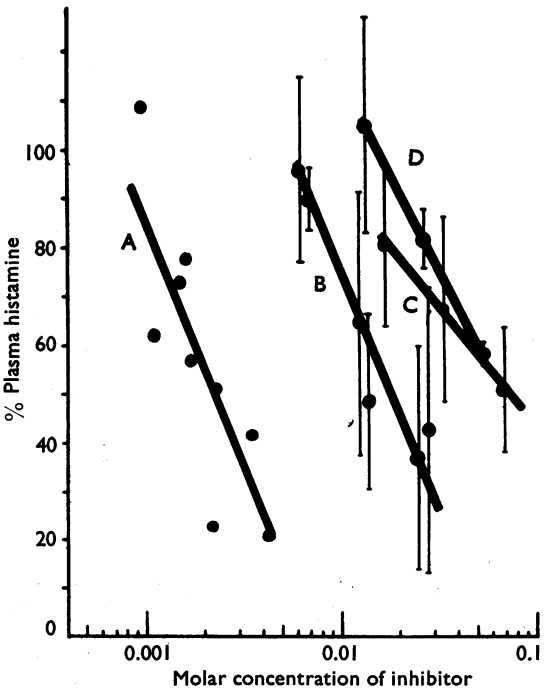

FIG. 1.—Inhibition of the *in vitro* anaphylactic release of histamine from rabbit blood cells. Blood from sensitized rabbits was incubated with the specific antigen (horse serum 1: 250) in the presence of inhibitors for 15 min. at 37.5° C. The plasma histamine level obtained in the presence of antigen+drug was compared with that found after treatment with antigen only (=100%). A, 3-hydroxy-2-phenylcinchoninic acid; B, sodium salicylate and sodium benzoate; C, sodium chloride; D, potassium chloride. Standard deviations are shown by vertical bars.

Sodium salicylate, sodium chloride and sodium benzoate at the highest concentrations used in these experiments did not interfere with the extraction and assay of histamine (approx. 1 μg./ml.) from dilute plasma. Mean estimates of plasma histamine found in the presence of drugs expressed as percentages of that in untreated plasma were as follows: sodium salicylate 104% (4 expts.), sodium benzoate 98% (2 expts.), sodium chloride 102% (2 expts.).

Difficulties were experienced when testing 3-hydroxy-2-phenylcinchoninic acid (HPCA) for inhibitory activity since low recoveries of histamine were obtained when it was present in plasma. Attempts to remove the compound from plasma were unsuccessful, and its interfering effects were therefore neutralized by using "paired" samples of blood in the histamine release reaction. One sample contained HPCA (as the Na salt in 0.85% w/v NaCl, *p*H 7.1–7.4) during the incubation period when antigen was present, and the same amount of HPCA was added to the other sample only after further histamine release had been prevented in both samples by adding sodium oxalate 0.02 M. Of the compounds tested, the most effective in preventing histamine release was HPCA, which was about eight times as active as sodium salicylate. Even HPCA is, however, only active at high concentrations.

Confirmation of the inhibitory action of sodium salicylate on histamine release was obtained by determining the amount of histamine retained in the cells following *in vitro* anaphylactic reactions; the cellular fraction of blood showed in each case a marked fall in histamine concentration which corresponded to the increase found in the plasma. In the presence of sodium salicylate, this fall was either prevented or reduced (Table I), and in two samples of blood treated with varying concentrations of the salt graded reductions were obtained.

None of the inhibitors used caused an alteration of plasma *p*H as determined by indicator papers reading to 0.3 of a unit. However, in view of the possibility that changes of *p*H not detectable in this way could have been sufficient to interfere with histamine release, experiments were carried out to determine the action of varying amounts of hydrochloric acid on the release reaction. Inhibitory effects were only observed when the plasma *p*H fell from its normal value of 8.3 to 7.4.

TABLE I

INHIBITION OF THE *IN VITRO* ANAPHYLACTIC RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD BY SODIUM SALICYLATE

Blood from sensitized rabbits was incubated with the specific antigen (horse serum 1:250) for 15 min. at 37.5° C. Cellular or whole blood histamine was extracted by Code's method

Rabbit	Total Blood Histamine (μg./ml.)	Cellular Histamine (μg./ml. Blood)		Concentration of Sodium Salicylate (mg./ml.)	After Treatment with Antigen + Sodium Salicylate. Cellular Histamine (μg./ml. Blood)
		Control	After Treatment with Antigen		
7	7·0	5·7	2·8	4	6·1
9	1·9	1·4	1·4		
5	2·0	1·5	0·24	4	0·57
3	2·4	1·8	0·28	4	1·8
5		1·8	0·37	2	1·1
				4	1·5
3	1·7	1·3	0·55	1	0·50
				2	1·2
				4	1·6

In order to test the possibility that sodium salicylate might exert a permanent inhibitory action on the cells, dilute heparinized blood from a sensitized rabbit was incubated for 15 min. with sodium salicylate 4 mg./ml. The salicylate-containing plasma was then replaced by fresh plasma from the same sample of blood. The specific antigen was added and incubation was continued for a further 15 min. after which a sample of plasma was removed for testing. Knowing the histamine content of each plasma sample and the volume occupied by the cells, it was possible to calculate the total quantity of histamine liberated at each stage. When sodium salicylate was present only during the first incubation period, the quantity of histamine released during the second period (Table II) was comparable with that released by antigen in the absence of drug. Since very little histamine was released when antigen and sodium salicylate were present during both incubations this release must have taken place during the second incubation. It can be concluded, therefore, that the presence of sodium salicylate does not permanently interfere with the capacity of cells to release their histamine.

TABLE II

THE TRANSIENT INHIBITORY ACTION OF SODIUM SALICYLATE ON THE *IN VITRO* ANAPHYLACTIC RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD

Treatment	Histamine Released During Incubation Periods (μg./ml. Blood)		
	Rabbit 5 (Duplicates)		Rabbit 14
Blood incubated with specific antigen and sodium salicylate (4 mg./ml.) for two 15-min. periods	−0·05	−0·07	0·41
Blood incubated with specific antigen for two 15-min. periods	2·2	2·5	1·2
Blood incubated with sodium salicylate (4 mg./ml.) during the first 15 min. Salicylate-containing plasma was then replaced by fresh plasma plus the specific antigen and incubation continued for 15 min.	2·1	2·1	1·1*

* Antigen also present during the first incubation.

Histamine release in normal rabbit blood can be initiated by incubation with preformed washed antigen-antibody precipitate (P. B. Dews, personal communication), and experiments were carried out to determine whether sodium salicylate, dextran sulphate and heparin would inhibit this release. The antigen-antibody precipitate always caused a marked increase in the histamine concentrations in the plasma, and this was completely prevented by sodium salicylate 4 mg./ml. and by dextran sulphate I 2–4 mg./ml. and partially by heparin 2 mg./ml. (Table III). The histamine was not derived from the antigen-antibody precipitate itself, since incubation of the precipitate with rabbit plasma for 30 min. did not increase the concentrations of plasma histamine (the mean value in untreated plasma was 0.31 μg./ml. (5 determinations) and after incubation with the precipitate was 0.33 μg./ml.).

These facts suggested that the antigen-antibody precipitate was causing activation of the plasma, and this was tested experimentally. When normal blood was incubated with its own "activated"

TABLE III

THE *IN VITRO* RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD AND ITS INHIBITION BY SODIUM SALICYLATE, HEPARIN, AND DEXTRAN SULPHATE I

Normal dilute heparinized rabbit blood with or without inhibitor was incubated with washed antigen-antibody precipitate for 15 min. at 37.5° C. Histamine in the supernatant dilute plasma was extracted by McIntire's method

Expt.	Plasma Histamine (μg./ml. Blood)					
	Control	Blood Incubated with Antigen-Antibody Precipitate				
		Alone	+ Sodium Salicylate (4 mg./ml.)	+ Heparin* (2 mg./ml.)	+ Dextran Sulphate I	
					(4 mg./ml.)	(2 mg./ml.)
1	0·27 0·21	1·5 1·9	0·30 0·33			
2	0·30 0·37	1·4 2·2	0·23 0·46			
3	0·27 0·32	1·9				
4	0·60	1·8		0·82		
5	0·20 0·15	1·2 1·1		0·70 0·57		0·17 0·17
6	0·37	1·7			0·29	

* 93 units/mg.

plasma (see Methods), there was almost always a marked increase in the histamine concentration of the plasma (Table IV). This release of histamine was inhibited by sodium salicylate 4 mg./ml., but heparin 2 mg./ml. did not appear to be effective.

This activation of rabbit plasma may be distinct from that which occurs during anaphylatoxin formation. Tests on isolated guinea-pig ileum showed that neither rabbit serum nor heparinized plasma

TABLE IV

THE *IN VITRO* RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD BY "ACTIVATED" RABBIT PLASMA AND THE EFFECTS OF SODIUM SALICYLATE AND HEPARIN ON THE REACTION

"Activated" plasma was prepared by incubating rabbit plasma with antigen-antibody precipitate. At the end of the incubation period the precipitate was removed and the plasma incubated with dilute heparinized blood from the same rabbit

Experiment	Plasma Histamine μg./ml. of Blood				
	Blood Incubated with Untreated Plasma		Blood Incubated with "Activated" Plasma		
			Alone	+ Sodium Salicylate (4 mg./ml.)	+ Heparin (2 mg./ml.)
1	0·33		1·0 1·1	0·49 0·52	
2	0·56 0·87		1·9	0·56 0·64	
3	0·37		0·54	0·36	
4	0·60		0·64	0·19	
5		0·31 0·30	0·60 0·71		0·76 0·52
6		0·68	1·2		1·4
3		0·37	0·54		0·51
4		0·60	0·64		0·48
Mean percentage	58	67	100	44	96

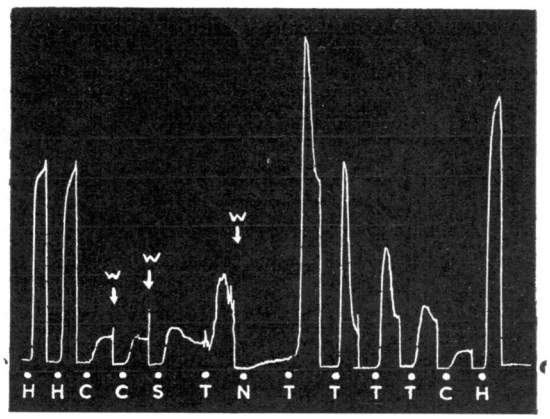

FIG. 2.—Inhibition of the action of rat serum agar anaphylatoxin on guinea-pig ileum by sodium salicylate. Isolated guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. at 36° C. H, histamine (as acid phosphate) 0.4 μg./ml.; C, control rat serum, 0.008 ml./ml.; W, organ-bath fluid replaced by fresh Tyrode solution; S, sodium salicylate added to bath fluid giving final concentration of 4 mg./ml.; T, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.008 ml./ml.; N, NaCl added to bath fluid giving final molarity equivalent to sodium salicylate 4 mg./ml.

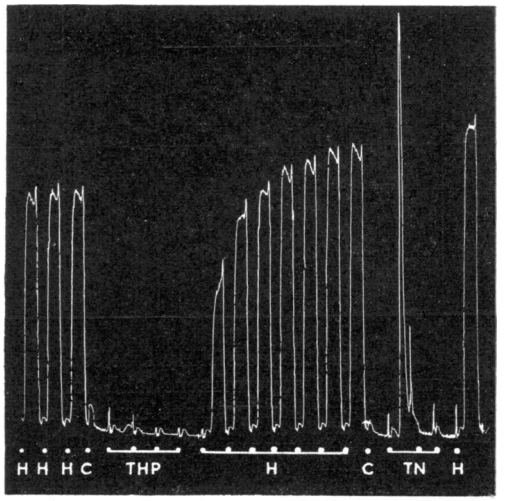

FIG. 3*a*

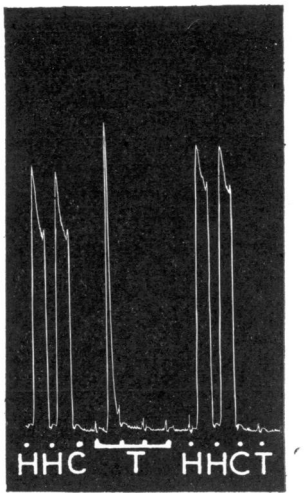

FIG. 3*b*

FIGS. 3*a* and 3*b*.—Inhibition of the action of rat serum agar anaphylatoxin by hydroxyphenylcinchoninic acid. Isolated guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. at 36° C. H, histamine, 0.03 μg./ml.; C, control rat serum, 0.004 ml./ml.; T, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.004 ml./ml.; THP, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.004 ml./ml. plus hydroxyphenylcinchoninic acid 0.5 mg./ml.; TN, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.004 ml./ml. plus NaCl of equal molarity to hydroxyphenylcinchoninic acid 0.5 mg./ml.

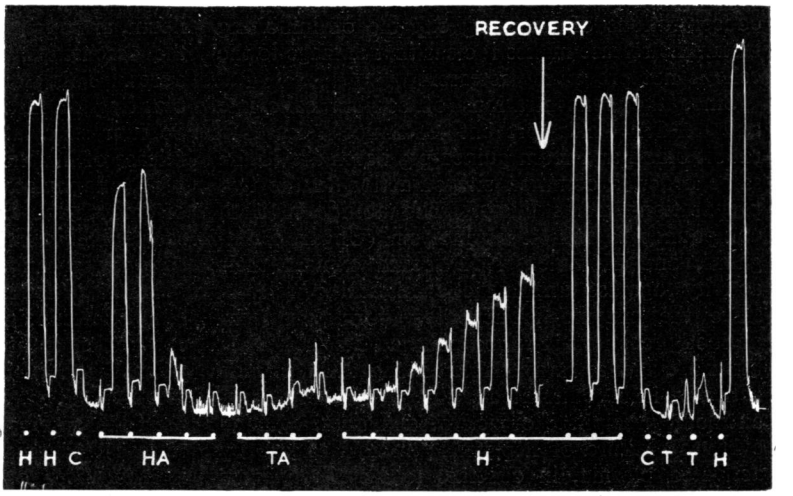

FIG. 4.—Inhibition of the effect of rat serum agar anaphylatoxin on guinea-pig ileum by mepyramine maleate. Isolated guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. at 36° C. H, histamine (as acid phosphate) 0.03 μg./ml.; C, control rat serum, 0.004 ml./ml.; HA, histamine (as acid phosphate) 0.03 μg./ml. plus mepyramine maleate 0.005 μg./ml.; TA, rat serum after incubation with New Zealand agar (0.83 mg./ml.) 0.004 ml./ml. plus mepyramine maleate, 0.005 μg./ml.; T, rat serum after incubation with New Zealand agar (0.83 mg./ml.) 0.004 ml./ml. *Recovery*, doses of histamine 0.03 μg./ml. until recovery of the gut had taken place.

(heparin 1.5 units/ml.) gave rise to an anaphylatoxin when incubated for 2 hr. with dextran sulphate D/3 0.1 mg./ml. or agar 0.83 mg./ml. although these concentrations were effective in producing anaphylatoxin when rat serum was used.

Both sodium salicylate and HPCA inhibited the action of rat serum agar anaphylatoxin on isolated guinea-pig ileum. Fig. 2 shows that sodium salicylate 4 mg./ml. prevented the action of anaphylatoxin without having any permanent inactivating effect on the gut; lower concentrations were not effective. The action of anaphylatoxin could be completely prevented by HPCA at a concentration of the order 0.5 mg./ml. but not without interfering with the response to histamine. Fig. 3a shows that in the presence of HPCA 0.5 mg./ml., 4 doses of anaphylatoxin failed to cause a contraction of guinea-pig ileum although, after recovering its sensitivity to histamine, the preparation responded to the same dose of anaphylatoxin. In a control experiment using a different strip of the same gut, complete desensitization was obtained by one dose of anaphylatoxin (Fig. 3b). This action of HPCA is in contrast to that of mepyramine maleate, which also prevented anaphylatoxin from producing a contraction but which rendered the gut insensitive to subsequent doses of anaphylatoxin after it had recovered its previous sensitivity to histamine (Fig. 4).

Sodium salicylate 4 mg./ml. and HPCA 1 mg./ml. did not prevent the development of an active anaphylatoxin when added to rat serum before its incubation with agar.

DISCUSSION

Available evidence suggests that the initial reactions leading to histamine release in rabbit blood occur in the plasma. Histamine release takes place when blood to which antibody has been added, or blood from a sensitized animal, is incubated with the specific antigen (Katz, 1940; Dragstedt, 1941; McIntire, Roth, and Sproull, 1950; Spain, Strauss, and Neumann, 1950), but the interaction between antigen and antibody need not occur in the presence of cells (McIntire *et al.*, 1950) although plasma is essential (Humphrey and Jaques, 1955). This suggests that release is initiated either by some action of the antigen-antibody complex upon the cells, or as a result of plasma activation, which could be brought about by the antigen-antibody complex itself, or by some reaction occurring during its formation.

The present results show that plasma which has been incubated with antigen-antibody precipitate acquires the ability to release a histamine-like substance in blood, and confirms that a similar release occurs when antigen-antibody precipitate is incubated with blood. However, any wettable surface may release histamine in rabbit blood (Code, 1952), and serum or plasma of various species is known to be activated by incubation with particulate matter (Wilson and Miles, 1946) so that the properties of preformed antigen-antibody precipitate do not necessarily represent those of the complex formed during *in vitro* anaphylactic reactions.

Since sodium salicylate inhibits histamine release either by antigen in blood from a sensitized animal, or by antigen-antibody precipitate or activated plasma in normal blood, it is unlikely that its action is due to interference with antibody or antigen-antibody complex (Coburn and Kapp, 1943). Further, the ability of cells to respond normally to antigen or anaphylatoxin after treatment with

concentrations of the drug capable of inhibiting histamine release makes it clear that damage to the cells is not preventing release.

Since the concentrations of sodium salicylate and HPCA which prevent histamine release in rabbit blood and from guinea-pig ileum are of the same order, a common site of action in the two preparations seems likely. The ability of both compounds to inhibit fibrinolysin (Ungar, Damgaard, and Hummel, 1952) could explain their activity against anaphylactic histamine release, since the enzyme is activated during antigen-antibody reactions in rabbit plasma (Geiger, 1952); but no evidence is available to suggest that it is activated in blood treated with preformed antigen-antibody precipitate or activated plasma. However, the failure of heparin to inhibit release by activated plasma, in contrast to its effectiveness against release due to antigen-antibody precipitate, is of interest, since heparin may prevent the activation of fibrinolysin although it is ineffective against the activated enzyme (Ungar and Mist, 1949).

Although activation of rabbit plasma bears a superficial resemblance to anaphylatoxin formation, failure to demonstrate activation of rabbit plasma or serum by agar and dextran sulphate D/3 under conditions in which rat serum was effective points to some fundamental difference between the two reactions. It seems unlikely that antigen-antibody precipitate incubated with blood forms an anaphylatoxin, since heparin, which partially prevents the resulting histamine release, does not prevent anaphylatoxin formation (Rocha e Silva, 1952). If protease activation is concerned in anaphylatoxin formation it should be possible to demonstrate it. Dextran sulphate D/3, dextran D, and agar, all release histamine in rabbit blood, and form anaphylatoxins when incubated with rat serum; but whereas dextran sulphate D/3 activates protease in serum from rats and rabbits, dextran D does not, and agar is ineffective in rat serum under comparable conditions (Haining, 1955, 1956).

The failure of sodium salicylate and HPCA to prevent anaphylatoxin formation is in contrast to the action of sodium citrate; this stops the activation of rat serum by agar and yet is ineffective against formed anaphylatoxin (Rocha e Silva, 1952). The mode of action of sodium salicylate and HPCA in preventing anaphylatoxin-induced contraction of the guinea-pig ileum is quite distinct from that of mepyramine maleate. Anaphylatoxins are known to release cellular histamine (Rocha e Silva, 1952). But in the presence of a specific histamine antagonist they fail to contract guinea-pig ileum, although histamine is released, as shown by the desensitization of the preparation to further doses. This can presumably be attributed to the blocking of histamine receptors by mepyramine. Sodium salicylate and HPCA must prevent the release of histamine since, in the presence of either drug, anaphylatoxin does not cause a contraction of smooth muscle and does not desensitize it. It seems likely that both drugs exert their inhibitory actions by an extracellular mechanism, since guinea-pig ileum which has been treated with anaphylatoxin, in the presence of sufficient of either compound to prevent a spasm, does not contract when the bath fluid is replaced by fresh Tyrode solution. It may be that in the presence of either drug the anaphylatoxin is not able to initiate the release mechanism because some factor in serum is inactivated or prevented from reaching a cell site. However, the possibility of these drugs having a transient intracellular action cannot be ruled out.

Summary

1. Factors influencing the *in vitro* release of histamine in rabbit blood are described.

2. Anaphylactic histamine release in rabbit blood is reduced by a lowering of plasma *p*H and by suitable concentrations of sodium chloride, potassium chloride, sodium benzoate, sodium salicylate, and 3-hydroxy-2-phenylcinchoninic acid. The most effective compound was 3-hydroxy-2-phenylcinchoninic acid, which is approximately eight times as active as sodium salicylate or sodium benzoate.

3. Sodium salicylate, heparin, and dextran sulphate of low molecular weight, inhibit, in normal blood, the histamine release due to incubation with washed antigen-antibody precipitate. Sodium salicylate is effective against release due to plasma activated by antigen-antibody precipitate.

4. Sodium salicylate and 3-hydroxy-2-phenylcinchoninic acid do not prevent the formation of anaphylatoxin but inhibit its action on isolated guinea-pig ileum. This property is shown not to depend upon an antagonism to histamine.

5. The significance of the results is discussed.

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