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ACID GASTRIC SECRETION IN THE RAT AND ITS INHIBITION BY PHLOXIN

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Two years ago, when the experiments reported in this paper were started, it was assumed by many workers that the histamine present in blood was localized in the eosinophils. This assumption prompted us to test whether histamine could serve as the "eosinophilic substrate" of eosinophilic leucocytes. As a first step we investigated the reaction between eosin and histamine *in vitro*. When histamine (as free base, dihydrochloride or diphosphate) is added to an aqueous solution of eosin, a dense precipitate forms with a carmine-red colour distinctly different from the colour of eosin. This reaction is not due to a change in *p*H: if one titrates an aqueous solution of eosin (sodium salt) with hydrochloric acid, an orange-red precipitate appears at *p*H 5.3, whereas with histamine diphosphate a carmine precipitate forms at *p*H 6.8. Substituting for eosin (Na salt of tetrabromofluorescein) the related compound "phloxine" (Na salt of tetrabromo-tetrachlorofluorescein, C.I. No. 778), a dye now sometimes used in the haematoxylin-"eosin" staining method, an even more rapid reaction with histamine is obtained.

Although recent experiments indicate that platelets rather than eosinophils may be the source of the histamine present in human blood (Code, 1952), the pronounced "histaminophilia" demonstrated by eosin and phloxin *in vitro* induced us to study the reactions between histamine and these two dyes in biological systems.

When an aqueous solution of eosin (10^{-3}M) was added to the same volume of an equimolar aqueous solution of histamine dihydrochloride a very slight cloudiness developed in about 48 hr.; on assaying this mixture on the isolated guinea-pig ileum a loss of biologically active histamine was found. When in a similar experiment an aqueous solution of eosin (1.1×10^{-2}M) was added to an equal volume of an equimolar aqueous solution of acetylcholine chloride no cloudiness or precipitation was observed after 48 hr., and the effect of the acetylcholine on the isolated guinea-pig ileum was unimpaired (van Noordwijk and Eichholz, 1952).

In view of the rapidity with which phloxin combines with histamine it was decided to investigate whether it would influence the stimulating effect of histamine on acid gastric secretion, an effect not inhibited by the commonly used antihistaminic drugs such as diphenhydramine and mepyramine (Loew, 1947).

METHODS

Rats of about 200 g. were fasted for 24 hr. Under pentobarbitone sodium anaesthesia (40–45 mg./kg.) a transverse incision of 1.5–2 cm. was made in the right upper quadrant of the abdomen, just below the costal arch. The oesophagus and duodenum were ligated—the latter about 1.5 cm. distal to the pylorus. A glass cannula was introduced into the stomach through an incision in the duodenum. Before closing the abdominal incision the stomach was rinsed with physiological saline, warmed to body temperature, so as to remove any solid contents. A few ml. of saline was left in the stomach, and a short rubber tube was mounted on the glass cannula and clamped off. Half an hour later the stomach was rinsed three or four times with saline (20 ml. in all), and the washings were collected in an Erlenmeyer flask. This was the control lavage. In the following experiments all injections, with the exception of the first injection of phloxin, were given after the control lavage. A second and third lavage were done half an hour and one hour later respectively.

The total base-equivalent of the fluid obtained with each lavage was determined by titration with 0.01N-NaOH, with phenol red as indicator. Before titration the washings were heated to boiling point to expel any dissolved CO_2. When, in experiments with phloxin, the washings were tinted red a potentiometric titration was carried out. The base-equivalent of the fluid first withdrawn was used as a control value for each animal.

Histamine, as the dihydrochloride, was given subcutaneously, the dose indicated being the total dose, which was administered in six doses given at 10 min. intervals, with the first dose immediately after the control lavage. The phloxin used was the dye commercially supplied by the firm of Brocades, Stheeman, and

Pharmacia (Amsterdam). When phloxin was given intravenously, the first dose was given 10 min. before the control lavage, and six maintenance doses, each equal to half the first dose, were given at 10 min. intervals thereafter. Pilocarpine hydrochloride was given as a solution (20 mg./ml.) in distilled water. All doses are expressed in mg./kg. of body weight.

RESULTS

The Spontaneous Secretion of HCl.—This was defined as the secretion taking place in animals prepared according to the above technique without any other stimuli being applied. Four series of representative results are shown in Table I. The

TABLE I

THE SPONTANEOUS SECRETION OF HCl IN THE RAT STOMACH

Rat No.	Base Equivalent of Control in ml.-0·01 N-NaOH	Base Equivalent as % of Control Value (1st Half Hr.)	
		2nd Half Hr.	3rd Half Hr.
1	3·12	51	53
2	2·91	67	23
3	9·50	71	47
4	3·48	35	32

wide variation in the control values obtained in these and subsequent experiments may have been due in part to variations in the depth of anaesthesia and in the temperature of the rat, as these have been shown to influence gastric secretion (Code, 1951).

The spontaneous secretion during the second and third half-hour of the experiment is markedly lower than the control value.

The Influence of Histamine.—Starting immediately after the control lavage, 25 mg. of histamine dihydrochloride (0.136 m.mole histamine base) per kg. body weight was injected subcutaneously in six single doses. Table II shows that the secretion

TABLE II

THE INFLUENCE OF HISTAMINE ON THE SECRETION OF HCl

25 mg. of histamine diHCl/kg. was injected subcutaneously in six doses.

Rat No.	Base Equivalent of Control in ml. 0·01N-NaOH	Base Equivalent as % of 1st Half Hr.	
		2nd Half Hr.	3rd Half Hr.
5	4·89	84	128
6	4·20	91	111
7	3·28	120	163
8	2·62	87	133

during the second half-hour was higher than the spontaneous secretion over this period, and that the secretion during the third half-hour was still higher.

The Effect of Phloxin on Gastric Secretion.—Phloxin (2% in distilled water) was given intravenously in a loading dose of 55 mg. (0.067 m.mole)/kg. 10 min. before the control lavage, and in maintenance doses of half this amount at 10 min. intervals (in all 220 mg. or 0.269 m.mole/kg.) for 70 min. As shown in Table III, the effect was a marked inhibition of the secretion of HCl.

TABLE III

THE EFFECT OF PHLOXIN ON THE SECRETION OF HCl

220 mg. of phloxin/kg. (2% in distilled water) administered in seven intravenous injections.

Rat No.	Base Equivalent of Control in ml. 0·01N-NaOH	Base Equivalent as % of 1st Half Hr.	
		2nd Half Hr.	3rd Half Hr.
28	1·18	3·0	0
29	4·28	1·9	0
30	2·36	1·2	0

The Influence of Phloxin on the Stimulating Effect of Histamine.—Ten minutes before the control lavage 120 mg. of phloxin (0.145 m.mole)/kg. body weight was injected into a jugular vein; immediately after the control lavage and the first injection of histamine a maintenance dose of phloxin, equal to half the initial dose, was given and was repeated at 10 min. intervals (a 6% solution of phloxin in distilled water was used in this experiment). In the course of the experiment (70 min.) the animal thus received $120+(6\times 60)=$ 480 mg. phloxin/kg. These maintenance doses

TABLE IV

THE INFLUENCE OF PHLOXIN ON THE STIMULATING ACTION OF HISTAMINE ON GASTRIC SECRETION

480 mg. of phloxin/kg. (6% in distilled water) was administered in 7 intravenous injections; 25 mg. of histamine diHCl was given in 6 subcutaneous injections starting after the second dose of phloxin.

Rat No.	Base Equivalent of Control in ml. 0·01N-NaOH	Base Equivalent as % of 1st Half Hr.	
		2nd Half Hr.	3rd Half Hr.
9	5·04	49	33
10	4·71	23	35
11	9·66	22	6
12	14·52	30	27
13	8·53	25	16
14	8·35	20	24

were given because phloxin appeared in the urine a few minutes after the first injection. Table IV shows that the stimulating effect of histamine was now abolished.

When 200 mg. phloxin/kg. was administered in one single intraperitoneal injection 4 hr. before the operation there was hardly any spontaneous secretion during the control period. Nevertheless, 25 mg. of histamine dihydrochloride injected subcutaneously after the control lavage still elicited a marked secretion in the subsequent half-hour

periods (Table V). This shows that the inhibition of gastric secretion caused by the preliminary injection of phloxin cannot have been due to an irreversible injury of the oxyntic cells.

TABLE V

THE EFFECT OF HISTAMINE ON GASTRIC SECRETION IN RATS PREVIOUSLY INJECTED WITH PHLOXIN

200 mg. of phloxin (2% in distilled water) was injected intraperitoneally 4 hr. before the expt.; 25 mg. of histamine diHCl was injected subcutaneously in six divided doses after the control lavage.

Rat No.	Dose of Phloxin	Base Equivalent in ml. 0·01N-NaOH		
		1st Half Hr. (Control)	2nd Half Hr.	3rd Half Hr.
5	0 (See Table II)	4·89	4·11	6·25
6		4·20	3·81	4·67
7		3·28	3·93	5·34
8		2·62	2·27	3·50
34	200 mg./kg.	0·69	1·64	2·04
35		0·00	1·84	2·16
36		0·09	0·29	1·12
62		0·00	0·30	1·15
63		2·37	3·68	4·52
64		0·00	0·04	0·19
65		0·23	2·37	2·70
66		0·27	1·43	1·80
67		0·10	0·25	0·67
68		0·35	1·50	2·88
69		0·12	0·15	0·60

The Effect of Phloxin on Gastric Secretion Induced with Pilocarpine.—As phloxin appeared to influence the spontaneous secretion of HCl as well as the secretion due to histamine, it was decided to investigate its effect on the secretion induced with the parasympathomimetic drug pilocarpine. Table VI shows that 20 mg. (0.082 m.mole) of pilocarpine HCl/kg., injected subcutaneously immediately after the control lavage, stimulates the secretion of HCl and that this effect is greatly reduced by phloxin injected in the usual way.

The Toxicity of Phloxin.—The influence of phloxin on gastric secretion may consist of either a specific inhibition of the effect of histamine, or a non-specific toxic action. Thompson and Vane (1953) have shown that a decrease of the blood flow through the gastric vessels of the cat is followed by a decrease in gastric secretion. Thus a substance might lower the rate of gastric secretion by its effect on the circulation. Hence a series of experiments was performed to study the influence of phloxin on blood pressure.

TABLE VI

THE EFFECT OF PILOCARPINE, AND OF PILOCARPINE WITH PHLOXIN, ON THE SECRETION OF HCl

20 mg. of pilocarpine HCl was injected subcutaneously; phloxin (2% in distilled water) was given at the same time in seven intravenous injections.

Rat No.	Drug Injected	Base Equivalent of Control in ml. 0·01N-NaOH	Base Equivalent as % of 1st Half Hr.	
			2nd Half Hr.	3rd Half Hr.
15	Pilocarpine	2·72	316	379
16		2·48	196	167
17		2·40	225	341
18		9·80	82	88
19		7·61	78	99
20		3·98	163	136
21	Pilocarpine and phloxin 220 mg./kg.	7·63	54	8
22		3·47	64	23
23		12·57	22	5
24		8·81	33	8
25		2·02	138	43
26		3·92	47	21
27	Pilocarpine and phloxin 120 mg./kg.	3·96	82	34

The arterial blood pressure of rats was recorded on a smoked drum by a double membrane tambour connected to an external carotid artery. Phloxin (6% in distilled water) was injected through a second cannula into an external jugular vein. In each of five rats 55 to 60 mg. phloxin/kg. caused a temporary fall of blood pressure during the injection, followed by a recovery, to near the initial level, within 2 min. This is illustrated in Fig. 1.

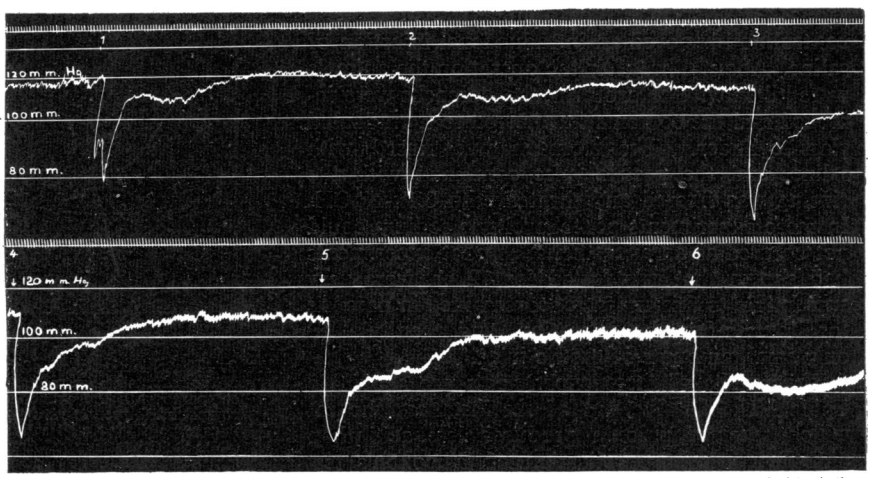


FIG. 1.—Carotid arterial pressure of a rat which received phloxin intravenously. At 1, 60 mg. of phloxin/kg. (6% phloxin in distilled water). At 2, 3, 4, 5, and 6, 30 mg./kg. of phloxin was injected. Time, 5 sec.

On the basis of these blood-pressure experiments it was considered unlikely that the results shown in Table III were due to a general hypotension.

The Effect of Phloxin on Gastric Secretion in the "Shay" Rat.—Although it was considered unlikely that the effect of phloxin on gastric secretion was due to an effect on the blood flow through the gastric vessels, another way of administering phloxin was sought which would be less likely than intravenous injection to influence the general circulation. Local application of phloxin to the gastric mucosa was ruled out, because Robertson and Grossman (1952) reported that the gastric secretion of the dog may be inhibited by the local application to the gastric mucosa of several substances which do not display this effect on parenteral application. Injection of phloxin through the duodenal wall was adopted, and, to prevent regurgitation of duodenal contents into the stomach, the pylorus was ligated before phloxin was injected into the duodenum through a No. 18 hypodermic needle. To prevent leakage of the injected fluid into the peritoneal cavity a second ligature was tied immediately caudal to the site of injection, care being taken not to obstruct the bile duct.

Shay, Komarov, Fels, Meranze, Gruenstein, and Siplet (1945) showed that pyloric ligation in the rat may be followed by a marked secretion of gastric juice. Hence this technique may be used to study inhibitory effects on gastric secretion.

In our experiments the rats were fasted for 72 hr. during which time they had free access to water with 0.4% NaCl, as used by Donald and Code (1952). The ligation of the pylorus and the injection of phloxin (300 mg./kg.; 6% phloxin in saline) were carried out under light ether anaesthesia. A littermate of each animal was used as a control and received an intraduodenal injection of saline. About 6 hr. after the operation the animals were killed with chloroform, the stomachs were removed and the contents transferred to a graduated glass cylinder. The stomach was rinsed with saline, and the gastric juice combined with the rinsing was filtered and titrated with 0.1N-NaOH. In these experiments pentobarbitone sodium could not be used as an anaesthetic, as pilot experiments had shown that rats fasted for 72 hr. were very susceptible to this anaesthetic, remained in narcosis for hours after the operation, and then yielded only small amounts of gastric juice.

The results of these experiments are shown in Table VII. In five out of six instances the rat which received phloxin produced less acid than did the control. Application of Wilcoxon's test shows that the hourly production of HCl is indeed

TABLE VII

THE EFFECT OF PHLOXIN IN THE SHAY RAT

The pylorus was tied, and phloxin 300 mg./kg. or 0·9% NaCl was injected at the specified time before the rats were killed and the gastric contents collected.

Rat No.	Injected With	Time between Operation and Death in min.	Volume Gastric Juice in ml.	*p*H of Gastric Juice	Total Production of Acid in m.equiv. × 100	Acid Production per Hour in m.equiv. × 100
101	Phloxin	405	2·5	2 55	12·5	1·85
102	Saline	409	4·5	2·08	27·5	4·03
103	Phloxin	394	0·1	3·99	2·2	0·34
104	Saline	384	9·0	1·53	69·5	10·78
105	Phloxin	373	3·0	2·35	13·5	2·17
106	Saline	366	9·5	1·41	72·8	11·92
107	Phloxin	403	13·0	3·57	6·58	0·98
108	Saline	380	6·0	2·02	37·7	5·95
109	Phloxin	380	1·3	3·60	5·45	0·86
110	Saline	370	3·5	2·20	18·61	3·01
111	Phloxin	360	3·0	2·25	17·21	2·87
112	Saline	367	3·0	2·35	11·44	1·87

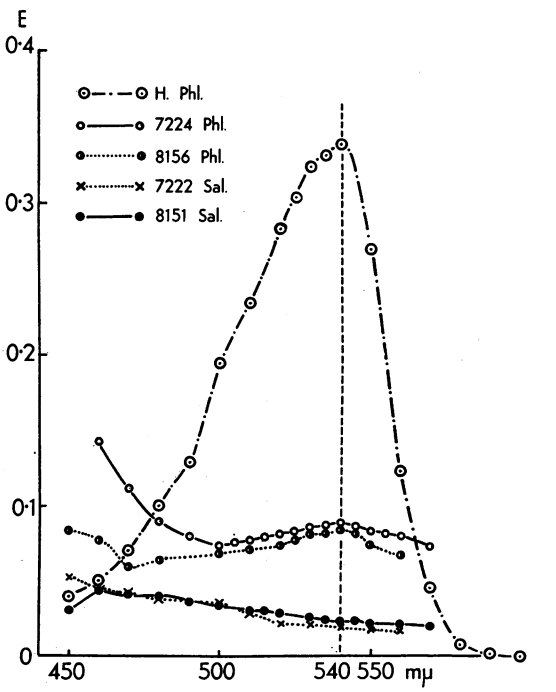


FIG. 2.—The extinction curve of the gastric juice of rats after ligation of the pylorus and intraduodenal injection of phloxin or saline, compared with the extinction curve of the histamine-phloxin compound formed *in vitro* (⊙—·—⊙). All samples of gastric juice were diluted with saline. Rats 7224 (O——O) and 8156 (◑·····◑) received 200 mg. of phloxin/kg. (dilution factor of gastric juice 1: 214 and 1: 5 respectively). Rats 7222 (X···X) and 8151 (●——●) received 1 ml. of saline (dilution factor 1: 1.6 and 1: 30 respectively).

significantly lower in the animals treated with phloxin than in the control animals ($P_2 = 0.0086$).

No sign of intoxication which could be ascribed to the phloxin was seen. When pairs of rats were subjected to the same procedure, and the blood pressure was measured four to five hours after the pylorus had been tied, a pressure of 105 to 120 mm. Hg was found both in the control rats and in those injected with phloxin. When the same dose of phloxin was given by stomach tube to normal rats of the same sex and age, no toxic signs were observed; the rectal temperature was 37–38° C. Absorption of the phloxin was proved by its appearance in the urine, and by the rosy colour of the ears, nose, and feet.

In some animals the gastric juice also was tinted red. We suspected this to be due to excretion of phloxin into the stomach; on analysis with a Coleman junior spectrophotometer it was found that this red gastric juice had an extinction peak at a wavelength of 540 mμ (Fig. 2), whereas phloxin in aqueous solution has a maximal extinction at 525 mμ (Fig. 3). When blood was withdrawn from a carotid artery of a rat four hours after the intraduodenal injection of 300 mg. phloxin/kg., diluted 100 times with saline and centrifuged, the extinction curve showed a slight elevation at a wavelength of 525 mμ, indicating that the phloxin was taken up from the duodenum as such (Fig. 3). Although the nature of the phloxin-like substance in the gastric juice has not been elucidated as yet, it is interesting to observe that when histamine is added to an aqueous solution of phloxin *in vitro*, a complex is formed which also has an absorption maximum at 540 mμ (Fig. 2).

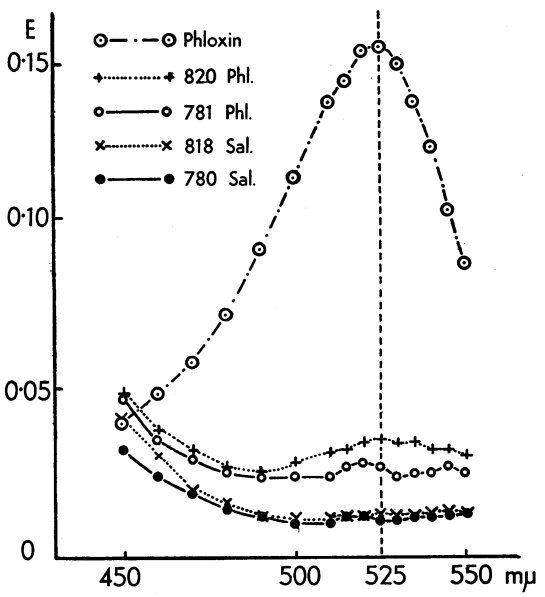


FIG. 3.—The extinction curve of rat blood (diluted 1 : 100 with saline) 4 hr. after ligation of the pylorus and intraduodenal injection of 200 mg. of phloxin/kg. in rats 781 (O——O) and 820 (+ · · · · +) or 1 ml. of saline in rats 780 (●——●) and 818 (X · · · · X), compared with the extinction of phloxin in saline (⊙—· —⊙).

DISCUSSION

The observation that in a number of rats injected with phloxin the gastric juice was tinted red is interesting in connexion with the finding of Bradford and Davies (1950), that some acid dyes are transported across the isolated gastric mucosa of the frog, toad, cat, and polecat but not concentrated in the gastric juice, in contrast with basic dyes. The acid dyes tested by these workers included phenolphthalein and fluorescein.

The similarity between the extinction curves of the "red" gastric juice and of the histamine-phloxin complex (in aqueous solution) obtained *in vitro* suggests that these samples of gastric juice contain phloxin in the form of a similar complex with histamine. MacIntosh (1938) found a substance with the properties of histamine in the gastric juice of dogs, and Kahlson (1948) found histamine in the gastric juice of the cat, but to our knowledge no data are available about the occurrence of histamine in the stomach of the rat.

Although gastric secretion in the rat is predominantly influenced by the vagus (Shay *et al.*, 1945), it is clear from the experiments described above that histamine may cause a significant increase in the amount of HCl secreted by the rat stomach under the present experimental conditions. It is also evident that phloxin inhibits this stimulating effect of histamine. This inhibitory effect may be due to the general toxicity of phloxin, to a more specific inhibition of gastric secretion irrespective of the eliciting agent, without affecting other functions, or to a specific inhibition of the effect of histamine on gastric secretion.

Although the fall in blood pressure caused by phloxin itself is too brief to explain the almost complete inhibition of gastric secretion during the second half-hour (Table III), it might be argued that its depressor effect may cause a secretion of adrenaline, and that the inhibition of gastric secretion observed is due to the latter. However, we feel that although adrenaline may contribute to the inhibitory effect it cannot be the sole cause; in the first place because adrenaline may both increase and decrease the blood flow through the gastric vessels, so that an increased rate of secretion may also be recorded (Ivy and Javois, 1924; Thompson

and Vane, 1953); in the second place because the degree of inhibition due to adrenaline recorded by previous investigators (Hess and Gundlach, 1920; Thompson and Vane, 1953) was of the order of 66% at the most—far less than the almost complete inhibition found in our experiments. In the experiments in which the pylorus was ligated no toxic signs were observed.

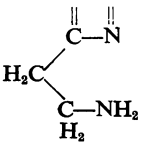
At first sight the present results seem to be in agreement with the hypothesis that phloxin inhibits gastric secretion irrespective of its cause, for it inhibits both the spontaneous secretion of acid in the stomach and the secretion elicited by pilocarpine and by histamine. However, these results may also be explained by assuming that in all these types of gastric secretion histamine is involved, and that phloxin exerts a more or less specific antihistamine effect. Results of experiments on rat's isolated ileum favour this possibility. Thus 120 μg. of phloxin inhibited the effect of 80–160 μg. of histamine, but not the effect of 0.5 μg. of acetylcholine or 0.08–0.24 μg. of carbachol (all per ml. of fluid in the organ bath) (van Noordwijk, 1954).

The finding of gastric juice with an extinction curve very similar to that of the histamine-phloxin complex formed *in vitro* is also relevant, although of course it does not provide conclusive evidence that the gastric juice contains the same histamine-phloxin complex.

The depressant effect of phloxin on pilocarpine may be explained by assuming either that histamine is involved in the chain of reactions which follows its administration and leads to the secretion of HCl, or that pilocarpine stimulates the oxyntic cells directly just as histamine does and that phloxin combines with pilocarpine or histamine in an identical way.

It is interesting to recall that both histamine and pilocarpine possess an imidazole ring, and that the drug tolazoline (benzazoline, "priscol") which is also reported to stimulate the secretion of HCl, in a manner similar to histamine (Thiele 1940), is an imidazoline derivative. We found that 75 mg. (0.382 m.mole) of tolazoline/kg. did indeed stimulate the secretion of HCl in the rat stomach, although this effect was less constant than that of histamine or pilocarpine. These facts suggest that the imidazole or imidazoline ring may be linked with the secretion of HCl in the stomach, and as phloxin is an acid dye it is likely that it reacts with one of the nitrogen atoms of the ring and hence prevents it from combining with the specific cell receptors. Such a reaction is made likely by the fact that both pilocarpine and tolazoline precipitate phloxin from aqueous or alcoholic solutions.

It will be recalled that Niemann and Hays (1942) suggested that the activity of histamine is linked with the following structure:



However, Grossman, Robertson and Rosiere (1952) tested a number of compounds, structurally related to histamine, for their ability to stimulate the secretion of HCl in the dog's stomach. They found 12 compounds to be active, but two of them did not possess the structure shown above. Besides these there were numerous compounds which were inactive in spite of having the required structure —such as, for example, 2 (β-aminoethyl)-pyridine. Furthermore, they found a discrepancy in the histamine-like activity of a number of compounds when tested on the guinea-pig's ileum or the cat's blood pressure on the one hand and the stimulation of gastric secretion on the other. Hence they concluded that the mechanism of the stimulating effect of histamine on gastric secretion is different from that of other actions of this drug.

The fact that substances with an imidazole, imidazoline, or a pyrazole ring (sometimes without a free aminoethyl group such as acetylhistamine or with no aminoethyl group at all) can affect gastric secretion may indicate that for this effect the presence of one or more nitrogen atoms in an aromatic ring is of greater importance than the presence of a free aminoethyl group.

It is tempting to link these compounds with the process of HCl formation outlined by Davies and Ogston (1950). According to their theory one of the fundamental reactions probably involved is the transportation of hydrogen across an intracellular barrier by a carrier. Perhaps this role of carrier may be fufilled by a heterocyclic compound, like histamine, which contains a basic N-atom.

It is felt that in this way the following separate observations may be correlated:

1. Histamine is a stimulant of the secretion of HCl in the stomach of many mammalian species.

2. Other compounds with an imidazole, imidazoline, or a pyrazole ring and sometimes lacking a free aminoethyl group (pilocarpine, tolazoline, acetylhistamine, 4 - *iso*propylamino - imidazole, Grossman *et al.*, 1952) may also stimulate the secretion of HCl.

3. Phloxin reacts with pilocarpine, tolazoline, and histamine *in vitro*, and *in vivo* it blocks the stimulating effect of pilocarpine and histamine on the secretion of HCl.

4. Phloxin also inhibits the normal secretion of HCl in the rat stomach.

Summary

1. Histamine, as well as pilocarpine, stimulates the gastric secretion of HCl in rats under pentobarbitone sodium anaesthesia.

2. Phloxin (tetrabromo - tetrachlorofluorescein) inhibits the normal gastric secretion of HCl in the anaesthetized rat and in the "Shay" rat.

3. Phloxin also inhibits the stimulating effect of histamine and of pilocarpine on the gastric secretion of HCl.

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