PHARMACOLOGY OF THE MALPIGHIAN TUBULES OF *RHODNIUS* AND *CARAUSIUS*: THE STRUCTURE–ACTIVITY RELATIONSHIP OF TRYPTAMINE ANALOGUES AND THE ROLE OF CYCLIC AMP

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INTRODUCTION

Recently it has been shown that 5-hydroxytryptamine (5-HT) even at concentrations of the order of 10^{-8} M/l will stimulate fluid secretion by the Malpighian tubules of two insect species, *Rhodnius prolixus* Stål and *Carausius morosus* (Maddrell, Pilcher & Gardiner, 1969).

In order to find out more about this interaction the activity of a range of substances chemically related to 5-HT has been studied. This has yielded information about the nature and the behaviour of the sites where 5-HT interacts with the cells of the Malpighian tubules.

Apart from its intrinsic interest such a study may throw light on the nature of the naturally occurring diuretic hormones. There are several pieces of information to show that 5-HT is not the diuretic hormone extractable from the nervous systems of *Rhodnius* or of *Carausius* (see p. 799). Nonetheless, as will be shown in this paper, inhibitors of 5-HT action also antagonize the action of these diuretic hormones. Since this suggests that the hormones and 5-HT act on the same sites, information about these sites should also tell us something about the possible structure of at least the activity-inducing parts of the hormones.

The second part of this paper examines the possible involvement of the cyclic nucleotide, 3′,5′-adenosine monophosphate (cyclic AMP) in the chain of events which link the primary stimulation by 5-HT, or by the hormones, to the actual mechanisms producing secretion. Cyclic AMP is thought to behave as an intracellular mediator for the action of many hormones (see for example Robison, Butcher & Sutherland, 1968).

Most of the experiments described are for Malpighian tubules of *Rhodnius*. Many experiments have, however, also been carried out using tubules from *Carausius*, and these provide a most valuable comparison with the experiments on *Rhodnius* tubules.

MATERIALS AND METHODS

For these studies *in vitro* preparations of isolated Malpighian tubules were used. For details of this preparation and for details of preparing extracts of the nervous system rich in diuretic hormone see Maddrell (1969) for *Rhodnius*, and Pilcher (1970*a*)

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for *Carausius*. The tubules were bathed in large drops of Ringer's solution containing known concentrations of the substances under test, and the secretory rate of the tubules was observed to see whether it was accelerated. Substances which failed to elicit such a response were usually further tested to see if they had interfered with the tubules' ability to respond to the diuretic hormone or to 5-HT. In these experiments a solution containing either 5-HT or the diuretic hormone was added directly to the test solution bathing the tubules. The test solution was judged to contain an inhibitor if there was not a prompt and large increase in the rate of secretion.

The technique for measuring trans-wall potentials of Malpighian tubules is described in Maddrell (1971).

Part I. *The effects of 5-HT and compounds of similar structure*

5-Hydroxytryptamine is a potent stimulator of secretion by Malpighian tubules of both *Rhodnius* and *Carausius*, measurable effects being produced at a concentration as low as 3×10^{-8} M/l. With small bathing drops at low concentrations the burst of
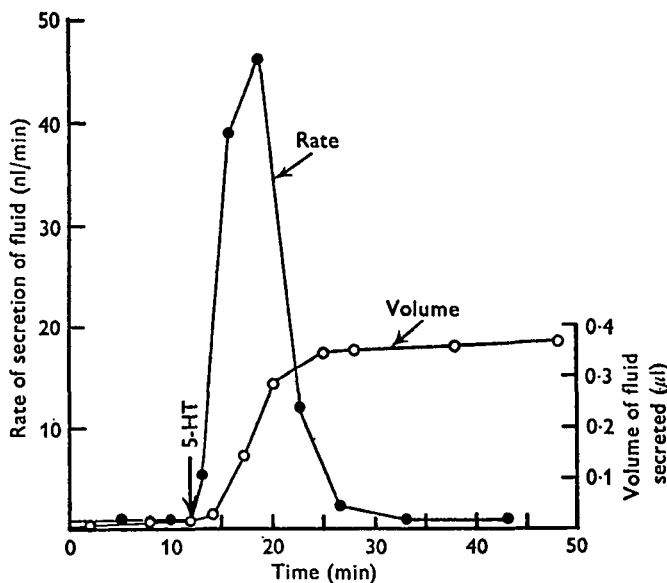
Fig. 1. The effect of a small dose of 5-HT (a final concentration of 10^{-7} M/l) on the secretion by a tubule from *Rhodnius* contained in 3 μl of solution.

secretion is short lived (Fig. 1). This may be due to a breaking down or elimination of 5-HT; it is known that Malpighian tubules will break down the diuretic hormone (Maddrell, 1964; Pilcher, 1969). Similarly the effects on the trans-wall potential of cannulated and perfused *Rhodnius* Malpighian tubules are quickly over just as they are after treatment with threshold concentrations of the diuretic hormone itself. At higher concentrations of 5-HT the rates of secretion are higher and longer lasting. The response of the tubules of both insects to various concentrations of 5-HT are plotted in Figs. 2 and 3.

Following on from this discovery it became of interest to ascertain which parts of

the 5-HT molecules were of importance to its secretion-inducing capacity. Accordingly various compounds related to 5-HT were tried at a range of concentrations to see whether or not they were active in causing isolated Malpighian tubules to secrete, or in interfering with the diuretic response brought on by 5-HT. The results are listed in
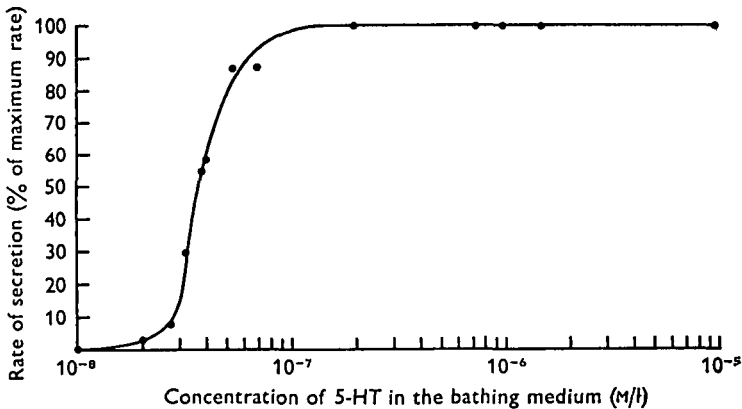

Fig. 2. Dose/response curve for the effects of 5-HT on secretion by Malpighian tubules of *Rhodnius*.
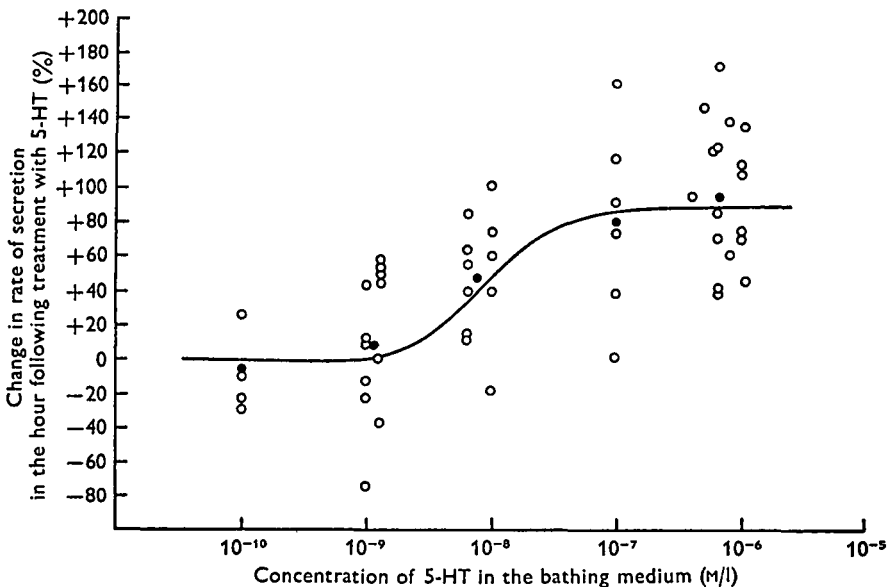

Fig. 3. Dose/response curve for the effects of 5-HT on secretion by Malpighian tubules of *Carausius*. The solid circles are the mean values for each set of points.

Table 1 and summarized in Fig. 4. It is clear that only very few of the compounds structurally related to 5-hydroxytryptamine will stimulate secretion. The loss of the hydroxyl group causes the loss of all activity as does substitution of the hydrogen atom in this group as in 5-methoxytryptamine and 5-benzyloxytryptamine. Moving the hydroxyl group into either the four or six position also abolishes the activity. Substitution in the terminal amino group depends on the substituent; methylation has a
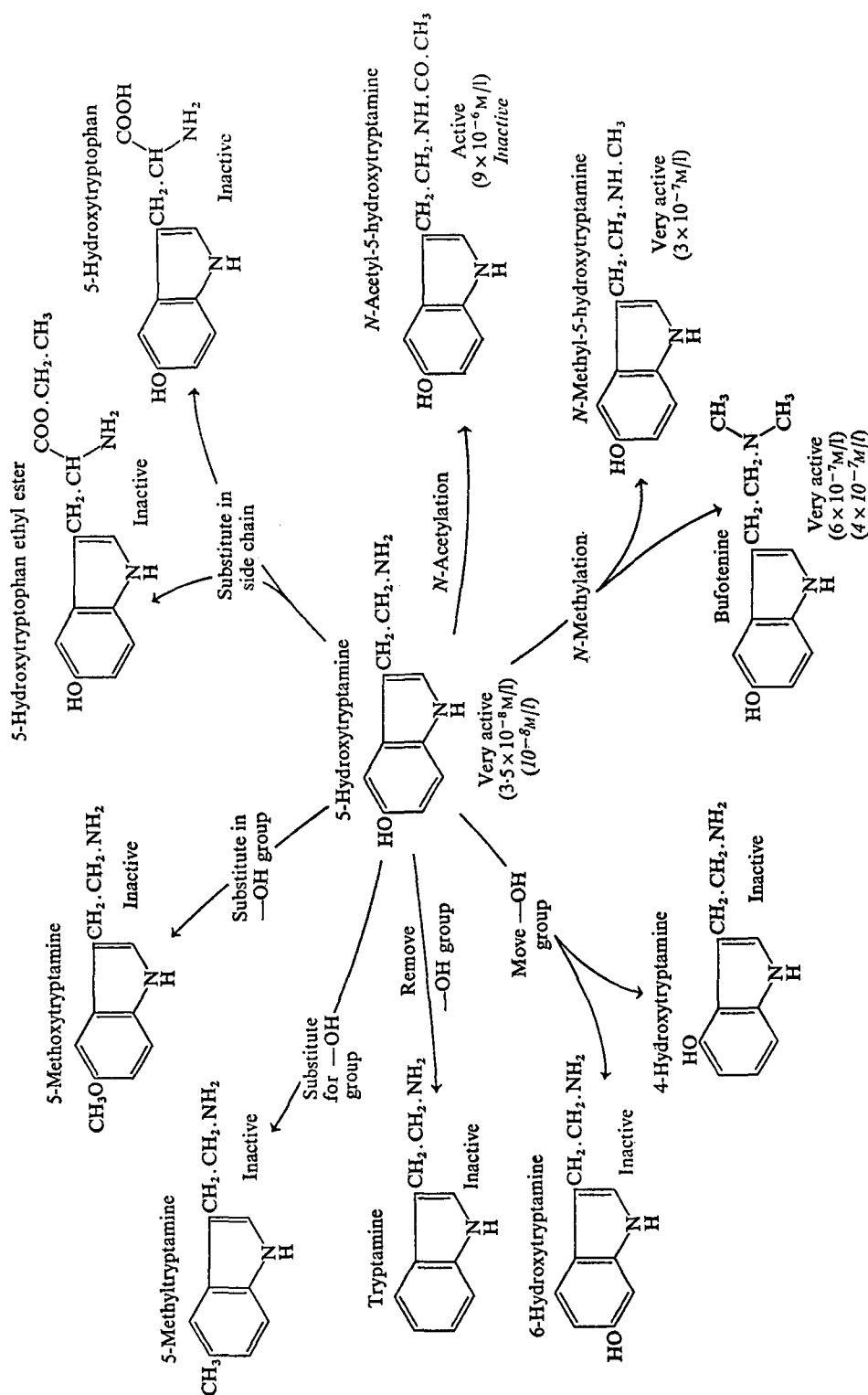
Fig. 4. The effects of structural changes in the 5-HT molecule on its ability to stimulate secretion by tubules of *Rhodnius* and *Carausius*. The figures below the active compounds are the concentrations needed to ensure 50% maximal secretion. Figures or comments in italics refer to the behaviour of *Carausius* tubules where they differ from those of *Rhodnius*.

relatively slight effect, but acetylation reduces the activity by 300 times on *Rhodnius* tubules and abolishes it on *Carausius* tubules (Fig. 4). The compounds that do stimulate the secretion will cause as high rates of secretion as will 5-HT, that is, the apparent intrinsic activity is unity (this point is discussed on p.). This narrow spectrum of compounds possessing a stimulatory action is rather rare among systems responsive to 5-HT. For example, the molluscan heart (Greenberg, 1960) and the insect tissue which has so far been the most extensively studied, the dipteran salivary gland (Berridge, 1971), are both stimulated by a much wider range of compounds.

One persistent worry in these experiments has been the possibility that some compounds may have as trace contaminants small amounts of 5-HT itself. Thus the activity of *N*-acetyl serotonin on *Rhodnius* tubules could have been due to the presence of 5-HT as an impurity to the extent of 0·3 %. In this particular case this is not the explanation of activity, because it would be equally active when tested on *Carausius* tubules; in fact *N*-acetyl serotonin does not stimulate these tubules at all. However, some (but not all) experiments on *Rhodnius* tubules with 4-hydroxytryptamine (4-HT) and 6-hydroxytryptamine (6-HT) have shown measurable stimulation at concentrations just less than those at which these substances act as inhibitors. The fact that attempts to plot these stimulant effects as a dose/response curve fail because of their inconsistency leads one to believe that they are explicable as effects of small amounts of 5-HT present as an impurity (to the extent of about 0·01 %). Whether any stimulation occurs or not depends on the thresholds for stimulation by 5-HT and for inhibition by 4-HT or 6-HT for the particular isolated tubules. In any case it is clear that 4-HT and 6-HT are at least 5000 times less active than 5-HT.
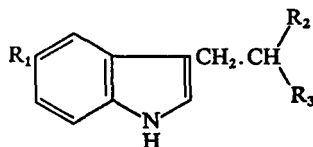
The action of tryptamine derivatives and other amines as inhibitors of Rhodnius *tubules*

Although rather few tryptamine derivatives will stimulate fast secretion by *Rhodnius* tubules, it is most striking that a large number of such derivatives will interfere with stimulation by 5-HT or by the diuretic hormone, by acting as inhibitors (Table 1). Pre-treatment of Malpighian tubules with such an inhibitor before, say, the diuretic hormone is added prevents or reduces the prompt and rapid acceleration of secretion which would otherwise follow (Fig. 6). The addition of an inhibitory compound to tubules already secreting fast in a solution of 5-HT very quickly slows the rate of secretion (Fig. 7). These effects depend on the concentration of the inhibitor, and it is possible, as shown in Fig. 8, to construct a dose/effect curve for the inhibitor concerned.

The list of compounds which act as inhibitors (Table 1) contains not only tryptamine derivatives but also other aromatic amines such as tyramine and iproniazid. Clearly, for a substance to act as an inhibitor it need not conform structurally to such a narrow set of conditions as are called for from compounds which behave as stimulants. Nevertheless all the inhibitors in Table 1 share with 5-HT the possession of an amino group on a two-carbon side chain projecting from an aromatic nucleus. It is possible, however, that aliphatic amines might act as inhibitors but these have not been tried. For a compound to act as an inhibitor it seems to be important that no negatively charged group is situated near the amine group, which is of course itself positively charged. As an example of this it has been found that lysergic acid is not an inhibitor though bromo-lysergic acid diethylamide (BOL) is a potent inhibitor. Similarly, while tryptamine is an inhibitor, tryptophan does not interact with the system at all, and
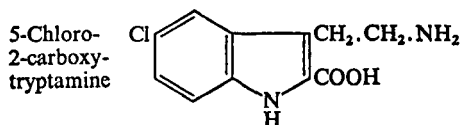
Table 1. *The effects of various pharmacologically active substances on secretion by the Malpighian tubules of* Rhodnius *and* Carausius

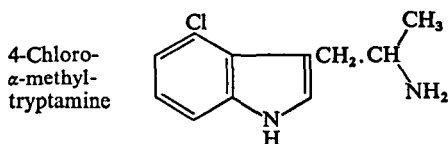A. Substances related to 5-hydroxytryptamine

				Rhodnius		Carausius	
	R_1	R_2	R_3	Stimulant	Inhibitor	Stimulant	Inhibitor
5-Hydroxytryptamine	—OH	—H	—NH$_2$	Yes	No	Yes	No
N-monomethyl-5-hydroxytryptamine	—OH	—H	—NH.CH$_3$	Yes	No	—	—
Bufotenine	—OH	—H	—N(CH$_3$)$_2$	Yes	No	Yes	No
N-acetyl-5-hydroxytryptamine	—OH	—H	—NHCOCH$_3$	Yes	No	No	—
5-Hydroxytryptophan	—OH	—COOH	—NH$_2$	No	No	No	—
5-Hydroxytryptophan ethyl ester	—OH	—COOCH$_2$CH$_3$	—NH$_2$	No	Yes	No	—
Tryptamine	—H	—H	—NH$_2$	No	Yes	No	—
5-Methyltryptamine	—CH$_3$	—H	—NH$_2$	No	Yes	No	Yes
5-Methoxytryptamine	—OCH$_3$	—H	—NH$_2$	No	Yes	—	—
5-Benzyloxytryptamine	—OCH$_2$C$_6$H$_5$	—H	—NH$_2$	No	Yes	—	—
N-acetyl-5-methoxytryptamine	—OCH$_3$	—H	—NHCOCH$_3$	No	—	No	—
5-Chlorotryptamine	—Cl	—H	—NH$_2$	No	Yes	—	—

B. Other substances
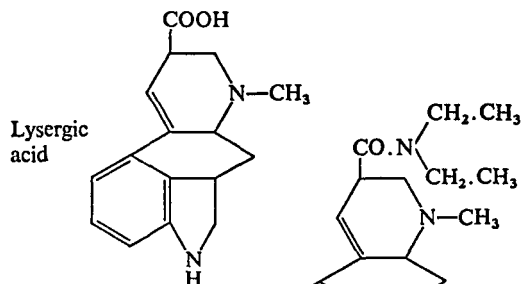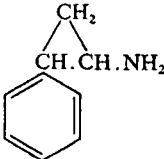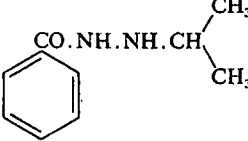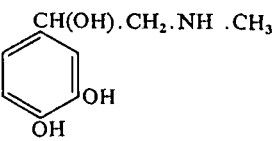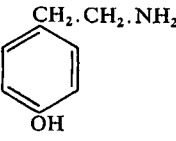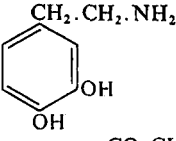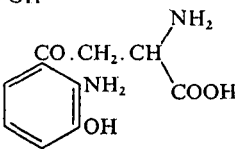
	Rhodnius Stimulant	Rhodnius Inhibitor	Carausius Stimulant	Carausius Inhibitor
5-Chloro-2-carboxy-tryptamine	No	No	—	—
4-Chloro-α-methyl-tryptamine	No	Yes	—	—
Lysergic acid	No	No	No	—
Bromo-lysergic acid diethylamide	No	Yes	No	[Yes]
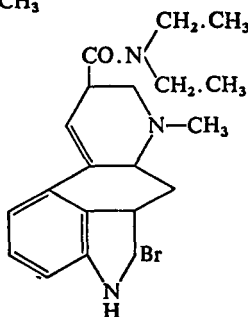
Table 1 (*cont.*)

	Rhodnius		Carausius	
	Stimulant	Inhibitor	Stimulant	Inhibitor
Tranylcypromine	No	Yes	—	—
Jproniazid	No	Yes	—	—
Adrenaline	No	No	No	—
Tyramine	No	Yes	—	—
Dopamine	No	No	No	—
3-Hydroxykynurenine	No	No	No	—

5-chlorotryptamine inhibits while 5-chloro-2-carboxytryptamine does not. Perhaps the clearest case of this is the ability of the ethyl ester of 5-hydroxytryptophan to act as an inhibitor though 5-hydroxytryptophan itself cannot. It is interesting that while tyramine behaves as an effective inhibitor neither dopamine nor adrenaline do – these latter compounds have an extra hydroxyl group in the *meta* position. These points are summarized in Table 2.

The conclusion which comes from the experiments so far described is that in both *Carausius* and *Rhodnius* only a very limited range of compounds closely related to 5-HT will stimulate an acceleration of secretion. At least in *Rhodnius*, however, a wide range of related compounds will interfere with secretion by acting as inhibitors.
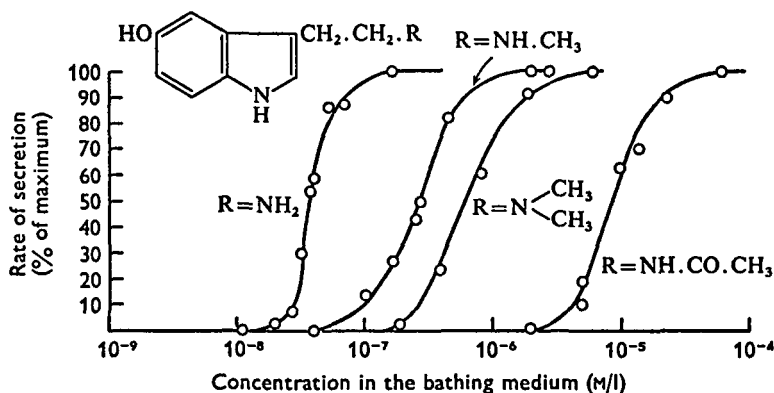
Fig. 5. The effects on the dose/response curve for 5-HT on secretion by Malpighian
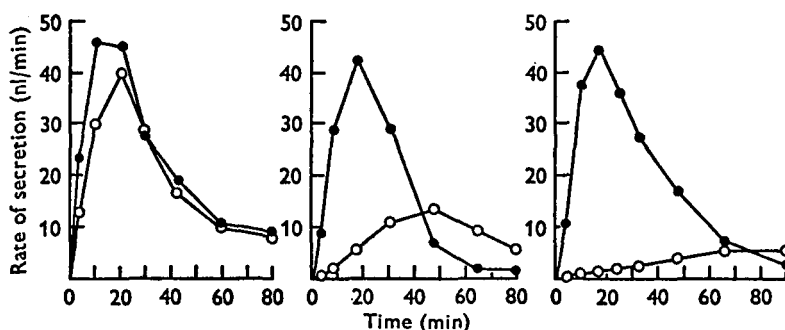tubules of *Rhodnius* of substitution in the terminal amino group.

Fig. 6. The response of Malpighian tubules of *Rhodnius* to a small dose of the diuretic
hormone (0·05 μl of an extract of a mesothoracic ganglionic mass/100 μl) contained in 18 μl
of Ringer's solution. The solid circles refer to determinations made on control tubules bathed
beforehand in Ringer's solution. The open circles refer to determinations made on tubules
soaked previously *for 45 min in tryptamine solutions, at a concentration of* 10^{-4} *M/l in the
left-hand graph, of* 3×10^{-4} *M/l, in the middle graph and of* 10^{-3} *M/l in the right-hand graph.*
Each point is the mean of four determinations.

The nature of the inhibition

The question now arises as to whether the various inhibitors described in the pre-
vious section can accurately be described as competitive inhibitors of 5-HT stimu-
lation. That is, if one supposes that 5-HT molecules interact with the cells at specific
receptor sites, do the inhibitors also have their effects at the same sites? *A priori*, it
seems very probable that this is the case because of the strong similarity in structure
between 5-HT and the inhibitors described.

The basic difference in the behaviour of competitive and non-competitive inhibitors
of enzymes is that with competitive inhibitors an increase in the concentration of the
substrate leads to a decrease in the extent of the inhibition (Dixon & Webb, 1964).
Accordingly, a series of experiments were carried out using tryptamine as an inhibitor
and 5-HT as a stimulant at two widely differing concentrations (2×10^{-7} M/l and
2×10^{-4} M/l) to see whether the dose/response curve for the inhibitor was affected by
the concentration of the stimulant. The results are plotted in Fig. 9. Although the
variation between tubules obscures the results somewhat, it appears that tubules

subject to a higher dose of 5-HT are less inhibited. That a high concentration of 5-HT does in fact decrease inhibition is more clearly shown by the results of a differently designed experiment. Here each of ten tubules was subjected in turn to the different concentrations of 5-HT while the concentration of tryptamine was held
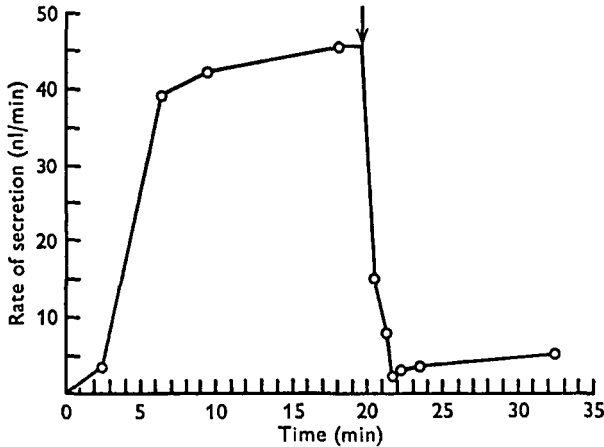
Fig. 7. The effect on the rate of secretion of a Malpighian tubule of *Rhodnius* secreting fast in a solution of 5-HT (10^{-5} M/l) of adding to it tryptamine to a final concentration of 2×10^{-4} M/l at the point indicated by the arrow.

Fig. 8. Dose/response curve for the effect of tryptamine in inhibiting secretion of Malpighian tubules of *Rhodnius* in the presence of 2×10^{-4} M/l 5-HT.

constant. The results from four such tubules are shown in Fig. 10. Under these conditions with the effects of different concentrations of 5-HT tried on the same tubules, it becomes plain that the degree of inhibition suffered by the tubules depends on the concentration of 5-HT. A control experiment showed that the alternating rates of secretion are not to be explained by the lower concentration of 5-HT being too low to elicit the maximum rate of secretion in the absence of the inhibitor. For this experiment

eight tubules were subjected in turn to the same concentrations used in the experiment illustrated in Fig. 10. This time the rate of secretion was not affected by the alternating concentration of 5-HT.

Table 2. *Five pairs of compounds, in each of which one acts as an inhibitor of secretion by the Malpighian tubules of* Rhodnius *and the other does not*

Inhibitors	Non-inhibitors

Bromo-lysergic acid diethylamide (BOL)

Lysergic acid

Tryptamine

Tryptophan

5-Chloro-tryptamine

5-Chloro-2-carboxy-tryptamine

5-Hydroxy-tryptophan ethyl ester

5-Hydroxy-tryptophan

Tyramine

Dopamine

This result, while it does not prove the point,* is consistent with the suggestion that tryptamine acts as a competitor for the site of action of 5-HT. If this is the case then on the face of it it is puzzling that inhibition should be relatively so little affected by the concentration of 5-HT. The explanation of this is to be found in the fact that tryptamine (and other inhibitors) are much less easily washed off the tubules than are stimu-

* In a situation where there are certainly several intervening steps between the interaction of the cells with a stimulant and the observed secretion of fluid, one cannot entirely exclude the possibility that this result follows from an interaction of inhibitors at some point other than at the receptor site for the stimulant, in a manner which simulates competitive inhibition at the stimulant receptor site.
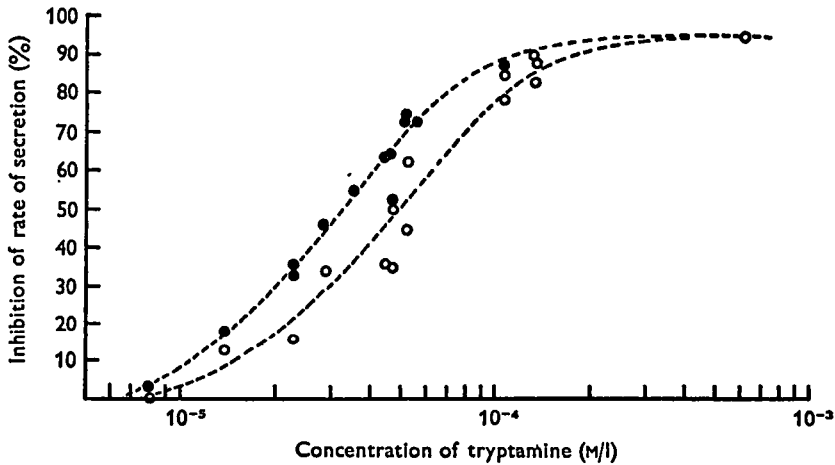
Fig. 9. Dose/response curves for the effect of tryptamine in inhibiting secretion by Malpighian tubules of *Rhodnius* in the presence of 2×10^{-4} M/l 5-HT (open circles) or 2×10^{-7} M/l 5-HT (solid circles).

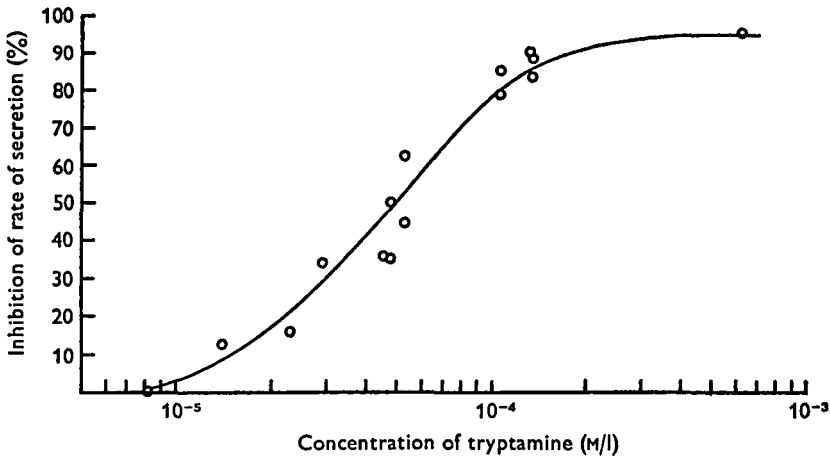
Fig. 10. The effect on the rate of secretion of Malpighian tubules of *Rhodnius* induced to secrete in 4×10^{-4} M/l 5-HT (upper graph) or 4×10^{-7} M/l 5-HT (lower graph) of adding tryptamine to a final concentration of 4×10^{-5} M/l; and then, while holding the tryptamine level constant, of alternating the level of 5-HT between a lower concentration of 2×10^{-7} M/l and a higher concentration of 2×10^{-4} M/l.

lants such as 5-HT and the diuretic hormone. They behave as if they remain associated with the receptor sites for longer periods of time. It is well known of course that the inhibition finally produced by irreversible competitive inhibitors of enzymes is not susceptible to changes in the substrate concentration (Dixon & Webb, 1964). While, for example, tryptamine is not an irreversible inhibitor of secretion, it none the less
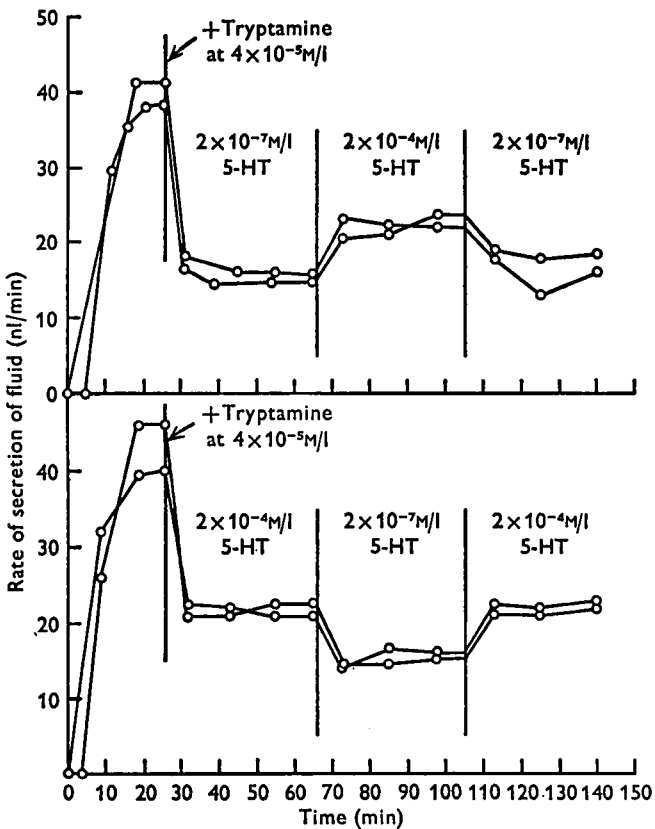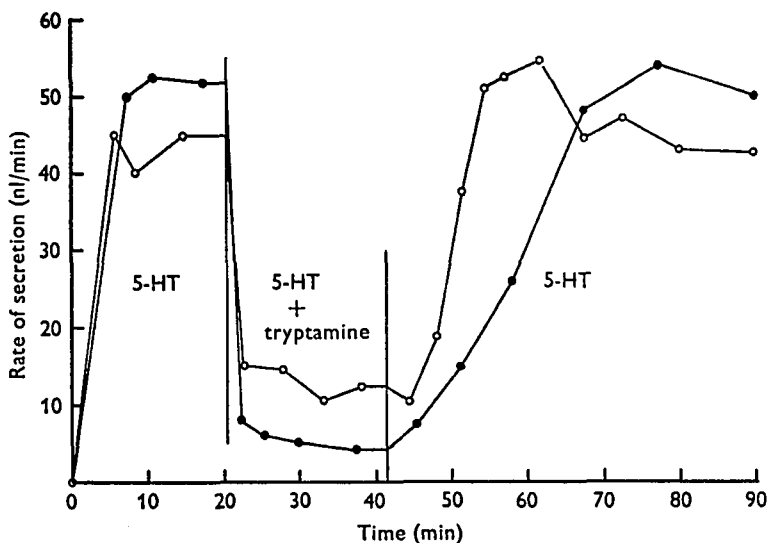

Fig. 11. The effect on the rate of secretion of Malpighian tubules of *Rhodnius* induced to secrete in 10^{-5} M/l 5-HT of a 20 minute period of immersion in 8×10^{-5} M/l tryptamine (open circles) or $6 \cdot 5 \times 10^{-4}$ M/l tryptamine (solid circles). Note that the rate of secretion recovers more slowly in the latter case.
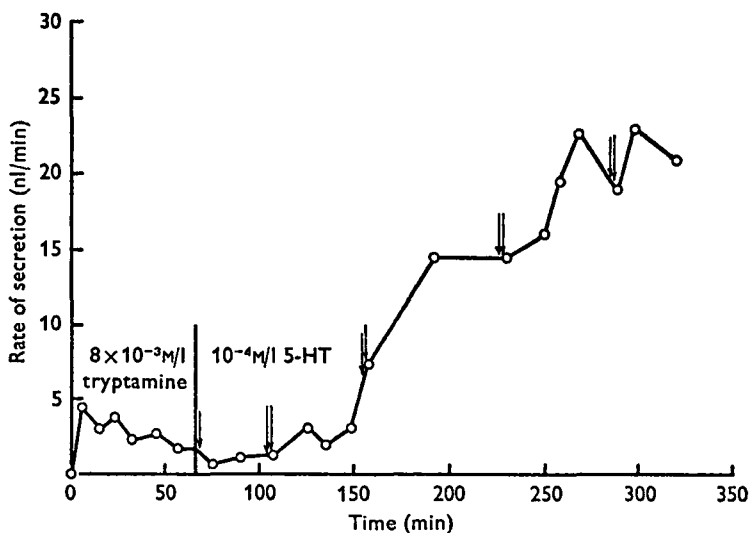
Fig. 12. The slow attainment of a high rate of secretion by a Malpighian tubule of *Rhodnius* in 5-HT at 10^{-4} M/l after 66 min in tryptamine at 8×10^{-3} M/l. The tryptamine solution was first changed for the 5-HT solution at the time indicated by the vertical line. Subsequent changes of fresh 5-HT solution are indicated by the arrows.

behaves as if its effects were only rather slowly reversible, as is shown in the section below. It therefore follows that we should expect that, even if it is a competitive inhibitor, the inhibitory effect of a substance such as tryptamine will be much less affected by the concentration of 5-HT than it would be if it were much more freely dissociable from the receptor sites.

Washing-off experiments with Rhodnius tubules

In these experiments tubules were isolated in stimulant-containing solutions. With secretion well under way an appropriate concentration of an inhibitor (usually tryptamine, 5-methyltryptamine or BOL) was introduced into the drops of solution bathing
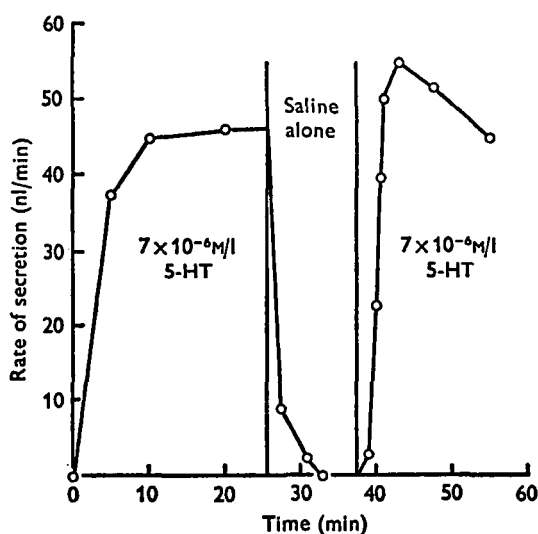

Fig. 13. The rapid recovery of a high rate of secretion by a Malpighian tubule of *Rhodnius* in 5-HT at 7×10^{-6} M/l after secretion had been stopped by immersing the tubule in Ringer's solution alone for 12 min.

the tubules. The degree of inhibition was observed from the decrease in the rate of secretion. After about 20 min the tubules were washed in fresh stimulant-containing solution and their rates of secretion were observed so that their recovery from inhibition could be followed. The results of some typical experiments are plotted in Figs. 11 and 12. As will be seen, the inhibitors are not very readily washed off the tubules except after treatment with concentrations in which the tubules continue to secrete at a modest level. At very high concentrations of inhibitor even repeated washes only slowly produce an increase in the secretory rate (Fig. 12). By contrast, tubules whose secretion was stopped merely by washing them in stimulant-free solution start secreting again promptly when replaced in a stimulant-containing solution (Fig. 13).*
The difference in the rate of recovery of secretion in this experiment and in the

* It is at first sight puzzling from Fig. 13 that secretion reaches a high level so much more quickly when the tubule is placed in the stimulant solution for the second time than at the start of the experiment. The explanation of this is that the lumen of a freshly isolated tubule is collapsed so that a considerable volume of fluid has to be secreted to fill the lumen before any emerges at the cut end of the tubule.

preceding ones can reasonably be attributed to the slowness with which tryptamine detaches itself from the receptors.

That 5-HT and the diuretic hormone detach quickly from the receptors can be shown in two ways. First, the rate of secretion drops to a low level within 2 min or so
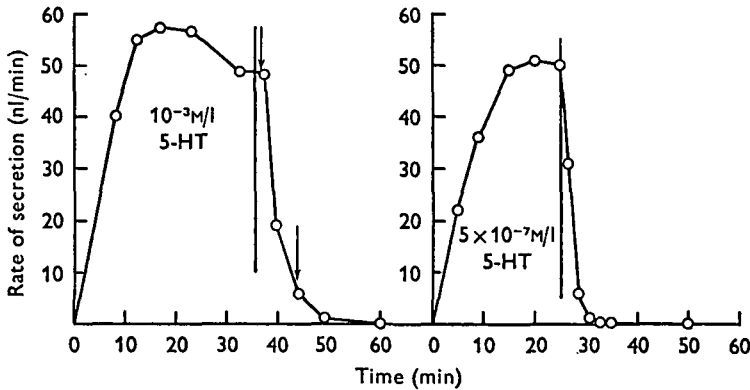
Fig. 14. The rapid slowing of secretion by Malpighian tubules of *Rhodnius* when the bathing solutions containing 5-HT at 10^{-3} M/l (left-hand graph) or 5×10^{-7} M/l (right-hand graph) were replaced by stimulant-free Ringer's solutions. The first such change is indicated in each case by a vertical line and any subsequent washes by arrows.
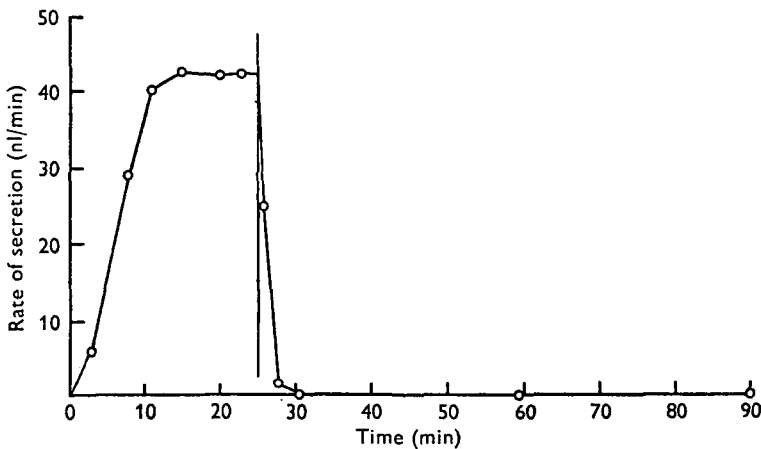
Fig. 15. The rapid slowing of secretion by a Malpighian tubule of *Rhodnius* when the bathing solution containing diuretic hormone (0·5 mesothoracic ganglionic masses/100 μl) was replaced with a stimulant-free Ringer's solution at the time indicated by the vertical line.

when tubules bathed in solutions containing either 5-HT or the diuretic hormone are transferred to stimulant-free solutions (Figs. 13–15). Secretion rapidly stops even when the tubules have been bathed in very high concentrations of stimulant (Fig. 14) though in this case more than one wash is required. Second, if a competitive inhibitor is added to a stimulant-containing solution in which the tubules are secreting fast, secretion slows very quickly indeed (Figs. 7, 11). This shows that the receptor sites are very quickly available to the inhibitor, presumably because the association of

5-HT or the diuretic hormone with the receptor sites does not occupy long periods of time.

It is paradoxical enough that 5-HT which will stimulate secretion at a concentration as low as 3×10^{-8} M/l, washes off very fast, while tryptamine which begins to act as an inhibitor only at concentrations higher than about 10^{-5} M/l, washes off a good deal more slowly.

The explanation of this apparent anomaly is crucial to an understanding of the events involved in the stimulation of secretion. From the facts one has to assume that after an initial association of stimulant molecules with the receptor sites there rapidly follows a sharp fall in the affinity between them. This seems more likely to be due to a change in the site than a change in the stimulant molecule, if only because the significance of the interaction is essentially in its effect upon the cell and not necessarily upon the stimulant molecule. One can envisage a sequence of events along the following lines:

Stimulant molecule
attaches to receptor

Stimulant molecule interacts
with receptor so that affinity
between stimulant and
receptor falls

Receptor is
reactivated

Stimulant molecule
leaves receptor
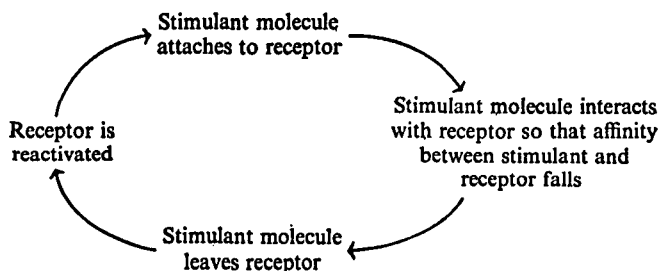
To explain why inhibitory molecules behave as inhibitors one must propose that it is the continuous and rapid repetition of the above cycle of events which generates rapid secretion. Inhibitory molecules leave the receptor sites reluctantly so that the cycle can only proceed slowly. There are obvious parallels in this system with the effects of competitive inhibitors on enzyme–substrate interactions.

In principle, any of the events shown above could be responsible for initiating secretion. However, experiments with inhibitor substances suggest that the field can be narrowed. In one series of experiments tubules were removed from several insects and put either into drops of Ringer to which was then added 5-methyltryptamine at a high final concentration of 10^{-2} M/l or into control drops of Ringer alone. As can be seen from Fig. 16 in which the results are presented, the tubules subjected to treatment with 5-methyltryptamine did give a short-lived burst of secretion. A later repeat dose of the same amount of 5-methyltryptamine did not elicit any further secretion, though the control tubules responded to 5-methyltryptamine applied at the same time. It follows that stimulation is probably associated with the attachment and interaction of molecules with the receptor site rather than with the detachment of molecules or the reactivation of the receptor sites. The fact that the burst of secretion produced by 5-methyltryptamine is short-lived is a result of the fact that it binds so firmly to the receptors that the cycling of events which is prerequisite for secretion cannot proceed. Incidentally, as is clear from Fig. 16, a high concentration of 5-methyltryptamine prevents the tubules from secreting even at the slow rate characteristic of unstimulated isolated tubules. This point is discussed on p. 801. Tryptamine appears to bind less strongly to the receptors than 5-methyltryptamine. As a result not only

can it be washed off more readily than 5-methyltryptamine, but a high concentration of tryptamine induces a continuous though slow (*c.* 2 nl/min) secretion by the tubule. Presumably the cycling of events outlined above can go on but at a slow rate.

It is not possible to go further and decide whether stimulation is associated with attachment to the receptor or with the interaction causing reduced affinity. An inhibitor molecule after attachment might not be easily washed off the receptors either (*a*) because of a failure to interact and produce a lowered affinity, or (*b*) because although a change occurs which would cause the release of a stimulant molecule, it does not dislodge the inhibitor.
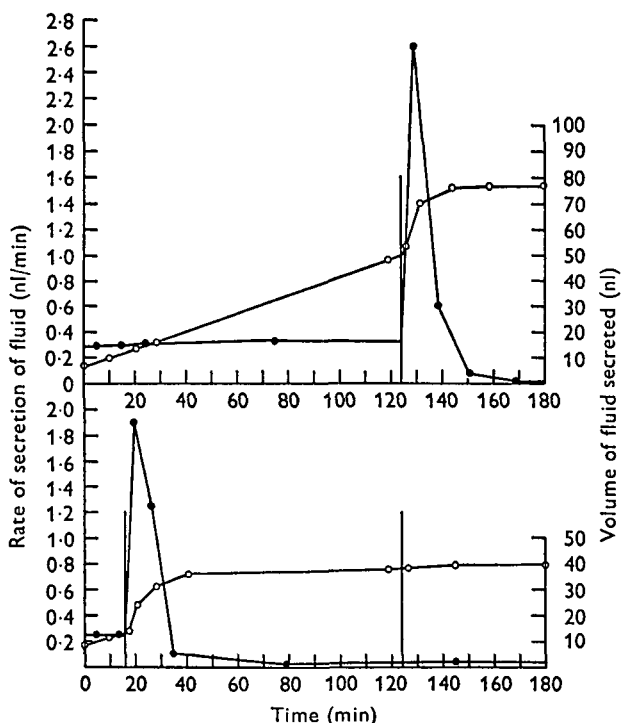

Fig. 16. The effect on secretion by Malpighian tubules of *Rhodnius* of replacing the bathing solution by one containing 10^{-2} M/l 5-methyltryptamine. In the lower graph two such doses of 5-methyltryptamine were applied at the times indicated by the vertical lines. In the upper graph the tubules were allowed to secrete slowly at the unstimulated rate for some time before treatment with 5-methyltryptamine (indicated by the vertical line). Each point is the average of three determinations. The volume of fluid secreted is indicated by the open circles and the rate of secretion by the solid circles.

Part II. *The role of cyclic AMP in the control of secretion*

There is increasing evidence that the action of many hormones, both in vertebrates and invertebrates, may involve the intracellular mediation of the cyclic nucleotide, $3',5'$-adenosine monophosphate (cyclic AMP). For example, adrenaline and glucagon, both of which cause hyperglycaemia *in vivo*, stimulate the synthesis of cyclic AMP by liver homogenates, and cyclic AMP stimulates the formation of active phosphorylase in the liver (Rall & Sutherland, 1958). These reactions are thought to be achieved by the stimulation of adenyl cyclase which catalyses the formation of cyclic AMP from

intracellular ATP. Theophylline, aminophylline, caffeine and other methyl xanthines competitively inhibit the enzyme, cyclic nucleotide phosphodiesterase, which catalyses the breakdown of cyclic AMP (Butcher & Sutherland, 1962). If there is a slow synthesis of cyclic AMP in unstimulated cells such an inhibition will lead to an increase in the level of intracellular cyclic AMP.
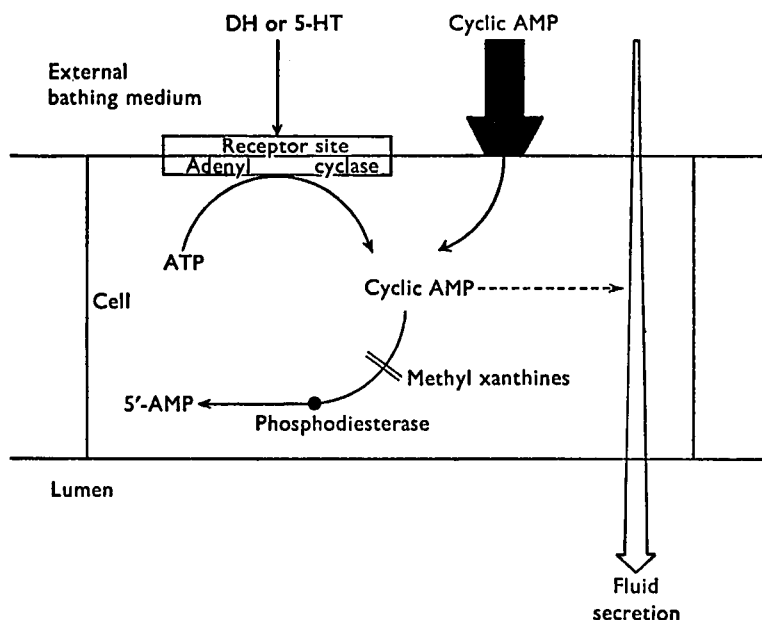

Fig. 17. Possible intracellular events involved in the stimulation of secretion by the Malpighian tubules of *Rhodnius* and *Carausius*. This construction is based on work on other systems (see text).

In line with this idea it has been shown that theophylline has an effect qualitatively like that of vasopressin or externally applied cyclic AMP on water movement in the toad bladder (Orloff & Handler, 1961) and like that of histamine or cyclic AMP on the frog gastric mucosa (Harris & Alonso, 1965; Alonso & Harris, 1965) and that it potentiates the effect of cyclic AMP in the latter (Harris & Alonso, 1965). Therefore, what one might predict for Malpighian tubules would be that both 5-HT and the diuretic hormones would act by causing an increase in the intracellular concentration of cyclic AMP. One would expect that an increase in secretory rate would be induced not only by 5-HT or the diuretic hormones, but also by cyclic AMP and by inhibitors of cyclic nucleotide phosphodiesterase. These proposed relationships are illustrated in diagrammatic form in Fig. 17.

The effects of cyclic AMP and of methyl xanthines on the Malpighian tubules of *Carausius* and *Rhodnius* were tested. Cyclic AMP stimulates fluid secretion in both species, with a threshold concentration of 1×10^{-4} M/l in the former and 4×10^{-5} M/l in the latter, which makes it one of the systems most sensitive to this substance. The rather high concentration of cyclic AMP needed to produce a response, which has been noted in other systems (e.g. Berridge & Patel, 1968), is thought to be due to the relative impermeability of the cell to cyclic AMP. The intracellular level by contrast is of the

order of 10^{-8} to 10^{-6} M/l (Butcher & Sutherland, 1962). The dose/response curve for *Rhodnius* tubules is given in Fig. 18. In *Rhodnius*, cyclic AMP appears to induce secretion marginally more quickly than does 5-HT, which possibly means that it acts at a different point in the chain of events. The rate of secretion which can be induced with *Rhodnius* tubules by even high concentrations of cyclic AMP is no more than can be induced by 5-HT.
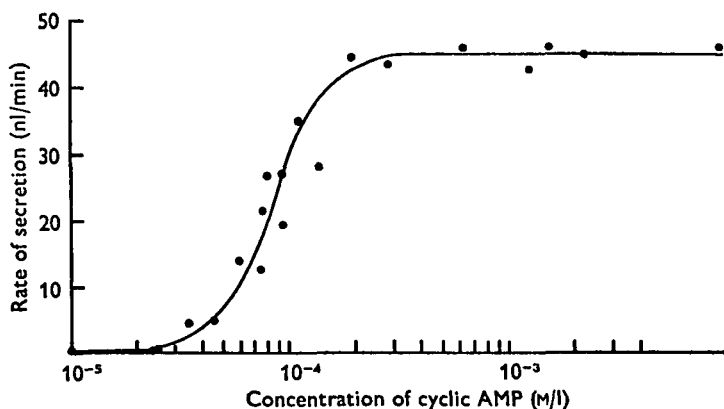

Fig. 18. Dose/response curve for the stimulation of secretion by the Malpighian tubules of *Rhodnius* by cyclic AMP.

Aminophylline (theophylline ethylene diamine) at a concentration of 10^{-4} M/l stimulates secretion by isolated *Carausius* tubules. This is in line with the idea that, in this insect, cyclic AMP may act as an intracellular mediator. In *Rhodnius*, however, the situation is different in that neither aminophylline (at any of the following concentrations: 10^{-5} M/l, 5×10^{-4} M/l, 2×10^{-3} M/l, 5×10^{-3} M/l or 2×10^{-2} M/l), nor theophylline (at 4×10^{-3} M/l, or 2×10^{-2} M/l) nor caffeine (7×10^{-3} M/l or 7×10^{-4} M/l) will stimulate secretion. This could mean that the unstimulated rate of formation of cyclic AMP is very low in *Rhodnius*, so that the partial inhibition of phosphodiesterase activity might have very little effect on the intracellular concentration of cyclic AMP. It is worth pointing out that in the absence of their respective diuretic hormones, the Malpighian tubules of *Rhodnius* secrete a good deal slower than do the tubules of *Carausius*. Consequently, it is a distinct possibility that *Rhodnius* tubules have a much lower intrinsic rate of formation of cyclic AMP and so a lesser sensitivity to inhibition of phosphodiesterase. Also in line with this suggestion is the fact that aminophylline at 10^{-3} M/l does not sensitize the Malpighian tubules of *Rhodnius* to cyclic AMP, the dose/response curve being unaffected by such pre-treatment with aminophylline. However, secretion induced by 5-HT does not persist for longer when *Rhodnius* tubules are washed with 5-HT-free theophylline solution than it does when the tubules are washed in solutions lacking both 5-HT and theophylline; in both cases secretion stops promptly. It is possible in all these experiments with *Rhodnius* tubules that any response to theophylline is lost in the adverse reaction of the cells to high concentrations of it. Tubules in 4×10^{-3} M/l of theophylline and a high concentration of 5-HT will only secrete at about a quarter of the expected rate; at 2×10^{-2} M/l of theophylline they are slowed even more.

On the scheme outlined in Fig. 17 one would expect that cyclic AMP would still be able to stimulate secretion in tubules which have been exposed to competitive inhibitors of 5-HT and of the diuretic hormone. Surprisingly, with *Rhodnius* tubules this proves not to be the case. Substances like tryptamine, 5-methyltryptamine, tyramine and BOL all interfere with the response of the Malpighian tubules to cyclic AMP. What
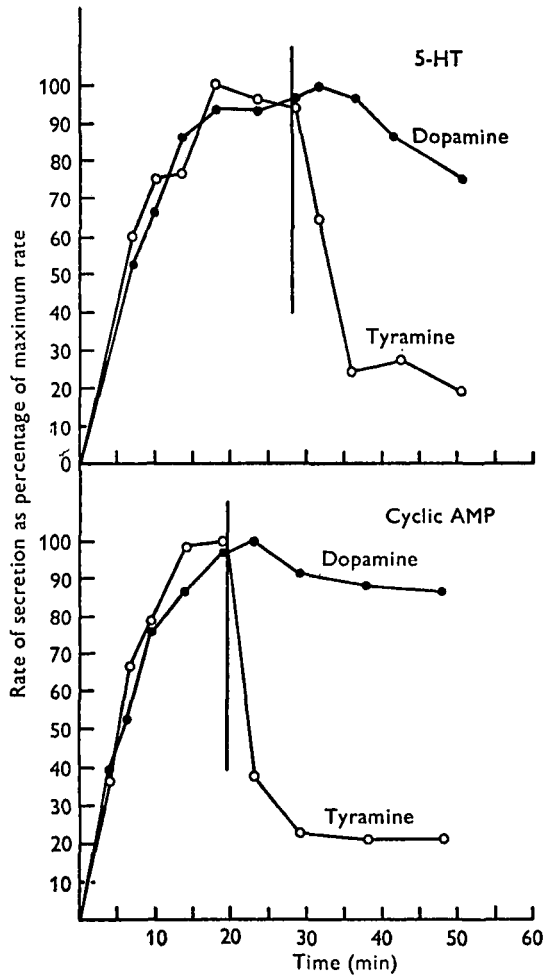
Fig. 19. The effect on secretion by Malpighian tubules of *Rhodnius* of treatment with either 2×10^{-3} M/l of tyramine (open circles) or of 2×10^{-3} M/l of dopamine (solid circles). In the upper graph the tubules were induced to secrete with 10^{-4} M/l 5-HT and then were treated with either tyramine or dopamine at the time indicated by the vertical line. The lower graph is similar except that in this case the tubules were stimulated with 2×10^{-3} M/l of cyclic AMP. Each point is the average of two determinations.

is more, the threshold concentrations needed to inhibit secretion in solutions containing cyclic AMP are the same as those required to inhibit secretion in solutions containing 5-HT, while intermediate concentrations have similar-sized effects (Fig. 19). In principle this could mean one of two things. *Either* these inhibitory compounds have some general adverse action on the cells, or more specifically on some event

linking primary stimulation to secretion, and this is responsible for secretion failure; *or* the action of cyclic AMP depends in some way on the site where 5-HT and the diuretic hormones act. In the first case the structural similarity between inhibitory compounds and stimulatory compounds like 5-HT would be merely coincidental. It would be unclear, for example, why tryptamine but not 5-methoxy-*N*-acetyl-tryptamine; 5-chloro-tryptamine but not 5-chloro-8-carboxy-tryptamine; and tyramine but not dopamine should interfere with secretion. Further, bromolysergic acid diethylamide (BOL) is quite a large molecule and so one would expect it to enter the cell rather slowly – yet it inhibits secretion (whether caused by the diuretic hormone, 5-HT or cyclic AMP) very quickly.

These considerations make the second explanation, although surprising, the more likely one. This is that the action of cyclic AMP depends in some way on the site where 5-HT and the diuretic hormone interact with the cell. If this site is blocked, then externally applied cyclic AMP is not able to have its effect. Possibly cyclic AMP enters the cells at or near the 5-HT sites, or cyclic AMP action within the cell depends on the 5-HT sites in some way. This point is discussed further on p. 802.

DISCUSSION

Very broadly, the results presented in this paper are consistent with the idea that 5-HT and other stimulatory molecules (including the diuretic hormones) interact with the cell at specific sites, probably on the cell membrane facing the haemolymph, and that as a result of this interaction secretion is induced, perhaps through the mediation of intracellular cyclic AMP produced as a response. To take these events in order, the extremely specific nature of the receptors is shown by the very narrow range of compounds which will stimulate secretion and by how little structural change in the 5-HT molecule can be accommodated without loss of activity (see for example Fig. 4). This specificity, it turns out, is not due to only very few molecules being able to bind to the receptors, because a comparatively wide range of amines will do this as judged from their ability to act as competitive inhibitors. Rather it is a consequence of a dual requirement for a stimulatory molecule, that it has not only to bind to the receptor but also to leave it again quickly after interacting with it. For the first event it appears that the terminal amino group is important, especially that it carries a net positive charge unobstructed by negative charges near it. For example, 5-hydroxytrytophan will not bind to the site though 5-hydroxytryptophan ethyl ester will. For the second event in which the molecule leaves the receptor, the hydroxyl group in the 5-position seems vital. It cannot be exchanged for a hydrogen atom (as in tryptamine), a methyl group (5-methyltryptamine), a methoxy group (5-methoxtryptamine) nor an aryloxy group (5-benzyloxytryptamine). The hydroxyl group cannot be moved without loss of activity; both 4-hydroxytryptamine and 6-hydroxytryptamine are inactive. These facts suggest that the negative charge carried by the oxygen atom in this position may be essential for the molecule to leave the receptor after stimulation. It is scarcely surprising that since the ability to sustain secretion depends on two different parts of the molecule (at least) and in all probability their spatial relationship as well, very few molecules are able to stimulate continued secretion.

From these findings one can suggest, as a speculation, that the receptor site has a negative charge to which the terminal nitrogen in the side chain of 5-HT is electrostatically attracted, and that there is also in the correct position relative to it a positive charge which holds the partially negatively charged oxygen atom found in the 5-position. Such a concept would explain, for example, the greater affinity for 5-HT than for tryptamine. For the stimulant molecule to be forced off the receptor after it has interacted, several suggestions can be made. One is that one at least of the two charges on the receptor changes sign, possibly by a conformational change, so that charges of the opposite sign become placed under the stimulant molecule. Alternatively it is possible that the stimulant molecule is broken down after interaction and the reaction products leave the site. It was argued on p. 793 that on *a priori* grounds, this is unlikely to be the case. This contention is supported by the fact that the quantity of secretion produced after treatment with diuretic hormone can be increased by treatment with amine-oxidase inhibitors (unpublished results of S. H. P. Maddrell and S. E. Reynolds). This suggests that the hormone, which is broken down by the tubules (Maddrell, 1964), may be destroyed by amine oxidases. The prolongation of secretion produced shows that the sites where the hormone is broken down and where it has its stimulant action are different.

Since so few substances will stimulate secretion by Malpighian tubules one may ask what is the nature of the diuretic hormone. Is it 5-HT? Several lines of evidence show conclusively that 5-HT and the diuretic hormone as extracted from the nervous system are not identical. They are, for the diuretic hormone of *Rhodnius:*

(1) 5-HT stimulates *Carausius* Malpighian tubules but *Rhodnius* diuretic hormone does not.

(2) *Rhodnius* diuretic hormone does not appear at effective concentrations in the fluid secreted by tubules bathed in even very high concentrations of the hormone. By contrast, 5-HT is present in the secreted fluid at about 10 % of its concentration in the bathing fluid. It is possible, for example, to set up five tubules arranged in series each secreting into the fluid bathing the next, and to stimulate all of them by adding a large dose of 5-HT only to the first, the 5-HT thus being able to cross the walls of four tubules in series.

(3) *Rhodnius* diuretic hormone tends to be destroyed or adhere to such charged surfaces as glass, chromatography paper and cellulose acetate. This, while it complicates attempts to characterize the hormone, clearly distinguishes it from 5-HT which can easily be entirely recovered from such materials.

(4) *Rhodnius* diuretic hormone in a brei of the mesothoracic ganglionic mass (Maddrell, 1963) is entirely destroyed if kept for 2 h at pH 8 and at 37 °C. 5-HT added to such a brei survives this treatment.

It is possible, using isolated preparations of both Malpighian tubules of *Rhodnius* and salivary glands of *Calliphora* as assay systems, to show that the diuretic hormone appearing in the haemolymph of *Rhodnius* after a meal is different from that extractable from the nervous system and different again from 5-HT. 5-HT is 10–20 times *more* potent in stimulating the salivary glands than it is in stimulating the Malpighian tubules, while active haemolymph samples are about two times *less* potent and ganglion extracts 25 times *less* potent in this differential assay (S. H. P. Maddrell, unpublished observations).

That the diuretic hormone of *Carausius* is not 5-HT is shown by arguments exactly similar to those described above (nos. 2, 3). Further, in *Carausius*, the diuretic hormone competes much more successfully with BOL for the receptor site than does 5-HT. At 10^{-4} M/l, BOL does not prevent the diuretic hormone at 0·2 pairs of corpora cardiaca/100 μl from causing a maximal acceleration of secretion, although it prevents 5-HT at 10^{-6} M/l from having any effect. Finally, the diuretic hormone of *Carausius* does not stimulate the muscular writhing of the Malpighian tubules of the same insect, although 5-HT will (Pilcher, 1970b).

So, although only very few compounds will stimulate Malpighian tubules to secrete, it appears that the naturally occurring hormones are not 5-HT. Yet they presumably stimulate the same receptors since competitive inhibitors of 5-HT also competitively inhibit the action of the diuretic hormone (one must accept this conclusion with some reservation, because of course the action of externally applied cyclic AMP is also inhibited by these competitive inhibitors). A rather similar situation exists in vertebrates where adrenaline (epinephrine) is found to stimulate many receptors and yet hormones (e.g. insulin, ACTH) exist which specifically activate only one or very few of the same receptors. An ingenious theory to explain this has recently been put forward by Maddaiah (1969). He suggests that a specific hormone receptor might exist next to the adrenaline-sensitive receptor. As a result of its attachment to this receptor, the polypeptide hormone might be held in such a way that suitable side chains would become situated over the adrenaline receptor so as structurally to simulate adrenaline and so to stimulate the receptor.

A somewhat similar system might exist for Malpighian tubules and their diuretic hormones. 5-HT and its relatives are very active compounds, and a diuretic hormone closely related to these molecules might produce an undesirably wide range of effects in many tissues. On the other hand if the diuretic hormones were made highly specific perhaps by a mechanism similar to that suggested by Maddaiah, the problem would be solved. In favour of such a scheme are the following pieces of evidence. (a) *Carausius* diuretic hormone does *not* induce writhing movements of the Malpighian tubules of *Carausius* but 5-HT does (Pilcher, 1970b). (b) *Rhodnius* diuretic hormone does *not* induce secretion by the Malpighian tubules of *Carausius* but 5-HT does. (c) Salivary glands of *Calliphora* are very sensitive to 5-HT (Berridge, 1970) but only respond to a very concentrated extract of the mesothoracic ganglionic mass of *Rhodnius* – this may well be due to trace amounts of 5-HT which are found in nervous tissues of other insects (Colhoun, 1967) and not to the diuretic hormone itself.

A system exactly similar to that proposed by Maddaiah involving additional hormone-site attractions seems unlikely for the *Rhodnius* diuretic hormone, because of the speed with which the hormone can be washed off the tubules. None the less if the hormone is a good deal larger than 5-HT and its relatives then this could explain its specificity. It could well be that the active part of the hormone molecule is very similar to 5-HT but that the shape of the rest of the molecule would allow the 5-HT-like part to engage only with the 5-HT-sensitive diuretic hormone receptor of Malpighian tubules; it would not readily interact with other 5-HT-sensitive tissues. In this connexion it will be recalled that the Malpighian tubules of *Carausius* are stimulated by fewer compounds than are *Rhodnius* tubules, and that *Carausius* tubules do not respond to the *Rhodnius* diuretic hormone, although *Rhodnius* tubules are stimulated

by the diuretic hormone of *Carausius*. Plainly the *Carausius* receptor imposes an even more rigorous set of conditions upon possible stimulant molecules.

The next point to be considered is the suggestion that the diuretic hormone receptors are on the cell membrane facing the haemolymph. *A priori*, one might expect a hormone receptor to be placed on the external surface of the cell nearest the source of the hormone. However, there is rather little direct evidence which bears on this point, though two pieces of evidence do support the suggestion. The first of these is that bromolysergic acid diethylamide (BOL) very quickly inhibits secretion induced by 5-HT or the diuretic hormone, although BOL is a relatively large molecule (mol. wt 347) which would be expected to enter the cell only relatively slowly. The second and more convincing piece of evidence is that concentrated solutions of 5-HT perfused through the lumen of a tubule do not stimulate the tubule to secrete – as judged by the absence of the marked electrical response which follows the application of 5-HT to the tubule from the haemolymph side (Maddrell, 1971). On these grounds it certainly seems that the cell membrane facing the haemolymph is the most likely site for the primary event in the interaction of the diuretic hormone and 5-HT with the cell.

The question now arises as to the possible involvement of cyclic AMP in the stimulation of secretion. The evidence for its involvement is as follows. First, cyclic AMP applied externally at quite high concentrations causes very similar accelerations of secretion in tubules of both *Rhodnius* and *Carausius* as do 5-HT and the respective diuretic hormones. Second, aminophylline at 10^{-4} M/l enhances the secretory rate of tubules of *Carausius* (but does not accelerate secretion by tubules of *Rhodnius*).

These results show that cyclic AMP may well be involved in the actions of the diuretic hormones and 5-HT. For a more definite statement to be made, measurements of the intracellular levels of cyclic AMP before and after stimulation would be required to see whether these react appropriately. The present results with their reliance on high extracellular concentrations of active chemicals highlight the difficulties of analysing the nature of intracellular processes from the wrong side of a relatively impermeable cell wall.

If the control system of Malpighian tubules is similar to that proposed for other systems (along the lines indicated in Fig. 17), then it may well be that the activity of the adenyl cyclase closely depends on the state of the receptor site. In *Rhodnius*, 5-methyltryptamine at high concentrations after provoking an initial short-lived burst of secretion prevents further secretion even at the low level (*c.* 0·25 nl/min) characteristic of unstimulated tubules (Fig. 16). If this low level of secretion is the result of a low rate of synthesis of cyclic AMP within the unstimulated cells, then the prevention of this synthesis by blocking the receptor site implies a tight coupling between this site and the adenyl cyclase responsible for synthesizing intracellular cyclic AMP. This could be most simply explained by assuming that the receptor site is actually part of the adenyl cyclase molecule itself. Just such an hypothesis is suggested by Robison, Butcher & Sutherland (1967) for the β-adrenergic receptor and adenyl cyclase of vertebrates.

The inhibition of secretion, by competitive inhibitors of 5-HT, opens up for insects the possibility of there being antidiuretic hormones which could not only oppose the action of the diuretic hormones, but which could also slow the unstimulated rate of

secretion. It also, however, suggests that care is needed in interpreting the results of treating Malpighian tubules with extracts of nervous tissues. Such tissues would be expected to contain amines which could act as antidiuretic factors though they may never naturally appear in the haemolymph. For such a substance reasonably to qualify as a hormone, it must be found to occur naturally in the haemolymph at concentrations which affect the tubules.

N-methyl-5-HT, N,N-dimethyl-5HT, N-acetyl-5-HT, 5-HT and the diuretic hormone of *Rhodnius* all cause isolated *Rhodnius* tubules to secrete at their maximum rate. This does not mean that their effects on the cells are all exactly of the same size. One can imagine, for example, that they all cause the accelerated synthesis of cyclic AMP but that the intracellular levels of cyclic AMP attained in each case differ. Secretion may still occur at identical rates, i.e. the *apparent* intrinsic activity be unity, because the level of cyclic AMP once it reaches a certain level is not limiting. Some other process – perhaps the rate of ion entry or the availability of energy – would then be the limiting step. Such an interpretation is made more reasonable by the fact that externally applied cyclic AMP cannot induce any faster secretion than can 5-HT.

A puzzling aspect of the work described in this paper is the inhibitory effect of tryptamine and other compounds on the response to cyclic AMP. It is proposed that this is due either to interference with the entry of cyclic AMP or to the intracellular action depending on the hormone receptor site. Against the first of these possibilities it can be argued that it would then be merely coincidental that the inhibitors have equal effects whichever of 5-HT or cyclic AMP is used to stimulate secretion. It seems more likely that some effect of cyclic AMP may depend on the condition of the hormone site. Such an effect may not be unlikely for M. J. Berridge and W. T. Prince (unpublished observations) have shown that 5-HT and cyclic AMP have different effects on the trans-wall potential difference of salivary glands of *Calliphora*. It seems that 5-HT may have an effect additional to that of inducing the synthesis of cyclic AMP, and this might be an increase in ion permeability. In this case, the difference does not limit the rate of secretion. It is conceivable, however, that in *Rhodnius* inhibition by tryptamine might lead to a decrease in ion permeability of the Malpighian tubule wall which might in this case lead to slowing of secretion. Such a slowing would be expected to affect stimulation by 5-HT or by cyclic AMP equally since a decrease in ion availability would act directly on the ion pumps controlling secretion.

SUMMARY

1. 5-Hydroxytryptamine (5-HT) and a few of its derivatives will cause an acceleration of secretion by the isolated Malpighian tubules of *Rhodnius prolixus* and *Carausius morosus*. Substitution in the terminal amino group of 5-HT causes little loss of effect, but changes in the indole ring and the hydroxy group in general abolish activity.

2. Most derivatives of 5-HT which do not stimulate secretion, and many other related amines, act as inhibitors of secretion in *Rhodnius*. This inhibition is probably of the competitive type.

3. Substances which stimulate secretion in *Rhodnius* are easily washed off the Malpighian tubules, although they will stimulate secretion at very low concentrations.

By contrast, competitive inhibitors wash away relatively slowly and inhibit only at much higher concentrations. It is suggested therefore that stimulation involves only momentary interactions between stimulant and receptor sites, but that competitive inhibitors spend considerably longer attached to the sites.

4. Cyclic 3′,5′-adenosine monophosphate (cyclic AMP) will stimulate secretion by tubules of both *Rhodnius* and *Carausius*. Tubules of both species are very sensitive to cyclic AMP, measurable effects being produced at concentrations of 4×10^{-5} M/l in *Rhodnius* and 10^{-4} M/l in *Carausius*.

5. Aminophylline (theophylline ethylene diamine) at a concentration of 10^{-4} M/l stimulates secretion by *Carausius* tubules. This compound and other inhibitors of phosphodiesterase have no stimulatory action on *Rhodnius* tubules, but any such effect may be masked by the adverse reaction of the tubules to the high concentrations necessary.

6. Inhibitors of 5–HT action such as tryptamine and tyramine also inhibit secretion by *Rhodnius* Malpighian tubules induced by cyclic AMP. This may be due to a dependence of the action of cyclic AMP on the state of the diuretic hormone receptor site.

7. The evidence is consistent with the idea that 5-HT, other stimulant molecules related to 5-HT and the diuretic hormones interact with Malpighian tubule cells of *Rhodnius* and *Carausius* at specific sites, probably on the cell membrane facing the haemolymph. As a result of this, secretion is induced, possibly through the action of intracellular cyclic AMP produced as a response.

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