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SUPERFUSION AS A METHOD FOR THE STUDY OF DRUG ANTAGONISM

BY

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In investigating constrictor substances in shed blood it became necessary to consider methods for the assay of 5-hydroxytryptamine (HT) in the presence of other pharmacologically active substances. Separation of some of these substances could be achieved by extraction with 95% acetone in which adenosine, substance P, and potassium salts are insoluble, whereas HT, histamine, acetylcholine, adrenaline, and noradrenaline are soluble (Amin, Crawford, and Gaddum, 1954). The problem thus became the determination of the amount of HT present in solutions containing histamine or ACh or both. Attempts were made to use the perfused rabbit ear (Holgate, 1949, 1953). With mepyramine 10^{-7} in the perfusate this preparation still showed a high degree of sensitivity to HT, but the method proved laborious for accurate assay. The rat uterus under conditions of superfusion showed great instability despite most careful prior preparation of the animal with stilboestrol. Very slight changes in experimental conditions gave rise to maximal contractions of the muscle invalidating all attempts at assay. The superfused rat colon gave good specificity for HT with high sensitivity, but with much variability between tissues obtained from different animals. The guinea-pig ileum was much more consistent in its responses, but contracted to HT, histamine, and ACh. A method involving the use of mepyramine and atropine was considered. This method had been used by Feldberg and Toh (1953) and by Dalgliesh, Toh, and Work (1953) in connexion with mixtures containing HT, histamine, and ACh together with substance P, the activity of the remaining substance P being determined after blocking HT with tryptamine (Gaddum, 1953). It was evident, however, there are conflicting opinions about the effect of atropine on the response of the ileum to HT. Some consider atropine to reduce markedly the response to HT in concentrations which also affect the response to ACh (Robertson, 1953; Rocha e Silva, Valle, and Picarelli, 1953), while others state that concentrations which abolish the effects of ACh and carbachol have but little effect upon the response to HT (Gaddum and Hameed, 1954; Gaddum, 1953). Similarly there is little information about the effect of mepyramine on the response of the ileum to HT, although Gaddum and Hameed (1954) state that it abolishes the effect of histamine in doses that have little or no effect on HT.

We were thus led to investigate the quantitative relationships of the antagonism by atropine and mepyramine of ACh, histamine, and HT. The technique of superfusion was used, since we had found this method both convenient and advantageous when studying the action of agonists (Cambridge and Holgate, 1954). The results obtained not only illustrate the limitations in the use of antagonists on the ileum for assay of HT in mixtures, but have yielded new information on the antagonism between atropine and HT.

METHODS

Superfusion Apparatus.—The apparatus used was modified from that described previously (Cambridge and Holgate, 1954), and is shown in diagrammatic form in Fig. 1. The tissue was suspended in air in a 15 cm. by 3 cm. water-jacketed chamber (K). This chamber was closed at the lower end by a perforated bung carrying a hook for tissue attachment, and, at the upper end, by a "Perspex" disc, slotted for the passage of a thread. A "Perspex" tube passing through the double wall of the chamber enabled a thermistor (S) to be placed below the tissue. Drugs and Tyrode solutions were contained in bottles (F and E) in the bath (G) and were driven from these by compressed air or oxygen respectively. These gases were fed in under pressure controlled by the water valves (BB) and recorded on manometers (CC), the actual entry to the bottles being determined by the opening of the electro-magnetic clamps (D). Passage of drugs and Tyrode from the bottles to the gut was through fine polyvinyl tubes contained in a small water jacket (H) to the water-jacketed glass warming

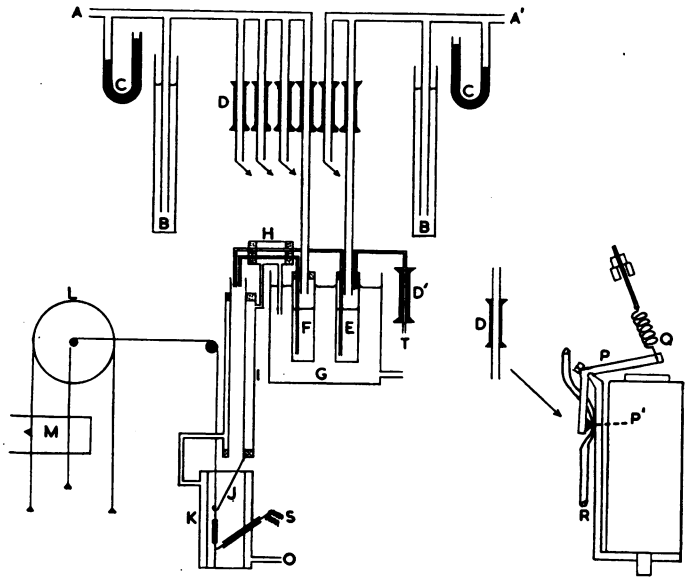

FIG. 1.—Diagram of superfusion apparatus. A, compressed air inlet, A', oxygen inlet; BB, water valves; CC, mercury manometers; D, electro-magnetic clamps controlling air and oxygen inlet to drug or superfusion fluid bottles; D', electro-magnetic clamp controlling pressure release; E, superfusion fluid bottles; F, drug bottles; G, warm water bath; H, small water jacket about outlet tubes; I, water-jacketed tube; J, glass finger; K, water-jacketed tissue chamber; L, pulley recording system; M, recording drum; O, inlet to water-jackets and bath; S, thermistor; T, pressure release tube. Inset: diagram of electro-magnetic clamp. P, armature with bar P'; Q, adjustable spring; R, tubing.

tube (I), and, finally, via a glass finger within the confines of the tissue chamber on to the upper thread attached to the tissue. Air and oxygen pressures were adjusted to give a final drop rate of 60/min., 20 drops being approximately equal to 1 ml. Viability of tissue for long periods was taken to indicate adequate oxygenation of superfusion fluid. Persistence of oxygen pressure in superfusion fluid bottles leading to "over-run" of fluid was eliminated by opening a pressure release clamp D' whenever D was closed. During the experiments the flow of superfusion fluid from bottles E was interrupted every 2½ to 3 min. for 40 sec., during which time 40 drops were applied to the gut from one of the drug bottles (F); the flow of Tyrode was then resumed. The flow of Tyrode actually ceased 3 to 5 sec. before the application of the drug. All operations were carried out automatically, using an apparatus developed from that described by Schild (1946, 1947). The time course of all events was further checked by direct observation.

The temperature in the bath (G) and the water-jackets was maintained at 30.0° C.

Recording System.—In previous experiments we used a frontal writing lever for recording gut movements, but in this series we have used a pulley system. The upper thread from the tissue, after leaving the chamber, passed over a "Perspex" pulley on pivot bearings, ran horizontally for 30 cm., was wound once round a further pulley 1.5 cm. in diameter to hang vertically and terminate in a counter-balance weight of 0.63 g. This second pulley was affixed to a large "Perspex" disc 15 cm. in diameter, drilled out for lightness and balance, the whole being mounted on pivot bearings. In a groove round the circumference of this disc ran a thread carrying a glass capillary writing point and, at its extremities, two counter-balance weights of approximately 1.0 g. to keep the thread taut. Both ends of this thread ran through slots in a horizontal brass bar which limited the excursion of the counter-balance weights so that the writing point did not pass beyond the upper and lower limits of the smoked drum. All grooves in the pulleys were of U-shaped cross-section to prevent binding of the threads. This system gave truly linear translation of gut contraction at a magnification of 10 : 1. A vibrator was bolted on to the main frame, this keeping the bearings free, and resulting in a high degree of sensitivity such that a weight of 0.1 g. applied to the thread attached to the gut gave a full excursion of the writing point.

Preparation of the Tissue.—In all experiments 3 to 4 cm. lengths of the terminal portion of the ileum of female virgin guinea-pigs weighing 150 to 250 g. were used, the pigs being starved 24 hr. before removal of the gut. After washing through with Tyrode, portions were tied with open ends. The use of animals within the weight range indicated gave a sensitivity to histamine similar to that found by Adam, Hardwick, and Spencer (1954), and higher than that previously reported by us.

Tyrode Solution.—The composition was (g./l.): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaHCO_3$ 1.0, NaH_2PO_4 0.05, and glucose 1.0. The *p*H lay between 8.0 and 8.4.

Drugs.—These were dissolved in Tyrode solution using the following salts: histamine acid phosphate, acetylcholine bromide, 5-hydroxytryptamine creatinine sulphate (kindly supplied by Dr. Merrell E. Speeter), atropine sulphate and mepyramine maleate. All concentrations referred to in this paper are in terms of ng./ml. of the base.

RESULTS

Antagonism of Histamine by Mepyramine

The antagonism between mepyramine and histamine lent itself best to preliminary investigation and will be described in detail as an example of the

methods used. Fig. 2 shows a typical example of the effect of one concentration of mepyramine on the response of the ileum to one concentration of histamine. The flow of superfusion fluid was interrupted every 2½ min. for the application (over a period of 40 sec.) of 2 ml. of Tyrode solution containing histamine (5 ng./ml.), thus producing the contractions shown at the beginning of the record. After at least 5 stable responses had been obtained antagonist solution was run over the tissue in place of Tyrode; thus, during the period M, the superfusing fluid, but not the histamine solution, contained mepyramine 0.35 ng./ml. The amine, as in the period marked B. In the experiment illustrated, superfusion with mepyramine-free Tyrode was resumed after 40 min., but in other experiments it was found that the size of the response to histamine in this equilibrium state persisted unchanged although application of antagonist was continued for as long as 2 hr. Responses recorded during this period showed no greater variation than those recorded in the absence of mepyramine. The first one or two responses to histamine immediately following the change back to mepyramine-free Tyrode were often smaller than those during the equilibrium

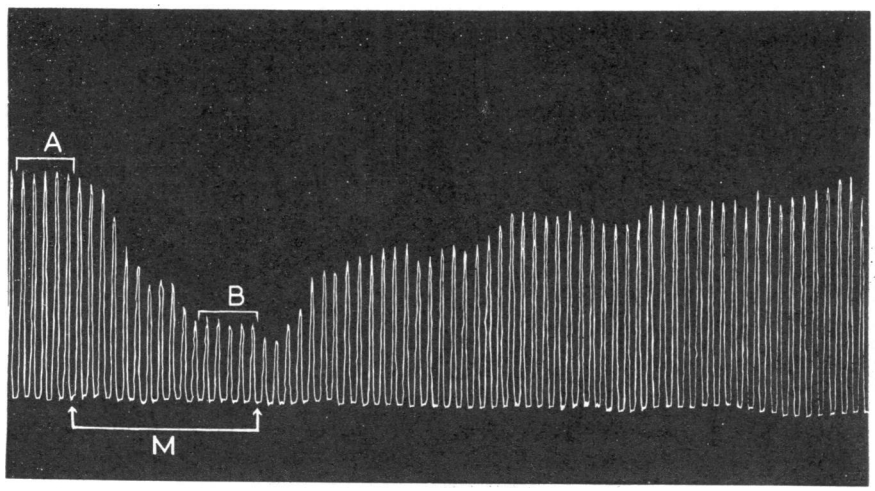

FIG. 2.—Typical record showing the depression of the response of the ileum to 5 ng./ml. histamine produced by 0.35 ng./ml. mepyramine in the superfusion fluid. Histamine application at 2½ min. intervals. Mepyramine applied during period indicated by M.

routine otherwise was unchanged and the decrease in the responses to histamine under these conditions is obvious. After replacement of the antagonist by mepyramine-free Tyrode the response to histamine increased gradually to its initial level.

The amount of drug given (40 drops) was well in excess of the minimum previously proved necessary for maximal accuracy (Cambridge and Holgate, 1954). At the beginning of every experiment a sensitizing concentration of agonist was chosen and repeatedly applied for 60 to 90 min. to ensure complete stability of response. Antagonists were not applied to the tissue until it had been under superfusion and drug stimulation for at least 2 hr.

Time Course of the Antagonism.—The effect of mepyramine applied in the way described increased, as in Fig. 2, over a period of 20 to 25 min. until the maximum reduction in the histamine response was obtained; the effect then remained stable during the continued application of mepyr-

state (Fig. 2), as was also observed by Schild (1947) using the bath technique. Thereafter the size of the contractions increased steadily, and after some 130 min. reached the initial size, at which point the tissue was ready for the application of another concentration of mepyramine.

Relationship Between Antagonism and Concentration of Mepyramine.—The results of observations such as those illustrated in Fig. 2 could be most conveniently expressed for many purposes in the following way. The average height of the contractions in the equilibrium period of antagonism (the mean of the 5 observations B) was expressed as a percentage of the initial height of contraction (the mean of the 5 observations A): in this example the effect of 0.35 ng./ml. of mepyramine was thus to reduce the response to histamine to 36%.

It was found that the effect expressed in this way did not vary appreciably in a large series of

tests using the same concentrations of both mepyramine and histamine on any one piece of gut, the sections being taken either from the same animal (within 10 cm. of the ileo-caecal junction) or from different pigs. When the concentration of mepyramine was varied, keeping that of histamine the same, the degree of antagonism produced by the mepyramine was in approximately linear relationship to the logarithm of the concentration—as in the example of Fig. 3, where 4 concentrations of mepyramine within a limited range were tested. Because of the constancy of behaviour of sections of gut from different guinea-pigs it was possible to use the same concentration of histamine in a large series of experiments. This concentration, and those of other agonists used, was chosen to give a response lying between 50 and 75% of the maximum possible contraction. In experiments where the effect of an antagonist on two agonists was investigated approximately equi-active concentrations of the agonists were used.

Some 22 experiments were performed in which different concentrations of mepyramine were tested on the contractions produced by 5 ng./ml. histamine, and the composite results of these experiments are shown in Fig. 4, line a. Scatter about the points has not been shown but was remarkably small, being of the order of $\pm 5\%$ except with very low concentrations of antagonist; in these circumstances it was frequently found that the mean response of the tissue to the agonist was uncertain

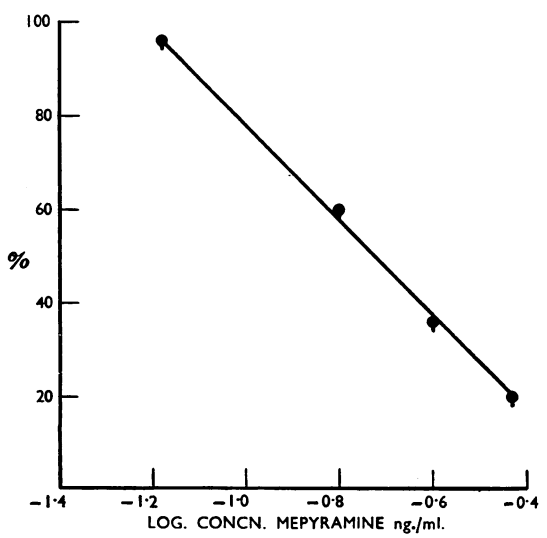

FIG. 3.—Graph showing the relationship between the log. concentration of mepyramine in the superfusion fluid and the depression of the response of the ileum to 5 ng./ml. histamine expressed as the percentage value described in text.

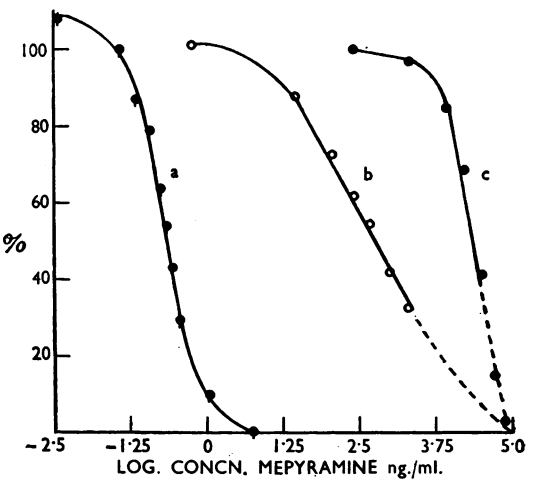

FIG. 4.—Graph showing the relationship between the log. concentration of mepyramine in the superfusion fluid and the depression in the response of the ileum to 5 ng./ml. histamine (a), 15 ng./ml. HT (b) and 5 ng./ml. acetylcholine (c), expressed as the percentage values described in text.

and was often slightly increased. Inconstancy of individual successive responses greater than that shown in the absence of inhibition was also a feature in the presence of low concentrations of antagonist.

Whereas in all preliminary experiments full recovery of response (as shown in Fig. 2) was obtained before application of a further concentration of antagonist, in later investigations into the same antagonist/agonist combination we found this to be unnecessary. In such investigations the first concentration applied was the lowest to be used and, after reaching equilibrium, a higher concentration was then applied, reducing the response to a new equilibrium state. By this means many more concentrations of antagonists could be tested within a limited time, the only assumption being that the sensitivity of the preparation to the agonist did not alter appreciably. That this assumption was valid was shown by the fact that curves obtained by this method did not differ from those obtained in the preliminary experiments.

In the foregoing experiments the same concentration of histamine was used throughout. Experiments were also performed in which contractions to 2 concentrations of histamine (in the example shown in Fig. 5—5.1 and 8.2 ng./ml.) were recorded alternately and the effect of different concentrations of mepyramine observed. In Fig. 5 the size of the mepyramine-reduced contractions (expressed as a percentage of the initial contraction to that concentration of histamine) is plotted

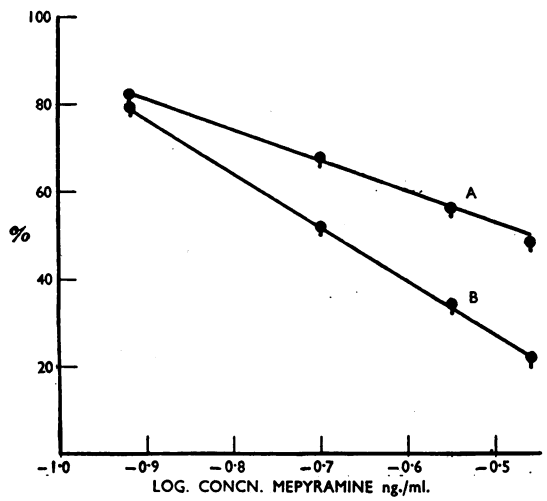

FIG. 5.—Graph showing the relationship between the log. concentration of mepyramine in the superfusion fluid and the depression of the response of the ileum to 8.2 (A) and 5.1 (B) ng./ml. histamine, the depression expressed as the percentage value described in the text.

against the log. concentration of mepyramine; in all such experiments the lines were not parallel.

Dose/Response Curve of Histamine in the Presence of Mepyramine.—Fig. 6 shows the result of an experiment in which the log. concentration/response curve of histamine was determined before and during the equilibrium state of depression produced by 0.3 ng./ml. of mepyramine; the two curves show no deviation from parallelism. The log. concentration/response curve plotted after recovery of the tissue from the action of mepyramine was not significantly different from the original, indicating the completeness of recovery under these experimental conditions.

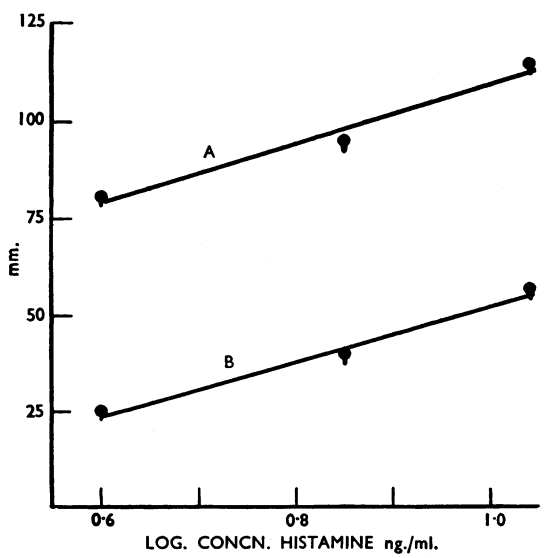

FIG. 6.—Log. concentration/response curves of histamine before (A) and during (B) the exposure of the ileum to mepyramine 0.3 ng/. ml. in the superfusion fluid.

pA Values for Mepyramine Against Histamine.—Schild (1947) has defined the pA_x value. Accurate application of this over a wide range of dose is only possible if the log. concentration/response curve of the agonist in the presence of the antagonist is parallel to the curve in the absence of antagonist; this has been shown above to be so for histamine and mepyramine.

We have determined *p*A values by the following procedure. Firstly, a log. concentration/response curve for histamine in the absence of mepyramine was constructed using doses of histamine of x, 0.5x, 0.2x, and 0.1x, the actual concentrations being chosen to lie on the linear part of the curve. From this curve it was found that the responses to 0.5x, 0.2x, and 0.1x were, in one example, 76%, 44%, and 20% of the contraction to x. Secondly, the effects of different concentrations of mepyramine were tested against responses produced by x ng./ml. of histamine (as a fixed concentration), so that a curve similar to that of Fig. 3 was plotted. From this line the concentrations of mepyramine necessary to reduce the response to x ng./ml. histamine to 76%, 44%, and 20% were 0.12 ng./ml., 0.24 ng./ml., and 0.39 ng./ml., and the negative logarithms to base 10 of the molar concentrations equivalent to these gave the pA_2, pA_5, and pA_{10} values in this test.

Schild (1947) and Reuse (1948) have measured *p*A values for histamine/mepyramine using the isolated gut in an organ bath. In their experiments the equilibrium state was not necessarily reached—that is, the size of the histamine contraction was still diminishing—so that it was necessary to define the time (2, 14, or 15 min.) after the introduction of the mepyramine at which the *p*A values were measured. In our experiments equilibrium was achieved and the values given in Table I might be designated pA_2E, pA_5E, or $pA_{10}E$ to

TABLE I

TABLE OF pA_xE VALUES FOR ATROPINE AND MEPYRAMINE AGAINST ACETYLCHOLINE, HT AND HISTAMINE

	Atropine			Mepyramine		
	ACh	HT	H	ACh	HT	H
pA_2	8·74	7·93	6·30	4·44	6·50	9·37
pA_5	8·37	6·00	5·95	4·14	6·04	9·08
pA_{10}	8·25	5·42	5·48	3·91	5·86	8·86

distinguish them from the pA_2 and pA_{10} (2 or 14 min.) values of Schild (1947) or the pA_2 and pA_{10} (15 min.) values of Reuse (1948). The values given in Table I do not differ greatly from those determined at the longer times of exposure by Schild and by Reuse.

Antagonism of Acetylcholine and 5-Hydroxytryptamine by Mepyramine

The action of mepyramine on the response of the ileum to ACh and to HT was studied by methods similar to the above. It was found advisable to make the applications of HT at intervals of 5 to 6 min. in order to avoid tachyphylaxis ; in practice these were alternated with applications of either ACh or histamine so that the depression curves for agonists could be determined simultaneously.

The time course of the antagonism of HT or ACh, using suitable concentrations of mepyramine, was similar to that of histamine ; thus, as before, the extent of the antagonism could be expressed as the percentage depression present at equilibrium. In Fig. 4 the effect of mepyramine against histamine (line a) is compared with its effect against HT (line b) in 11 experiments, and against ACh (line c) in 9 experiments. The interrupted sections of these lines indicate some degree of uncertainty as, at these high concentrations, mepyramine itself produced some contraction of the ileum. In these experiments the same concentration of agonist was given on every occasion, namely 15 ng./ml. for HT and 5 ng./ml. for ACh, these concentrations being approximately equal in activity to 5 ng./ml. of histamine.

Log. concentration/response curves for HT and ACh in the presence and absence of mepyramine were parallel in each instance. It was thus permissible to determine pA values by the procedure described above. These are given in Table I. The pA_2 and pA_{10} values for mepyramine/ACh are comparable with those reported by Schild (1947); the values for HT reflect its intermediate position between histamine and acetylcholine.

Antagonism of Acetylcholine, HT, and Histamine by Atropine

Acetylcholine.—Experiments as described for histamine/mepyramine using ACh and atropine gave very similar results. An equilibrium state was attained in which the depressed response persisted unchanged as long as atropine was continued ; there was a precise relationship between the degree of depression and the log. of the concentration of atropine used (Fig. 7, line a); log. concentration/response curves in the presence and absence of the antagonist were parallel, and

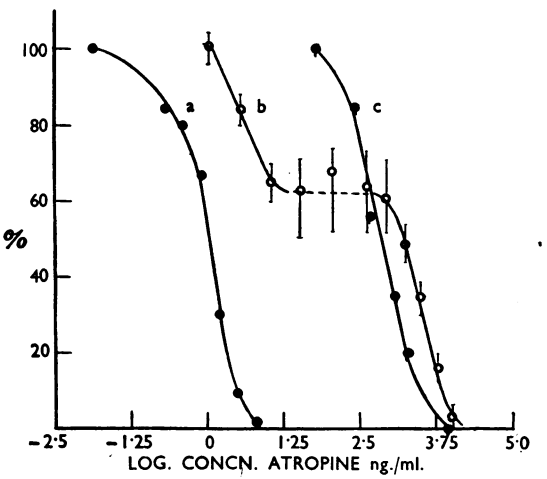

FIG. 7.—Graph showing the relationship between the log. concentration of atropine in the superfusion fluid and the depression of the response of the ileum to 10 ng./ml. acetylcholine (a), 20 ng./ml. HT (b) and 5 ng./ml. histamine (c) expressed as the percentage value described in the text.

depression/log. concentration of antagonist curves for 2 different concentrations of agonist were not parallel. The linear portion of the atropine/ACh curve (Fig. 7, line a) extends over a smaller range of atropine concentration and is somewhat steeper than the corresponding mepyramine/histamine curve (Fig. 4, line a). The lowest effective concentration of atropine is, at the same time, less than the corresponding mepyramine concentration, thus producing a final curve of somewhat different form. The pA values determined in the way described are given in Table I.

Owing to the period required for stabilization of response and for determining the dose/response relationship for one or more agonists, atropine was not applied until the tissue had been under superfusion for 2 to 3 hr. For this reason it was not established whether any change in sensitivity of the preparation to atropine occurred during the first 2 hr. following its removal from the animal, as reported by Flesch and Kun (1948). There was no change in sensitivity after this initial period as evidenced by the fact that the order in which the different concentrations of antagonist were applied made no difference to the final relationship obtained between the concentration of antagonist and the degree of depression of response to the agonist.

The equilibrium state was usually reached in 20 to 25 min. with mepyramine, and in 15 to 20 min. with atropine. Recovery from the effect of the antagonist was complete in 90 to 140 min. with

mepyramine and in 60 to 90 min. with atropine; presumably atropine diffused into and out of the tissue more rapidly than did mepyramine. Since in the above experiments the agonist solution did not contain antagonist there could have been some diffusion of the antagonist out of the tissue during the application of agonist. Addition of antagonist to agonist solutions in concentrations equal to those present in the superfusing fluid produced a slight reduction in the time necessary to reach the equilibrium state. With atropine, but not with mepyramine, this addition also produced a reduction in the response to the agonist greater than that produced when atropine was absent from the agonist solution. The difference, however, seemed too slight to warrant the technical inconvenience involved in changing the agonist solution every time a fresh concentration of antagonist was applied; this might, however, be necessary if any shorter acting antagonist were being tested.

Histamine.—As was expected, very high concentrations of atropine were necessary to produce reduction in the response to histamine. The form of the depression/log. concentration curve was similar to those previously determined and is shown in Fig. 7, line c; the *p*A values are given in Table I.

5-Hydroxytryptamine.—Experiments concerning the stability of the reduced response, and studies of log. concentration/response curves in the presence and absence of atropine, gave results similar to those for other agonist/antagonist combinations. However, the curve relating the depression of the response to HT to the log. of the concentration of the antagonist differs from the others (Fig. 7, line b). Six different sections of ileum were used. On each occasion depression of the response to HT by atropine began when the concentration of the latter reached approximately 1.0 ng./ml. With increasing concentration of atropine the depression increased until, when the concentration reached about 10 ng./ml., the response lay somewhere between 50 and 70% of the original. Increasing the concentration of antagonist over the range 10 to 1,000 ng./ml. produced no further depression of the response. Responses were, however, very variable in this range of atropine concentration. In those tissues where the response was 50% of the original in the presence of 10 ng./ml. of atropine, the response in the presence of concentrations up to 1,000 ng./ml. was also of the order of 50%. In tissues where the response had been reduced to 70% of the original by 10 ng./ml. of atropine this higher response was still seen although the concentration of antagonist was increased 100-fold. Increasing the concentration of atropine beyond 1.0 μg./ml. reduced the response to HT on a curve similar to that of the reduction of the response to histamine. The vertical limits on line b, Fig. 7, indicate the ranges in the 6 different tissues.

This characteristic behaviour of the response of the ileum to HT in the presence of different concentrations of atropine, as indicated by these results, has not previously been described. We feel that this should be taken into account in any consideration of the mode of action of HT upon the guinea-pig gut.

The "plateau" in the curve for depression described above is also indicated by the *p*A values (Table I). The pA_2 value lies near to that for atropine/ACh and the pA_{10} near to that for atropine/histamine. The pA_5 value, as it happens, lies near to that for atropine/histamine but, depending upon the exact position of the "plateau," might just as readily lie nearer to the pA_5 for atropine/ACh.

When using HT in concentrations above 20 ng./ml. some tachyphylaxis was observed unless applications were spaced at 6 min. intervals. In addition, when these higher concentrations of HT were used, it was frequently observed that they caused some interference with the response of the tissue to histamine. A second application of the same concentration of histamine following HT was always greater than the first, this occurring also in the presence of atropine. For this reason, as well as for that of tachyphylaxis, concentrations of HT above 20 ng./ml. were not used, and, moreover, concentrations at or below this figure were given alternately with ACh and not histamine.

Discussion

Use of the Ileum as an Assay Preparation for HT.—The development by Amin, Crawford, and Gaddum (1954) of a method for the extraction and separation of HT and substance P from tissues provides a means of overcoming the difficulty of assay of HT on the ileum in mixtures which might also contain substance P. The separation of the two substances by this method would appear to be almost complete, but the extract would be likely to contain, in addition to HT, histamine, ACh, adrenaline, and noradrenaline. If HT is to be assayed in the presence of histamine it can be seen from Fig. 4, lines a and b, that abolition of the effect of 5 ng./ml. of histamine can be achieved by a concentration of mepyramine which has practically no effect on the response of the ileum to

15 ng./ml. HT, hence the effect of HT could be satisfactorily separated from that of histamine in mixtures containing histamine and HT in the proportions of 1:3. But in rabbit's blood, for example, the proportion might be as high as 3:1 (Code, 1937; Holgate, 1953) and the concentration of mepyramine which would be necessary to abolish the activity of histamine under these conditions would very seriously reduce, if not abolish, the response of the ileum to the less active HT. Indeed, Cambridge and Holgate (1953) have shown that the remaining response in the presence of 10 ng./ml. mepyramine to a mixture of histamine and HT in the proportions of 1.5:1 was, in fact, due to histamine, the effect of HT having been completely abolished. Only if the content of histamine in the mixture were considerably less than that of HT would it be possible, using carefully chosen concentrations of mepyramine, to assay accurately the content of HT.

With regard to the assay of HT in the presence of ACh, from Fig. 7, lines a and b, it can be seen that the activity of 10 ng./ml. ACh would be abolished by a concentration of about 10 ng./ml. atropine, but the response to 20 ng./ml. HT would, in these circumstances, be reduced to about 60 to 70% of the original. In addition this response would be brought into the range of greatest variability.

Clearly, even without the complications due to the possible presence of adrenaline and noradrenaline, the guinea-pig ileum in the presence of atropine and mepyramine is a most unsuitable preparation for the assay of HT in mixtures containing ACh and histamine—unless the proportion of the activity due to HT is very high; even in this event the concentrations of the antagonists used must be chosen with care.

Methods for Expressing the Activity of Antagonists.—Gaddum (1926), Winder, Kaiser, Anderson, and Glassco (1946), Miller, Becker, and Tainter (1948), and Rapport and Koelle (1953) all used a fixed concentration of agonist and compared the effect of various antagonists by measuring the reduction they produced in the response of the tissue. For example, Winder *et al.* (1946) expressed the power of the antagonist as that concentration required to produce 70% depression in the response (the ED70 value), the concentration then being expressed in terms of diphenhydramine equivalents. Miller *et al.* (1948) devised the ED50 value—the concentration of spasmolytic agent capable of producing a 75% reduction of a maximal spasm in one half of the test preparations. The concentration of antagonist was expressed as the log. of its dilution, the *p*D value. Figs. 3, 4, 5, and 7 give results of experiments of this type; the ED70 value, for example, could be calculated from them. The choice of concentration of agonist has been made by different authors in different ways. Winder *et al.* (1946) speak of a "pre-determined standard spasm" and Miller *et al.* (1948) of a "maximal spasm." We chose a concentration which produced a response 50 to 75% of the maximal contraction possible in a 3 to 4 cm. length of ileum pulling against a load of 0.2 to 0.3 g. If a higher concentration of agonist was used (Fig. 5, line A) the same degree of antagonism could only be produced by using a higher concentration of antagonist, which would lead to a higher ED70 value. In the early phases of our investigations this fact led us to the use of what we termed the "R" value, which is the ratio between the concentration of the agonist and that of the antagonist required to produce a constant amount of reduction in the response. This value has more recently been better named the "drug ratio" by Gaddum, Hameed, Hathway, and Stephens (1955) and has been fully discussed by these authors. We noticed that this value was remarkably constant over a relatively wide range of concentration of stimulant drug and realized that it would form a useful way of comparing rapidly the activity of many antagonists against one agonist. All these expressions of activity are limited in their application and serve mainly in the comparison of potencies of different antagonists.

Some authors allowed the antagonist to act for arbitrarily chosen periods of time; thus, Winder *et al.* (1946) introduced the antagonist into the bath 1 min. before the agonist, a time which they regarded as suitable since the results obtained then represented "a combination of speed of penetration to the site of action with the intrinsic intensity of prophylactic action." Miller *et al.* (1948) measured the reduction of the maximal spasm after the antagonist had been present in the bath for 2 min. Rapport and Koelle (1953) measured the percentage reduction of the response produced by equi-active doses of ACh, HT, and histamine after exposure to known concentrations of the antagonists for 5 min., adding the agonist to the bath while the antagonist was still present. In some experiments the agonists were tested at 1 min. intervals in the presence of each other, while in other experiments the bath was washed out with the antagonist between each addition. Thus the period of contact with the antagonist increased during the test, and the authors comment on the significance of the duration of exposure to antago-

nists in such experiments. Owing to the cumulative and persistent action of atropine under these conditions, Rapport and Koelle were unable to demonstrate the gradual development of block with increasing concentration. It is clear from Fig. 2 that the duration of exposure will greatly influence the concentration of antagonist necessary to produce a given degree of depression, a fact clearly stated by Gaddum (1926) and by Schild (1947). The method of superfusion has the clear advantage over other methods in that the time course of antagonism can be followed and the equilibrium state can be appreciated.

The pattern of recovery of response, as illustrated in Fig. 2, has been used as a means of studying antagonists. For example, Rocha e Silva and Beraldo (1948), and Beraldo and Rocha e Silva (1949), investigated this aspect of the response to a standard dose of an agonist after exposure of the tissue to an antagonist for 1 min., the time for 50% recovery being the R50 value. From this type of experiment Rocha e Silva, Valle, and Picarelli (1953) concluded that the atropine antagonism of HT was similar to the antagonism of ACh by atropine because of the similarity of the recovery curves. From our experience the recovery curve seems to be a property of the antagonist itself rather than a reflection of the mode of action of the agonist.

The *p*A value of Schild (1947) is the most satisfactory term by which to express antagonism since it is independent of the concentration of agonist—provided this is submaximal and that the log. concentration/response curves in the presence and absence of the antagonist are parallel (Schild, 1949). Our results are *p*A values at the equilibrium state, which can be readily achieved with superfused tissues; the necessity of arbitrarily choosing the time of exposure to the antagonist is thus avoided.

Antagonism of 5-Hydroxytryptamine by Atropine.—Expressing the antagonism of HT by atropine, both in terms of the *p*A values and by the curve relating the depression of response to the concentration of antagonist, has revealed certain characteristic features. Depression of the response of the ileum to HT to about 60% of the original can be achieved by low concentrations of atropine, similar to those affecting equi-active concentrations of ACh. For complete abolition of response much higher concentrations of atropine are necessary, similar to those required for the abolition of the response to equi-active concentrations of histamine. These findings may reconcile the conflicting views of previous authors regarding this antagonism.

Summary

1. A method is described for the study of drug antagonism using the superfusion technique.

2. This method can be adapted to any of the standard means of expressing antagonism; it has the added advantages over the bath technique of (*a*) exact determination of equilibrium conditions, (*b*) improved viability of the tissue, and (*c*) the possibility of determining the effects of many concentrations of one or more antagonists on the same preparation.

3. In applying this method to the study of the antagonism of HT by atropine, the curve relating the degree of depression of response to the concentration of atropine has been shown to have a characteristic form not previously described. This must clearly be taken into account in any discussion concerning the mode of action of HT upon the isolated guinea-pig ileum.

4. The method has been used to investigate the value of the atropine- and mepyramine-treated guinea-pig ileum as an accurate assay preparation for the determination of HT in mixtures containing ACh and histamine. It is concluded that this preparation is unsuitable except for mixtures containing relatively large amounts of HT.

5. Means of expressing the activity of antagonists are discussed. It is concluded that the curve relating the degree of depression of the agonistic response to the concentration of the antagonist, taken in conjunction with the *p*A value at equilibrium, affords the most comparable way of expressing the activity of one or more antagonists against a group of agonists. The drug ratio, on the other hand, is more useful when comparing the activities of a number of antagonists upon one agonist.

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