THE REACTION BETWEEN ACETYL CHOLINE AND MUSCLE CELLS. Part II.

By A. J. CLARK.

(*Pharmacological Department, University of Edinburgh.*)

Section I. The destruction of acetyl choline by the frog's heart.

In a previous paper(1) the writer described the relation between the concentration and the action of acetyl choline on the isolated heart and on the rectus abdominis of the frog. Loewi and Navratil(2) have since shown that extracts of frog's tissues and particularly extracts of the frog's heart can destroy acetyl choline fairly rapidly. This suggested a possible source of error in the writers' calculations both regarding the relation between concentration and action, and the amount of drug fixed by the heart, and therefore experiments were made to eliminate these errors.

Experimental methods. The following methods were used:

(1) Immersed strip: a strip of ventricle was immersed in Ringer and the isometric response was recorded.

(2) Moist strip: a moist strip of ventricle was suspended in air and its response recorded isometrically. This method was used chiefly to estimate the amount of acetyl choline present in very small quantities of fluid.

(3) Irrigated strip: a strip of ventricle was arranged as above, but was irrigated by a fine jet of fluid driven by compressed air. Two capillary nozzles were arranged so that irrigation either with a solution of a drug or with Ringer's fluid could be alternated rapidly.

(4) Isometric ventricle: the response of the whole ventricle was recorded isometrically. The arrangement used is shown in Fig. 1.

The advantages of method (4) are that any quantity of irrigation fluid from 10 c.c. to 0·1 c.c. can be used, and can be changed quickly. The whole system outside the heart was filled with boiled saline (0·65 p.c. NaCl). The usual initial tension was 3 cm. of water, and this sufficed to produce a rapid diastolic filling. The ventricle was allowed to contract isotonically except for short periods when records were taken, and thus the heart received an adequate amount of aerated fluid.

In all four methods the heart was driven at a regular rate (about 15 per minute) by break induction shocks. The Ringer's fluid had the

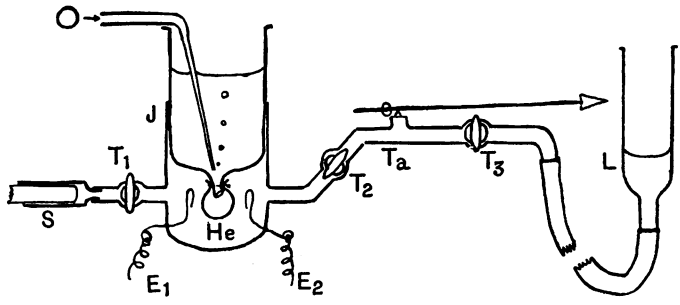

Fig. 1. Apparatus for measuring action of drugs on isometric response of frog's ventricle with varying initial fillings, or varying initial tensions.

He=heart; *O*=oxygen supply; T_1, T_2, T_3=glass taps; E_1, E_2=electrodes; *J*=ground glass joint; *Ta*=small rubber membrane tambour (diameter 5 mm.); *S*=all glass syringe, graduated to 0·01 c.c., for varying initial filling; *L*=moveable level for varying initial tension.

following composition: NaCl 0·65 p.c.; $CaCl_2$ 0·012 p.c.; KCl 0·015 p.c.; sodium phosphate at *p*H 7·5 0·05 p.c. A stock concentrated solution of phosphate at the desired *p*H was used.

The relation between concentration and action of acetyl choline. The irrigated strip method eliminated errors due to destruction of the drug, since the heart cells were irrigated continuously with fresh solution. The curve relating action and concentration obtained with this method was identical with that described in my previous paper(1), which was obtained with the immersed strip method. The only difference was that a given concentration of drug produced a much greater effect with irrigation than with immersion. For instance in one heart where the two methods were tested alternately, a 50 p.c. reduction in the force of contraction was produced by 10^{-7} molar with irrigation, whereas 10^{-6} molar was needed to produce the same effect with simple immersion. This difference depends presumably on the fact that with simple immersion the drug only diffuses slowly into the sponge-like tissue of the ventricle and as it is being broken down there continuously, the actual concentration on the cell surfaces is much less than that in the bulk of the fluid.

The measurements made from strips of ventricle are, however, open to the objection that the force of contraction only represents a small fraction of the maximum force the ventricle can exert. Experiments

were therefore made with the whole ventricle contracting isometrically. When the initial filling was adequate, the maximum systolic tension of a fresh heart was usually 70–90 cm. of water.

This method was found to give satisfactory results and the curves obtained relating concentration and action were the same as those described in my previous paper(1). Fig. 2 shows the results of a typical experiment.

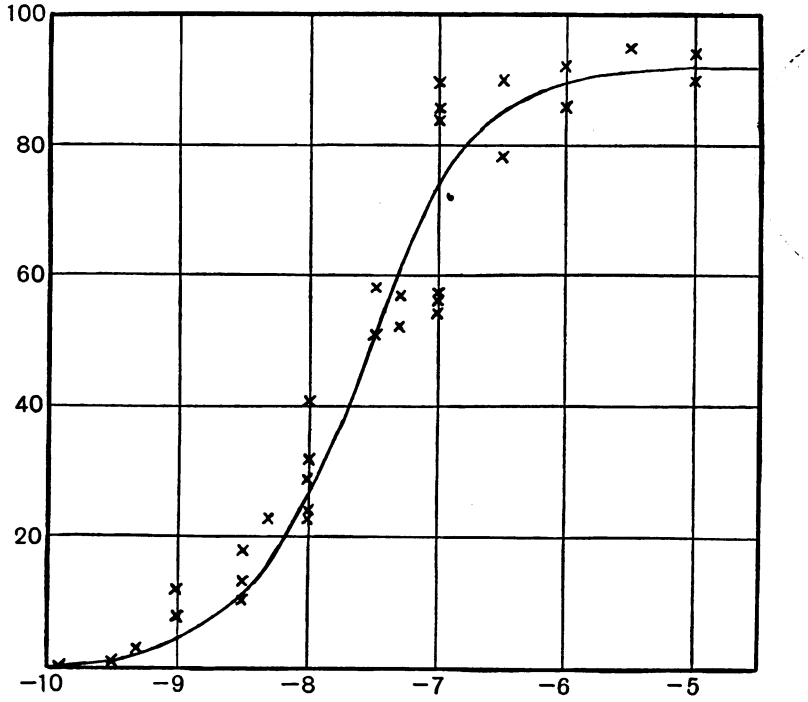

Fig. 2. Action of acetyl choline on the isometric response of the frog's ventricle.

Ordinate (y) = Reduction in force of contraction, expressed as percentage of normal contraction.

Abscissa = Logarithm of (x), the molar concentration of acetyl choline.

The crosses show the observed figures whilst the curve is drawn to the formula $k.x=\frac{y}{92-y}$. ($k=10^{7.6}$.) In the figure "x" is shown on the logarithmic scale.

The results obtained with the improved methods described agree therefore with the results obtained with the simpler strip method, but the more accurate methods of recording show that in most cases acetyl choline does not produce complete arrest of the heart, for usually there

is a small residual contraction which is not abolished even when very high concentrations of the drug are employed.

In my previous paper the formula adopted for interpreting the relation between concentration and action was $K \cdot x = \frac{y}{100-y}$ where K = constant, x = concentration and y = action produced by the drug expressed as p.c. of the maximum possible action: this last was taken to be complete arrest. The more recent methods show that the drug usually fails to produce complete arrest of the heart, however great the concentration, and therefore this formula must be modified to $K \cdot x = \frac{y}{A-y}$, where A = the maximum action the drug can produce, expressed as p.c. of complete arrest. Fig. 2 shows that the observations fit this formula fairly well. In this case the maximum action was taken as 92 p.c. diminution in response.

The destruction of acetyl choline by the heart. When a frog's heart is placed in contact with small volumes (0·5 c.c.) of acetyl choline solutions it recovers rapidly from the initial effects of the drug. This recovery, which is shown in Fig. 3, is quickest with weak solutions and slowest with strong solutions, and is presumably due to destruction of the drug by the tissues as described by Loewi and Navratil(2). In order to estimate the rate of this destruction tests were made of the acetyl choline content of the solution, by removing small drops (0·005 c.c.) and applying them to a moist ventricular strip preparation (method *A*). The response of the strip was standardised by applications of acetyl choline solutions of known concentration and thus a rough estimation of the content of acetyl choline was possible. The smallest concentration that could be detected by this method was about 5×10^{-7} molar. The results showed that acetyl choline disappeared from the solution as the heart recovered, and that the activity of the heart was roughly proportional to the content of acetyl choline remaining in the fluid.

Straub(3) described a similar type of recovery from muscarine in the hearts of aplysia, torpedo and the frog. He showed firstly that in the case of aplysia the drug passed into the heart and was stored there, and secondly that the heart when it had absorbed the drug became tolerant to further applications.

In the case of acetyl choline I was unable to detect any storage of the drug in the frog's heart. To test this point hearts were exposed to excess of acetyl choline (10 c.c. of 10^{-4} molar) for periods of one to twelve hours. At the end of such periods there was still a considerable concentration of drug remaining in the solution, but when the heart

was rinsed with Ringer's fluid and then ground up with sand, no acetyl choline could be detected in the emulsion.

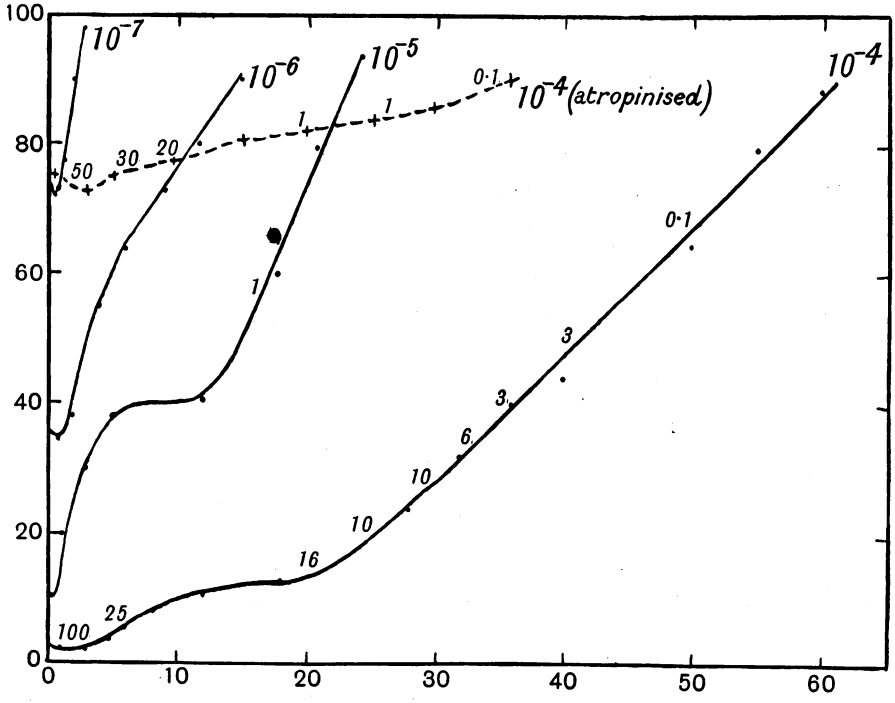

Fig. 3. The destruction of acetyl choline by the frog's ventricle.

Abscissa = Time in minutes.

Ordinate = Isometric contraction expressed as p.c. of normal.

In all cases quantities of 0·5 c.c. were introduced into a ventricle which weighed 100 mgm. (moist weight). The curves show the response of the heart after concentrations of acetyl choline varying from 10^{-7} to 10^{-4} molar, and also the response to 10^{-4} molar acetyl choline after the ventricle had been atropinised.

Drops of fluid were removed at intervals and their content of acetyl choline tested on a ventricular strip. The figures along the curves show the molar concentrations of acetyl choline $\times 10^6$.

The frog's heart acquires a certain amount of tolerance to acetyl choline on prolonged exposure to the drug. This effect, which will be discussed later, is a source of error in the calculation of the rate of destruction of the drug but does not affect the main conclusions that the frog's heart destroys acetyl choline at a fairly rapid rate.

In the experiment shown in Fig. 3, 10^{-7} molar acetyl choline produced a 30 p.c. reduction in the force of contraction of the heart and

the times taken by the heart to recover after varying doses of acetyl choline to a response 70 p.c. of normal, were as follows:

Initial molar concentration of acetyl choline	10^{-6}	10^{-5}	10^{-4}
Time in minutes taken to recover to 70 p.c. of normal response	8	19	52

The heart (which weighed 100 mgm.) therefore took eight minutes to reduce the concentration in 0·5 c.c. from 10^{-6} to 10^{-7} molar, and therefore took $19 - 8 = 11$ minutes to reduce the concentration from 10^{-5} to 10^{-6} molar, and $52 - 19 = 33$ minutes to reduce the concentration from 10^{-4} to 10^{-5} molar. From these figures it is possible to calculate the destruction in gram molecules of acetyl choline per unit weight of heart tissue per minute for a wide range of concentration.

The rate of destruction is fairly uniform for any given concentration in any particular heart, for the time taken for recovery with constant concentration varies as the quantity of the drug. This is shown by the following figures:

Volume in c.c. of 10^{-7} molar acetyl choline solution introduced	0·2	1·0	2·0
Time in minutes to reduce the concentration to 10^{-8} molar	10	45	80

A series of experiments was made in the manner described above to determine the rate of destruction of acetyl choline at varying concentrations. The results obtained are shown in Table I.

TABLE I. Time in minutes taken for reduction of molar concentration of acetyl choline. All figures reduced to common standard of 0·1 c.c. fluid in a ventricle weighing 100 mgm.

Date	10^{-3}–10^{-4}	10^{-4}–10^{-5}	10^{-5}–10^{-6}	10^{-6}–10^{-7}	10^{-7}–10^{-8}
16. xi. 26	—	—	—	5·7	2·8
17. xi. 26	7	6·2	3·3	4·5	2·0
25. xi. 26	—	8	3·5	2·4	1·6
9. xii. 26	6	6·6	2·2	1·6	—

These figures were confirmed by other experiments made upon isolated strips of ventricle to which drops of fluid were added. The rate of recovery of these strips indicated a destruction of acetyl choline at a rate of the same order as that described above. The figures in Table I show that there is a considerable individual variation in the rate of destruction of the drug, but that in all cases the time required to reduce the concentration to one-tenth is three or four times greater with the highest than with the lowest concentrations. This difference is remarkably small considering that the concentration varies ten thousand-fold.

TABLE II.

Range of molar conc. of acetyl choline	10^{-3}–10^{-4}	10^{-4}–10^{-5}	10^{-5}–10^{-6}	10^{-6}–10^{-7}	10^{-7}–10^{-8}
Gram molecules of drug destroyed per minute per mgm. of heart (moist weight)	2×10^{-10} to 10^{-10}	10^{-11} to 3×10^{-12}	4×10^{-12} to 10^{-12}	6×10^{-13} to 10^{-13}	10^{-14} to 2×10^{-14}

The average figures given in Table II show that within the limits of error the log. of the amount of drug destroyed plotted against the log. of the concentration of the drug gives a linear relation, and the relation between the amount destroyed (x) and the concentration (c) can be expressed by the formula $K.c^{1/n} = x$; where $K = 7 \times 10^{-8}$, and $n = 1{\cdot}2$.

The rate of destruction of acetyl choline varied considerably in different hearts, for some hearts destroyed the drug ten times as quickly as others. Variations in the rate of destruction of the drug did not bear any certain relation to variations in the sensitivity of the heart. One abnormal heart was found which was completely insensitive to acetyl choline even in concentrations of 10^{-4} molar, but the rate of destruction in this heart was of the same order as that in normal hearts.

The destruction of acetyl choline after atropinisation is also shown in Fig. 3. In this case the ventricle was exposed to atropine 10^{-5} molar; the atropine was then washed out, leaving the ventricle very insensitive, and the rate of destruction of acetyl choline was tested on a strip of ventricle. The rate of destruction in this case was within the limits of variation of figures obtained with normal hearts.

The fact that destruction of acetyl choline proceeds unaltered both in atropinised hearts and in hearts naturally insensitive to the drug suggests that there is no direct relation between the amount of drug destroyed and the amount of action the drug produces.

Loewi and Navratil(2) showed that emulsions of heart tissue destroyed acetyl choline, and that this action was abolished by heating to 56° C. They also showed that acetyl choline was destroyed by emulsions of liver and gut and to a lesser extent by emulsions of skeletal muscle. I confirmed these results as regards emulsions of the heart, liver, gut and skeletal muscle and also found that frog's serum had as powerful an action in destroying acetyl choline as the frog's heart, for the destruction by 0·001 c.c. serum per minute was of the same order as the destruction by 1 mgm. of moist ventricle per minute. My experiments confirm Loewi and Navratil's conclusion that the destruction of acetyl choline is due to a ferment, and that this ferment is widely

distributed in the frog's tissues, and is not confined to those tissues on which the drug produces a specific action. Many similar ferments that destroy other drugs are known. For example, the frog's heart, liver and serum contain a ferment which destroys atropine (4).

The destruction of acetyl choline by the heart appears to be due to an intracellular ferment, for I found that when 0·2 c.c. of fluid was kept in a heart for two hours and then removed, the fluid had no power to destroy acetyl choline. Moreover, the action is not due to any substance that can be washed out of the heart, for a heart that had been isolated for 24 hours and had its perfusion fluid changed at least one hundred times still retained its full power to destroy the drug.

The amount of acetyl choline reacting with the frog's heart. The writer has calculated (1) the amount of acetyl choline actually reacting with heart cells by comparing the effects of the drug upon a strip of frog's ventricle immersed in a large volume of solution with the effects produced when small quantities of drug are added to the moist ventricular strip. This method showed conclusively that the amount of drug actually reacting with the heart cells must be very small. The fact that the heart cells can destroy acetyl choline fairly rapidly further reduces the possible quantity of drug that can react with the cells.

Table III shows in line 2 the figures calculated in a previous paper (1) for the amount of drug disappearing from solution when the drug acted on heart cells, and in line 3 are shown the quantities which can be attributed to destruction of the drug by the ferment action already described. A comparison of the figures given in Table III shows that by this calculation the destruction of the drug would account for nearly the whole of the drug disappearing at the lowest concentration measured, but that at higher concentrations the amount destroyed is only a small fraction of the quantity that disappears.

TABLE III.

(1) Molar concentration of acetyl choline added	10^{-7}	10^{-6}	10^{-5}	10^{-4}
(2) Gram mols. acetyl choline disappearing per mgm. of dry tissue	5×10^{-14}	6×10^{-12}	$1{\cdot}6 \times 10^{-10}$	$1{\cdot}6 \times 10^{-9}$
(3) Gram mols. of drug destroyed within 1 minute per mgm. of dry tissue	3×10^{-14}	8×10^{-13}	3×10^{-12}	3×10^{-11}

It is of course unjustifiable to assume that the whole of the drug that disappears without being destroyed by the ferment action necessarily takes part in producing the specific action of the drug. A fixation or adsorption of pilocarpine and other drugs by serum and other tissues

on which the drugs exert no specific action has been described (Storm van Leeuwen(5), Beutner(6)).

The figures given in my previous paper for the maximum amount of drug that can possibly react with the tissues to produce the specific action of the drug are therefore too high, and there is a considerable probability that the true figures are very much smaller.

Rate of reaction and wash out of acetyl choline. Experiments with isolated strips upon which a jet of solution was played made it possible to measure approximately the rate of reaction and the rate of wash out of the drug. The following figures were obtained:

Molar concentration acetyl choline	10^{-5}	10^{-6}	10^{-7}
Time of half action	1·4″	2·0″	3·7″
„ full action	4″	6″	9″
„ half wash out	3″	<3″	—

These figures show that the combination between acetyl choline and the tissues occurs very rapidly and that the drug can be removed equally rapidly by washing out.

If the jet of acetyl choline solution be stopped, and the strip is not washed with Ringer, the heart recovers owing to the destruction of the drug. This recovery due to destruction of the drug is a much slower process than washing out, as is shown by the following figures:

Molar concentrations of acetyl choline	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Time for half recovery on washing out	—	<3″	3″	12″	24″
Time for half recovery due to destruction of the drug	10″	12″	25″	80″	117″

The rapidity with which the action of acetyl choline is produced on introduction of the drug, and is removed by washing out, supports the view that the drug acts on the surface of the cells.

Tolerance to acetyl choline. Straub(3) found that when the heart of aplysia was exposed to muscarine the drug was concentrated in the heart cells, and that the heart then became tolerant to the drug. He showed a similar tolerance in the frog's heart and concluded that the action of the drug depended on the difference of concentration without and within the cells. Gasser and Dale(7) found that the rectus abdominis of the frog became insensitive to acetyl choline upon prolonged exposure to the drug.

I observed a partial recovery of the frog's heart after exposure to a constant concentration of acetyl choline. This effect is shown in Fig. 4; in this case the quantity of solution employed was too great for the destruction of the drug by the heart to produce a significant alteration in the concentration. The figure shows that the drug produces its

maximum effect in from 15 to 30 seconds, and that this is followed by a partial recovery which is completed within about 10 minutes. This effect is seen also in Fig. 3, where there is a rapid initial recovery due to tolerance, which is followed by a slower continuous recovery due to destruction of the drug.

Other experiments showed that this tolerance only produced a partial recovery, and that, when a heart was exposed to the drug for periods of some hours, no further recovery occurred after the first ten minutes, provided of course that effects due to destruction of the drug were excluded.

It seems unlikely that this tolerance is due to storage of the drug in the heart, because, as has been previously mentioned, no such storage can be demonstrated, and moreover is very improbable, in view of the power of the heart to destroy the drug.

The full sensitivity of the heart to acetyl choline is recovered rapidly on washing out, and experiments made with rapidly moving drums showed that after washing out for even 30 seconds the full sensitivity of the heart was restored. It appears unlikely that it should be possible to remove the drug from the interior of the cells at this speed.

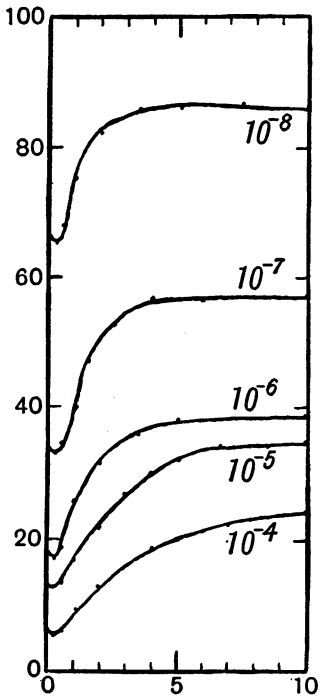

Fig. 4. Response of isometric ventricle after introduction of 10 c.c. acetyl choline solutions of varying concentrations. Ordinate and abscissa as in Fig. 3. The curves are marked to show the molar concentration of acetyl choline.

A partial recovery of activity on the part of tissues exposed to a constant concentration of drug has been observed by the author in the case of other drugs acting on the frog's heart, and also has been described as a feature of the action of adrenaline on a number of tissues. The phenomenon therefore is not peculiar to the case of acetyl choline. I am unable to explain this tolerance effect but my experiments make it improbable that it is due to the entrance of acetyl choline into the cells, as was suggested by Straub.

Another effect which I am unable to explain is that repeated applications of acetyl choline sensitise the heart to the drug. This action is shown by the following figures:

Time since heart isolated	20′	25′	30′	35′	120′
P.c. reduction produced by 10^{-6} molar acetyl choline	72	85	92	94	94

Hearts freshly isolated were found to be less susceptible to acetyl choline than hearts after a few hours' isolation, but it was found very difficult to distinguish with certainty between effects due to prolonged isolation and those due to repeated application of the drug.

In practice errors due to these causes were avoided by always ignoring the first few responses to acetyl choline given by a heart. A similar sensitisation of the heart after repeated administration of a drug, or after prolonged perfusion, occurs with other drugs and therefore this effect is not peculiar to acetyl choline.

Individual variations in sensitivity to acetyl choline. The response of frogs' hearts to acetyl choline is characterised by a remarkable individual variation. Experiments made upon 71 hearts gave the following results:

TABLE IV

Molar concentration of acetyl choline needed to produce 50 p.c. reduction in response		3×10^{-5}	3×10^{-5} to 3×10^{-6}	3×10^{-6} to 3×10^{-7}	3×10^{-7} to 3×10^{-8}	3×10^{-8} to 3×10^{-9}
Number of hearts	(1) Immersed ventricular strip	0	5	22	12	9
	(2) Isometric ventricle	2	0	2	11	7

In the first series the ventricular strip was immersed in the fluid and hence, for reasons already mentioned, the drug produced a more powerful action with the second than with the first method, but in both cases the figures show that the sensitivity of the hearts varies over at least a thousandfold range of concentrations.

In addition to this type of variation in sensitivity to acetyl choline the hearts also varied as regards the maximum effect that could be produced by the drug. In all cases the relation between concentration and action resembled that shown in Fig. 2. A concentration about one hundred times that needed to produce a 50 p.c. reduction produced an almost maximum action, and little further effect was produced however much further the concentration of the drug was raised. The maximum reduction produced was in most cases more than 90 p.c. of the normal beat, but in a few cases lower figures were obtained, and occasionally 60 p.c. reduction was the maximum effect that could be produced, even when the concentration of the drug was increased to several thousand times that sufficient to produce a reduction of 50 p.c.

I am unaware of any other case in which the natural susceptibility

of a tissue to a drug shows a similar range of variation. As far as I could determine the sensitivity of the hearts were not influenced by the season or by the sex of the animal. There are a large number of possible experimental errors that might cause apparent variation in susceptibility, but great care was taken to exclude all possible errors and the variation was found to continue unaltered.

Discussion. The experiments show that acetyl choline is rapidly destroyed by the frog's heart, and that this destruction must occur within or on the surface of the cells, since the ferment does not diffuse out into fluid kept in the heart. The relation between the destruction of acetyl choline and its concentration follows the usual adsorption formula. These facts suggest that the drug is adsorbed on the heart surface and there destroyed. Experiments made to measure the amount of drug adsorbed by the heart failed to show any certain relation between the amount adsorbed and the amount destroyed, but these experiments were of necessity subject to large experimental errors.

My experiments suggest that at least two independent processes occur when acetyl choline is brought in contact with tissues: firstly, an adsorption and destruction of the drug by the tissues, and secondly, a reaction between the drug and certain specific receptors. The latter process which produces the specific action is probably a reaction with receptors on the surface of the cells. The fact that fixation of a drug by cells can proceed independently of its specific action was shown by Cook(8) in the case of methylene blue acting on the frog's heart.

The action appears to be completely reversible since a similar effect can be produced and removed by washing out a hundred or more times on the same heart. Nevertheless, a certain amount of irreversible change occurs for the heart's sensitivity is permanently increased by the first few applications of the drug. On the other hand, when the heart is left in contact with the drug it establishes a certain degree of tolerance because the initial effect produced decreases after a few minutes. These facts indicate that the factors influencing the response of the heart to acetyl choline must be complex.

The curve relating the action of acetyl choline with the concentration of the drug can be explained most simply on the assumption that a freely reversible monomolecular reaction occurs between the drug and a limited number of receptors of uniform sensitivity.

Gaddum(9) found that the relation between the concentration of adrenaline and its action on the rabbit's isolated uterus followed a curve similar to the one shown in Fig. 2, and suggested that the relation might

be due to a frequency effect. The relation cannot be explained simply in such a manner, but it can be interpreted on these lines by assuming that the drug acts on a population of receptors that vary in sensitivity, and that this variation is of an extreme "skew" type such that the distribution of receptors plotted against the logarithm of their sensitivity to the drug gives a distribution curve of the usual type. This hypothesis is mentioned because it would accord with the remarkable variation in the response of individual frogs to acetyl choline shown in Table IV.

Section II. The influence of ions on the action of acetyl choline.

It is well known that the actions both of para-sympathetico-mimetic and of sympathetico-mimetic drugs are influenced by the ionic concentration of the milieu of the tissue upon which they act. This fact, coupled with the resemblance between the effects of vagal stimulation and of excess of potassium, has led to numerous speculations relating vagal action with the action of ions.

Unfortunately the evidence regarding the influence of ions on vagal action in the frog is conflicting. Some of this confusion may be due to the fact that, as shown by Ten Cate(10), the effects produced by the sympathetic and the vagus on the frequency of the frog's heart are not influenced by ionic changes in the same way as are the effects on the force of contraction of the heart. In this paper only effects upon the force of contraction will be considered.

There is a fairly good agreement that the vagus is paralysed by complete lack of potassium (Ten Cate(10)), and by complete lack of calcium (Ten Cate(10), Busquet and Pachon(11), Hagan and Ormond(12)), although Brine(13) denies this latter effect. Such a paralysis does not denote any specific action of these ions on the vagus because ionic changes of this extent also paralyse other nerve endings.

The chief evidence regarding the effect of slighter changes in ionic concentrations is as follows.

Loewi(14) and Kolm and Pick(15) state that the vagal excitability is increased when the calcium concentration is diminished but Asher(16) considers the evidence on this point to be doubtful.

Ten Cate(10) found that excess of calcium antagonised the action of the vagus, although Howell(17), Loewi(14) and Cori(18) found that this ionic change produced no certain action. Zwaardemaker and Lely(19), Asher(20) and Ten Cate(21) all agree that lack of potassium first augments and finally abolishes the action of the vagus. Excess of

potassium has a doubtful action. Ten Cate(10, 22) and Burridge(23) state that it diminishes the vagal action, but some of Ten Cate's figures(10) suggest the reverse effect. Reduction in the sodium chloride content paralyses the vagus (Witanowski(24)). Finally, Andrus(25) found that vagal stimulation produced a greater action on the tortoise heart in neutral than in alkaline (*p*H 8·0) Ringer.

The evidence is equally uncertain regarding the influence of ionic changes on the action of vago-mimetic drugs.

Excess of calcium antagonises muscarine on the frog's heart (Zondek(27), Loewi and Ischisaka(28)), although Loewi(14) had previously denied this, and Bouchaert(26) found that it did not affect the action of pilocarpine. Excess of calcium also antagonises acetyl choline. (Kolm and Pick(15) on frog's heart; Voss(29) on frog's vessels.)

Bouchaert(26) found that lack of potassium inhibited the action of pilocarpine on the frog's heart, and Voss(29) found that excess of potassium augmented the action of this drug on frog's vessels.

Witanowski(24) found that reduction in sodium chloride slightly reduced the action of acetyl choline on the frog's heart.

Andrus(25) found that acetyl choline produced a greater action on the rabbit's auricle at a *p*H of 7·0 than at a *p*H of 8·0. The author(1) stated that the effect of acetyl choline on the frog's heart was unaltered by changes in the reaction, but this conclusion, I have since discovered, was due to a technical error, for the experimental method did not ensure that the heart cells were bathed sufficiently thoroughly with fluid to demonstrate properly the effects of changes in reaction.

Voss(29) found that acetyl choline acted more powerfully on the frog's heart in alkaline solutions than in neutral solutions.

This summary of results shows the variety of opinions that exist regarding the influence of ions on the action of the vagus and of vago-mimetic drugs. Part of the confusion is due to the fact that the vagus, like other nerves, is paralysed by a large excess of potassium or by complete lack of calcium. The effects of such extensive changes cannot therefore be compared with the effects of moderate changes in ionic content. Even allowing for this fact the evidence is too conflicting to provide any certain conclusions.

Experimental methods. The methods described previously in this paper were used. In most cases the effects of changes in ionic content were determined on the isometric ventricle of the frog, using 10 c.c. of fluid. Ventricular strips irrigated with jets of fluid were used to determine the effect of change of reaction, since in this case it was important

to wash away rapidly from the cell surfaces any carbon dioxide that was formed. Ringer's fluid with a phosphate buffer was used for neutral or acid fluids, and borate was used as the buffer for alkaline fluid.

Calcium concentration and action of acetyl choline. Variations in calcium concentration affect the force of contraction of the frog's heart profoundly and this effect is even more apparent with isometric than with isotonic records. The normal Ringer contained one millimolar calcium, and reduction of this to one-quarter reduced the response about 90 p.c., whilst increase to two millimolar about doubled the force of contraction; further increase in calcium concentration produced little further increase in the force of contraction.

Table V shows that increase of the calcium content of the Ringer above normal reduces the sensitivity of the heart to acetyl choline, but that reduction of the calcium content below normal does not alter this sensitivity. The response of the heart when the calcium content was below 0·5 millimolar was so feeble that it was not possible to measure accurately the effect produced by acetyl choline, but no striking change in sensitivity was noted under these conditions.

TABLE V.

Molar calcium content $\times 10^3$		0·5	1	2	4
Molar conc. of acetyl choline $\times 10^8$ required to produce 50 p.c. reduction in response of frog's ventricle	(1)	8	8	—	40
	(2)	2·5	2·5	6·4	—

Although alterations in the calcium content produced considerable alterations in the sensitivity of the heart to acetyl choline, yet the relation between the concentration of acetyl choline and the action produced always followed the formula given with Fig. 2 and the effect of changes in calcium concentration was simply to alter the constant K.

The antagonism between acetyl choline and calcium excess is in accordance with most of the observations made regarding the effect of this ionic change upon the action of vago-mimetic drugs.

TABLE VI

Molar conc. KCl $\times 10^3$	0·5	1·0	2·0	4·0	8·0	12·0
Isometric response as p.c. of normal	188	150	100	77	36	6

Influence of potassium on the action of acetyl choline. The normal Ringer contained two millimolar potassium, and the effect of changes in this concentration are shown in Table VI. These results agree fairly well with figures that the writer previously has obtained with the ventricular strip method (30). The effects produced by changes in the potassium concentration on the response of the heart to acetyl choline

are shown in Table VII. This shows that reduction in the potassium concentration increased the sensitivity of the heart to acetyl choline whilst increase in the potassium concentration decreased this sensitivity.

TABLE VII.

Molar content of potassium $\times 10^3$	0·5	1·0	2·0	4·0
Molar conc. of acetyl choline ($\times 10^8$) which produced 50 p.c. reduction in response	1	3	5	8

Increase in the potassium chloride content above 0·004 molar produced so great a reduction in the force of contraction that it was difficult to measure the effect of acetyl choline, but no striking change in sensitivity to acetyl choline was observed with these higher concentrations of potassium. The fact that decrease in potassium increases the action of acetyl choline accords with the results of various workers who have shown that this change increases the sensitivity of the frog's heart to vagal stimulation.

The influence of reaction on the response to acetyl choline. Changes in hydrogen ion concentration produced a very marked effect on the isometric response of the whole ventricle. The carbon dioxide produced by the heart cells was a possible source of error since it tended to alter the reaction of the fluid in contact with the cells; therefore experiments were also made with ventricular strips irrigated with a jet of fluid. The two types of experiments gave concordant results and typical figures are shown in Table VIII.

TABLE VIII

Hydrogen ion concentration $\times 10^9$	0·1	1·0	6	30	60	300	600
Isometric response in cm. water	10	60	40	28	19	10	2

Experiments both with the strip method and with the whole ventricle showed that decrease in the hydrogen ion concentration antagonised the action of acetyl choline. Typical results are shown in Table IX.

TABLE IX.

Hydrogen ion concentration	10^{-7}	$1{\cdot}6 \times 10^{-8}$	10^{-9}
Molar concentration of acetyl choline $\times 10^8$ producing 50 p.c. inhibition	2	3·2	8

Accurate results could not be obtained with solutions with a *c*H above 10^{-7} because the heart beat feebly in such solutions, but the experiments showed that acidity did not alter the action of acetyl choline to any striking extent.

These results confirm the conclusions of Andrus(25) (acetyl choline on rabbit's auricle) but are opposed to those of Voss(29) (acetyl choline on frog's heart).

The figures given above do not show adequately the full changes produced by alkalinity on the response to acetyl choline. Not only does the heart become less sensitive as measured by the concentration needed to produce 50 p.c. of maximum inhibition, but also the maximum inhibition that can be produced by acetyl choline is reduced.

This difference is shown clearly in Fig. 5, which shows that when

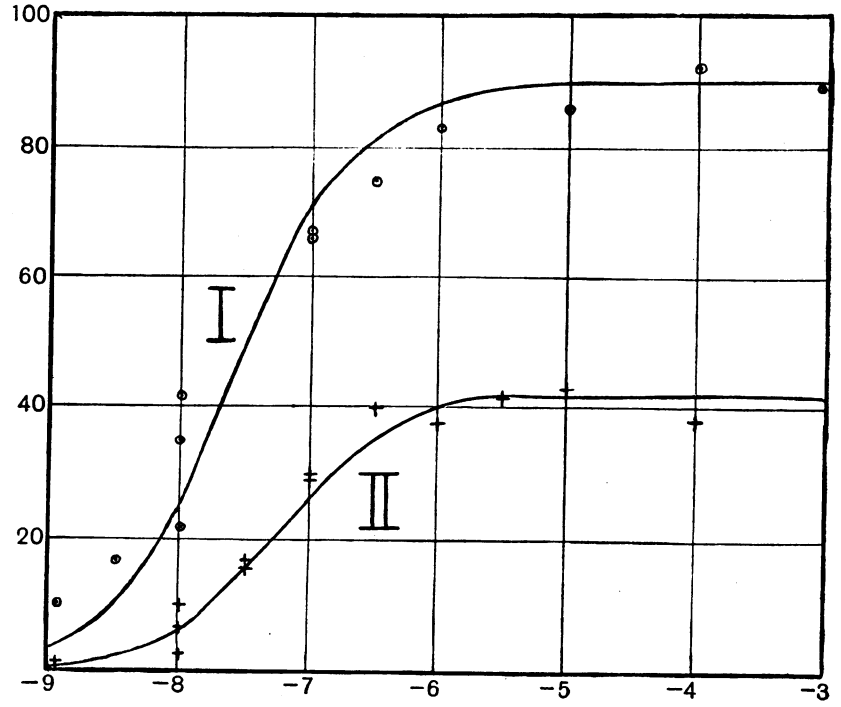

Fig. 5. Influence of reaction on the action of acetyl choline.

Ordinate and abscissa as in Fig. 2.

The crosses show the figures observed, whilst the curves were drawn to the following formulae. (x and y as in Fig. 2.)

Curve I. Phosphate buffer pH = 7·5. Formula $k.x = \frac{y}{90-y}$. (k = 40,000,000.)

Curve II. Borate buffer pH = 9·0. Formula $k.x = \frac{y}{42-y}$. (k = 20,000,000.)

the heart was perfused with Ringer pH 7·5 the maximum effect produced by acetyl choline was a reduction of 90 p.c. in the response, but that when the pH was 9·0 acetyl choline only produced a 40 p.c. reduction in the response.

Figures from other experiments showing the same effect are given in Table X.

TABLE X

Hydrogen ion concentration	Maximum reduction in response produced by acetyl choline (1)	(2)	(3)	(4)	(5)	Average
10^{-7} to 3×10^{-8}	100	95	90	95	—	95
$1{\cdot}6 \times 10^{-8}$ to 6×10^{-9}	65	92	50	90	90	77
10^{-9}	—	80	50	—	42	57

Increased alkalinity therefore makes a certain proportion of the heart cells completely immune to acetyl choline.

Discussion. My results show that the intensity of action of acetyl choline on the frog's heart is increased by reduction in the potassium content, and that it is reduced by increased potassium or calcium content or by increased alkalinity. As far as could be ascertained no marked effect was produced by decrease of calcium or by increased acidity.

Since acetyl choline is destroyed by the frog's heart, experiments were made to determine whether ionic changes altered the rate of destruction of the drug, since such alterations would be a possible cause for changes in the sensitivity of the heart to the drug. These experiments however all gave negative results.

The effects produced by ionic changes on the action of acetyl choline are of course of particular interest if we accept the hypothesis of Loewi(2) that stimulation of the vagus causes the release of acetyl choline, since in this case the changes observed in the action of acetyl choline should throw light on the relation between vagal action and the action of ions. The antagonism of acetyl choline by excess of calcium and by increased alkalinity is in accordance with the majority of observations regarding the effect of these changes on vagal action. These effects are moreover such as would be anticipated because excess calcium and increased alkalinity produce effects on the frog's heart almost exactly the opposite of those produced by either acetyl choline or by vagal stimulation. The effects produced by alterations in the potassium content on the action of acetyl choline are much more difficult to understand.

Diminution of potassium concentration definitely increased the action of acetyl choline, and several observers have shown that this ionic change also increases the action of the vagus. Increase in potassium content antagonised the action of acetyl choline, but the evidence regarding the influence of this change on the vagus is indecisive.

These effects are the opposite of those that might have been anticipated. In the first place excess of potassium depresses the contractility of the heart in a manner very similar to vagal stimulation, whilst lack of potassium produces a systolic effect somewhat resembling sympathetic stimulation. In the second place calcium and potassium are supposed to be antagonists and yet variations in these two ions produce a similar effect on the response to acetyl choline or to vagal stimulation. Calcium and potassium are therefore not antagonists as regards their effects on the response of the heart to acetyl choline. Other evidence is available showing that calcium is an imperfect antagonist of potassium. For example, calcium lack and potassium excess produce different changes in the electrical response of the frog's heart (Daly and Clark(31)), and on conduction in the tortoise auricle (Seliskar(32)). Moreover changes in the content of one ion can only be antagonised as regards their action on contractility over a fairly small range of concentrations (Clark(30)).

The effects produced by ionic changes on the response of the heart to acetyl choline and to vagal stimulation can be explained on the hypothesis that these latter actions are dependent upon the perfusion out from the heart of potassium. Howell and Duke(33) showed that vagal stimulation liberated potassium from the mammalian heart. This was denied by Hemmeter(34) who worked with elasmobranch hearts, but was confirmed by Asher(20) who used the frog's heart.

The passage out of potassium from the heart should be antagonised by excess of potassium in the Ringer and should be favoured by lack of potassium. The writer(35) has shown that perfusion of the frog's heart with potassium free Ringer results in a loss of potassium from the cells.

On the other hand, this loss of potassium would be antagonised by any change that made the cell wall less permeable, and excess of calcium and increased alkalinity are believed to produce this effect. Hence this hypothesis would explain why the two last mentioned ionic changes antagonise acetyl choline. Unfortunately this hypothesis is very difficult to reconcile with the fundamental fact that lack of calcium, excess of potassium and acetyl choline all produce a very similar depression of the contractility of the frog's heart.

My experiments support Ten Cate's(10) conclusion that the action of the vagus or of vago-mimetic drugs cannot be identified with the action of potassium in any simple manner, but that nevertheless there appears to be some special connection between vagal action and the distribution of potassium in the heart cells and in the surrounding fluids.

Conclusions.

Part I.

1. The relation between the concentration of acetyl choline and its action on the frog's heart has been tested with a variety of experimental methods and in all cases the same relation has been found as was described in a former paper.

2. The destruction of acetyl choline by the frog's heart resembles a ferment action and the relation between the amount destroyed in unit time by unit weight of tissue (x) and the concentration (c), over a range of concentrations from 10^{-3} to 10^{-8} molar, is given by the formula $K.c^{1/n} = x$.($K = 7 \times 10^{-8}$ and $n = 1{\cdot}2$).

3. Acetyl choline combines with or can be washed out of the heart in a few seconds, and the rate of action and rate of wash-out are of a similar order.

4. The presence of acetyl choline cannot be demonstrated within heart cells after prolonged exposure to the drug.

5. The amount of acetyl choline that produces the specific action of the drug is probably considerably smaller than the maximum amounts previously calculated.

6. The individual susceptibility of frogs' hearts to acetyl choline varies over a remarkably wide range.

Part II.

The action of acetyl choline on the frog's heart is modified by changes in the ionic content of the Ringer's solution.

The chief changes produced are as follows:

The action of acetyl choline is reduced by increased calcium or potassium content or by increased alkalinity.

The action of acetyl choline is increased by decreased potassium content.

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