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THE ACTION OF ACETYLCHOLINE ON THE RABBIT AURICLE*

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The problem of the exact site and mechanism of action of acetylcholine on cardiac muscle is a difficult one. It is probable that it will eventually be solved, not by one experiment, but by the gradual accumulation of accurate quantitative evidence and a consideration of this information against a physico-chemical background. It is doubtful whether there is as yet sufficient accurate information to solve this problem basically. The present investigation was an attempt to obtain such evidence as may be useful in the future for this general synthesis. Some slight advance has been made in this investigation and some confused points have been cleared up, but the ultimate answers are still far distant. It is only by a many-sided attack that we shall ultimately be able to solve such complex and fundamental problems, and it is hoped that the results of the present work will contribute to the final solution.

METHODS

The methods used and the manner of reporting the results are identical with those described in a previous report (Webb, 1950). All experiments were performed on rabbit auricles beating spontaneously in Tyrode solution at 30° C. The concentrations of such substances as acetylcholine and adrenaline are given on the weight/volume basis since this is customary, but it is felt that for many considerations the molarities should be used. For convenience, the equivalent molarities for the salts used are given here. Acetylcholine chloride ($1:10^7 = 5.5 \times 10^{-7}$ M), carbachol chloride ($1:10^7 = 5.4 \times 10^{-7}$ M), choline chloride ($1:10^4 = 7.2 \times 10^{-4}$ M), pilocarpine nitrate ($1:10^6 = 3.7 \times 10^{-6}$ M), physostigmine sulphate ($1:10^6 = 3.1 \times 10^{-6}$ M), adrenaline hydrochloride ($1:10^7 = 4.6 \times 10^{-7}$ M), and atropine sulphate ($1:10^6 = 3.0 \times 10^{-6}$ M).

RESULTS

Action of a standard concentration of acetylcholine on the auricle

The concentration of acetylcholine chloride usually employed in these experiments was 10^{-7}. This concentration produced in most auricles a definite depression of both the rate and the amplitude. During the course of the year, 260 tests with this concentration were made on the normal auricles from 39 rabbits; the mean percentage changes and their standard deviations were: rate, -14.2 and ± 9.51; ampli-

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tude -51.2 and ± 10.95. The distribution diagrams are shown in Fig. 1. The changes in rate and amplitude measured were the maximum effects produced by acetylcholine. In all auricles the effect upon the amplitude was greater than upon the rate. The

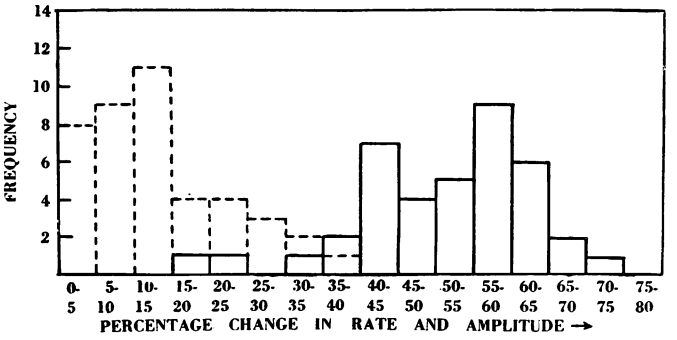

FIG. 1.—Frequency distribution diagrams for the changes in the rate and amplitude brought about by the addition of acetylcholine to normal rabbit auricles. – – – – – rate. ——— amplitude.

ratio of the percentage change in amplitude to the percentage change in rate (A/R) indicates that the amplitude was depressed about 3.6 times as much as the rate by acetylcholine at this concentration. It will be shown later that this ratio is of some significance in determining the relative activity of cholinesterase in the pacemaker cells and in the auricular contractile cells.

There was marked variation between the auricles from different rabbits in the responses of rate and amplitude to acetylcholine. This variation in response to acetylcholine has been observed in other tissues (see Clark, 1937, for references). The rate response varied more than the amplitude response. The standard deviation in the rate was 67 per cent of the mean, whereas the standard deviation in the amplitude was only 21 per cent of the mean, indicating that variation in the rate response was roughly three times as great as in the amplitude response. The ratio of the highest to the lowest concentrations of acetylcholine required to produce a standard depression of the rate was found to be approximately 300 in the series of auricles investigated, while the same ratio for the amplitude response was found to be about 40. Although there was not a sufficient number of experiments with pilocarpine, carbachol, or potassium to compare the results statistically with the acetylcholine variation, there seemed to be much less variation in the response of the auricles to these substances. In several experiments there was less variation in response to acetylcholine in the presence of physostigmine than when the acetylcholine was acting alone. Such results would indicate that variation in cholinesterase activity may contribute to the total variation in response to acetylcholine and this conclusion was substantiated in other phases of this work. The individual variations in rate and amplitude responses were also relatively independent, the ratio A/R varying widely between different auricles.

These deviations in response to acetylcholine were not correlated with seasonal changes as has been reported by several workers, particularly in frog hearts (see Cori, 1921, for early references; Applerot, 1930; Singh, Sehra, and Singh, 1945). The average responses during the four seasons are shown in Table I. It is very doubtful whether the small deviations are significant. However, it is not to be

TABLE I

ACTION OF ACETYLCHOLINE DURING THE VARIOUS SEASONS

Season	Animals	% change in rate	% change in ampl.
Autumn	7	−21.6	−53.7
Winter	10	−11.5	−50.0
Spring	11	−13.5	−49.9
Summer	11	−12.5	−51.9

expected that the tissues of the homoiothermic rabbit would show as marked seasonal changes as those of the poikilothermic frog.

The response of the auricles to acetylcholine was relatively constant for several hours after placing the tissue in the bath, but there was a tendency for the sensitivity to increase slightly with time. Such behaviour in the rabbit auricle was reported by Pines (1934). The increase in sensitivity observed here was never more than 10 per cent. It is possible that this is correlated with a steady decrease in the formation and release of acetylcholine in the tissue, inasmuch as endogenously produced acetylcholine will induce some development of tolerance to acetylcholine and thereby a decreased sensitivity.

Acetylcholine occasionally produced disturbances in the rhythm, the most common being the sudden appearance, as the rate and amplitude were falling, of an alternating type of beat in which the stronger contractions were of much greater amplitude than would have been expected if the rhythm had remained normal. The auricles from one animal were completely useless for quantitative investigation because of the development of such arrhythmias each time acetylcholine was added. On the other hand, if the auricles were beating abnormally in alternating rhythm, the addition of acetylcholine usually stopped this irregularity and allowed a more normal series of contractions.

Relation between concentration and action of acetylcholine

The concentration-action curve of acetylcholine for the normal rabbit auricle is typically sigmoid and similar in shape to the curves obtained on the hearts of other animals (Clark, 1927 ; Barlow, 1928 ; and others). The range of concentration over which acetylcholine acts is wide, the ratio between the maximal and minimal effective concentrations being in the rabbit auricle between four and ten thousand with respect to the amplitude response. This ratio is probably somewhat greater with respect to the rate response, but the actual value is impossible to determine inasmuch as the auricles were stopped when the amplitude dropped to zero, the pacemaker cells in all probability continuing to discharge impulses. In the presence of physostigmine (10^{-6}) the curves were shifted and their shapes changed. Not only were the auricles made ten to a hundred times more sensitive to acetylcholine, but the range of concentration over which acetylcholine acted was markedly shortened so that the ratio between maximal and minimal effective concentrations was now about two hundred. These results are illustrated in Figs. 2 and 3, where the effect of physostigmine on the concentration-action curves of acetylcholine may be seen.

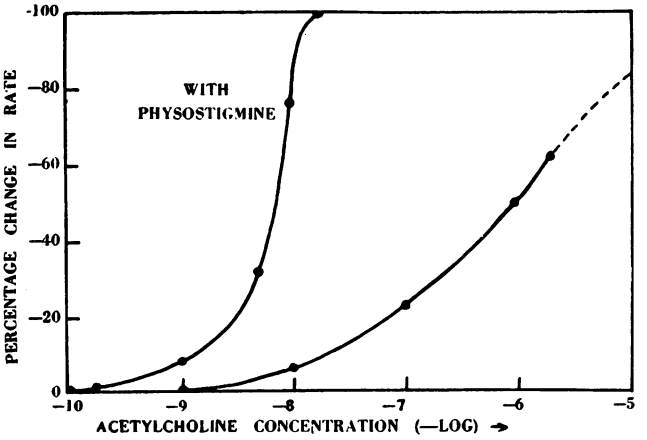

FIG. 2.—Concentration-action curves for acetylcholine on auricular rate: the right-hand curve is for a normal auricle, the left-hand one for the same auricle in contact with 10^{-6} physostigmine.

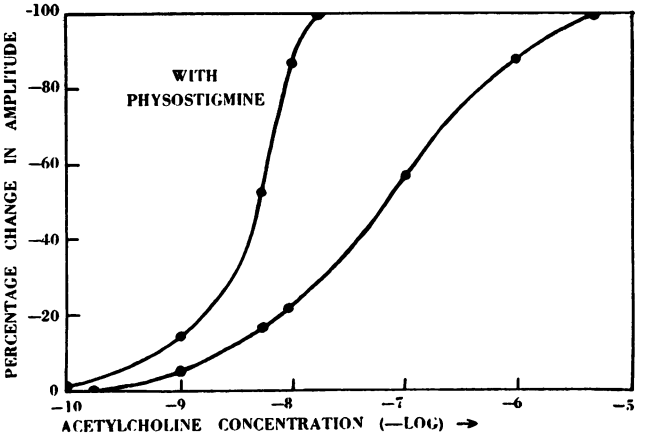

FIG. 3.—Concentration-action curves for acetylcholine on auricular amplitude: the right-hand curve is for a normal auricle and the left-hand one for the same auricle in contact with 10^{-6} physostigmine.

The marked effect of physostigmine on the response of rabbit auricles to acetylcholine indicates that cholinesterase plays a very important role in determining the sensitivity of the cardiac tissue to acetylcholine, the shape of the concentration-action curve, the difference in response between rate and amplitude, and, in general, in determining all quantitative relations between acetylcholine and the heart. The ratio A/R is 3.60 in normal auricles, but in the presence of physostigmine this ratio dropped to 1.61 (average of determinations on five pairs of auricles where the effect of the acetylcholine was approximately equivalent to that of a concentration of 10^{-7} in the absence of physostigmine). This change may be visualized by comparing the concentration-action curves for rate and amplitude responses in the presence of physostigmine. The curves are much closer together than before the addition of the physostigmine, the effect of acetylcholine on the rate and amplitude becoming quite comparable.

The most likely explanation of these phenomena is that the cholinesterase is effectively reducing in some manner the concentration of acetylcholine in the region

of the receptor groups with which acetylcholine combines to produce its typical inhibitory effect. One may visualize a screen of cholinesterase that hydrolyses a certain fraction of the acetylcholine molecules as they diffuse through, and because of this the concentration of acetylcholine beyond this screen, that is in the region of the receptor groups, is much lower than the concentration outside. It is also possible that the cholinesterase molecules are in close juxtaposition to the acetylcholine receptor groups and thereby reduce the concentration in the immediate vicinity of these groups. When acetylcholine is present in the medium at a concentration of C_m it is in dynamic equilibrium with the acetylcholine in the receptor group region, the concentration of which we shall call C_r. In normal auricles $C_m > C_r$, but when the cholinesterase is inhibited by physostigmine we may assume that $C_m = C_r$ approximately. From the curves in Figs. 2 and 3 we may calculate roughly the concentrations of acetylcholine in the receptor group regions when the external concentrations are at various levels. The curves in Figs. 2 and 3 are not average curves but represent the situation in the auricles from one rabbit; thus, the following calculated results are not to be considered as generally applicable. However, comparable relationships probably occur in all auricles, the difference in the behaviour of the auricles to acetylcholine being determined in large part by the effectiveness of the cholinesterase. In Table II are given the calculated concentrations of acetylcholine near the receptor groups at various levels of acetylcholine

TABLE II

CALCULATED CONCENTRATIONS OF ACETYLCHOLINE IN RECEPTOR GROUP REGIONS

External concentration C_m	Concentration at ampl. receptors $(C_r)_a$	Ratio $C_m/(C_r)_a$	Concentration at rate receptors $(C_r)_r$	Ratio $C_m/(C_r)_r$
10^{-9}	3.2×10^{-10}	3.1	2.0×10^{-10}	5.0
10^{-8}	1.6×10^{-9}	6.3	7.8×10^{-10}	12.8
10^{-7}	5.6×10^{-9}	17.9	2.9×10^{-9}	34.5
10^{-6}	1.1×10^{-8}	90.8	7.1×10^{-9}	141.0

in the medium. We must consider the rate receptors and the amplitude receptors separately, for the degree of effectiveness of the cholinesterase is quite different in the two situations. The concentration of acetylcholine is always less at the rate receptors than at the amplitude receptors in normal auricles, owing to the cholinesterase, but from the fact that the ratio A/R is 1.61 when physostigmine is present it is probable that the difference in response between rate and amplitude to acetylcholine is partly inherent in the sensitivity of the two mechanisms. In other words, there is a basic difference in rate and amplitude sensitivity, but this difference is accentuated by the presence of the cholinesterase. In Table II it will be seen that the ratio between the concentrations of acetylcholine in the medium and in the receptor group regions rises as the concentration of acetylcholine is increased. This is one reason why the effective concentration range of action of acetylcholine is so wide, the internal concentration not rising proportionately with the external concentration.

A striking proof of this action of cholinesterase in protecting the receptor groups from acetylcholine is afforded by the following experiment. If we add acetylcholine

at a concentration of 10^{-7} to a pair of auricles and allow them to reach a steady state of equilibrium, the rate and amplitude remaining reasonably constant and somewhat depressed below the normal level, we can demonstrate that cholinesterase is preventing the action of the acetylcholine by simply adding some physostigmine (10^{-6}). The auricles stop immediately. Owing to the inhibition of the cholinesterase, the concentration of acetylcholine at the receptor groups has been suddenly raised to a sufficient level to produce complete inhibition of the auricles.

If it is true that cholinesterase is partly responsible for the great width of the effective concentration range of acetylcholine, we should expect to find that other similarly acting cardiac depressants that are not destroyed by cholinesterase would have much narrower ranges of action. Concentration-action curves for carbachol and potassium acting on the rabbit auricle are given in Fig. 4, and it is evident that

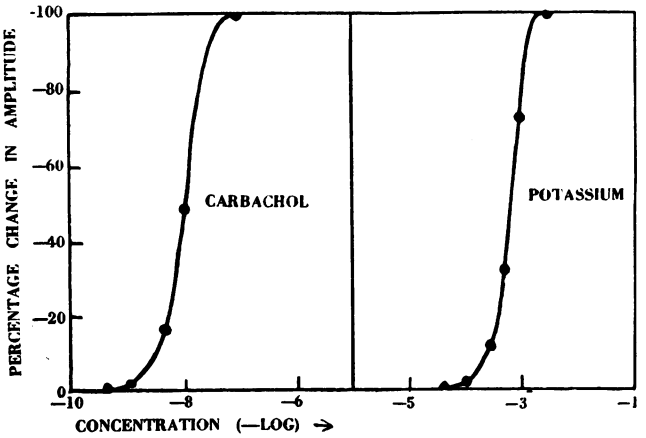

FIG. 4.—Concentration-action curves for carbachol and potassium on the normal auricular amplitude.

these curves are much steeper than the acetylcholine curves and, in fact, correspond quite closely in shape to the acetylcholine curve in the presence of physostigmine when the cholinesterase is inactive. It will be seen that when carbachol is compared with acetylcholine in the presence of physostigmine, it is about 1/3 as potent on the rabbit auricle, a figure quite comparable to that observed by Sachs (1937).

A possible complication in the interpretation of acetylcholine concentration-action curves is the ability of acetylcholine to stimulate cardiac tissue under certain conditions (Barlow, 1928 ; Dutant, 1937 ; Sachs, 1937 ; Spadolini and Domini, 1940a, b ; Hoffmann, Hoffmann, Middleton, and Talesnik, 1945 ; McDowall, 1946 ; Vane, 1948 ; Burn and Vane, 1949 ; and others). No stimulation of the rabbit auricle by low concentrations of acetylcholine was ever observed in the present experiments. Occasionally with the usual concentrations of acetylcholine a temporary and mild stimulation was seen before the depression occurred, but this had no effect on the ultimate degree of inhibition produced. In the presence of atropine (10^{-5}) and physostigmine (10^{-5}), a few auricles were slightly stimulated by high concentrations of acetylcholine ($10^{-5} - 10^{-4}$), but this was not the rule. On normal, fresh auricles, the stimulating action of acetylcholine seems to be quite unimportant until such high concentrations are reached. It was thus concluded that the concentration-action

curves were not influenced by such actions of acetylcholine under normal circumstances. In older or exhausted auricles the situation may well be different.

Variations in the ratio A/R

The ratio A/R represents the relative actions of acetylcholine on the amplitude and rate of the rabbit auricle. We have seen that it is dependent on at least two factors: (*a*) the inherent relative sensitivities of the two systems and (*b*) the differential activities of cholinesterase in the two regions. The ratio A/R is by no means a constant for a particular pair of auricles, but varies with the conditions under which the acetylcholine is acting. We shall discuss some of the general variations in this ratio and later we shall have occasion to observe how other factors may modify it.

The variation of the ratio A/R *with time*

The A/R ratios that are presented in this paper were calculated from the maximal effects of acetylcholine on the auricles. As will be shown in the next section, after maximal depression the amplitude begins immediately to recover in the presence of acetylcholine while the rate remains relatively depressed. This will produce a steady decrease in the ratio A/R after the point of maximal action. It is likely that the earliest actions of acetylcholine on the auricles show an A/R ratio that is higher than the figures given, for it seems that the effect on the amplitude occurs more rapidly than the effect on the rate.

The effect of acetylcholine concentration on the ratio A/R

Table III shows the effect of the concentration of acetylcholine on the relative responses of rate and amplitude. It is quite clear that as the concentration increases there is a decrease in the ratio A/R. At very low concentrations of acetylcholine A/R

TABLE III

VARIATION OF A/R WITH ACETYLCHOLINE CONCENTRATION

Acetylcholine concentration	Ratio A/R	Acetylcholine concentration	Ratio A/R
1×10^{-9}	∞	5×10^{-7}	2.24
5×10^{-9}	∞	1×10^{-6}	1.71
1×10^{-8}	11.0	2×10^{-6}	1.55
5×10^{-8}	6.20	5×10^{-6}	(1.00)
1×10^{-7}	2.93		

is infinity, for there are concentrations that produce a measurable effect only on the amplitude. On the other hand, at high concentrations the ratio will eventually reach 1.0 at a time when both rate and amplitude fall to zero ; however, this is an artefact, for it is likely that the pacemaker cells are still discharging after the amplitude has been reduced to zero. This change in A/R with concentration is due to the different shapes of the concentration-action curves for rate and amplitude and thus primarily depends on the relative activities of cholinesterase at the two sites, for

when the cholinesterase is inhibited the two curves are brought closer together and the ratio A/R is quite uniformly between 1.5 and 1.8.

The ratio A/R *for other cardiac depressants*

If the high A/R ratio of acetylcholine action is mainly due to the activity of cholinesterase, we should expect that substances not destroyed by this enzyme would exhibit lower ratios. This is what happens (Table IV); the ratios of A/R all lie

TABLE IV

THE RATIO A/R FOR OTHER CARDIAC DEPRESSANTS

Substance	Concentration	% change in rate	% change in ampl.	A/R	Auricles
Pilocarpine ..	10^{-6}	−32.9	−44.8	1.36	9
Choline ..	10^{-4}	−17.0	−20.0	1.18	1
Carbachol ..	10^{-8}	−32.3	−50.9	1.57	4
Carbachol ..	10^{-7}	−71.0	−87.5	1.23	2
Potassium ..	0.01 M	−34.0	−57.5	1.69	4

between 1.2 and 1.7 which is very similar to the range of A/R when acetylcholine acts in the presence of physostigmine. It is difficult to calculate exactly the A/R ratios for these substances because they act slowly and frequently the rate and amplitude change independently of each other. The figures in the table were calculated from the maximal changes in the rate and amplitude produced by the substances.

The time course of the action of acetylcholine

The average response of rabbit auricles to acetylcholine (10^{-7}) is plotted against the time in Fig. 5. There are four phases to the action: (1) a slight, temporary stimulation of the amplitude without change in the rate; (2) a rapid depression of rate and amplitude; (3) an immediate and slow recovery of the amplitude to a

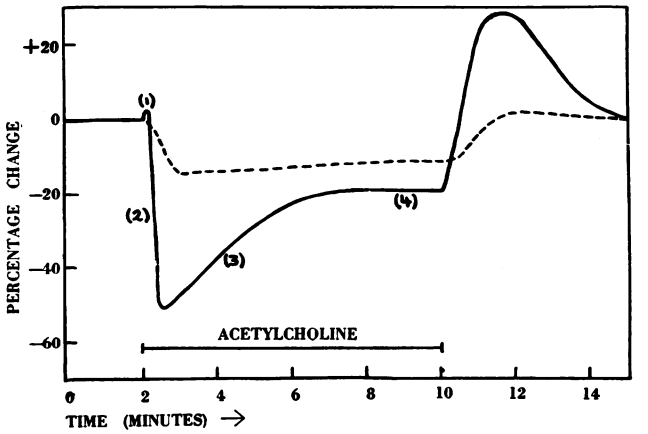

FIG. 5.—The time course of action of acetylcholine on the rabbit auricle, showing the four phases of action and the stimulation observed when the acetylcholine is washed from the bath. - - - - - - rate. ——— amplitude.

certain value, the rate recovering not at all or only slightly; and (4) a stationary phase of equilibrium during which the auricles remain relatively constant in a state of depression as long as the acetylcholine is present.

The brief initial stimulation of the amplitude was observed in only a certain fraction of the auricles. It was very seldom present in fresh auricles but developed with time, a fact noted by Graham (1949). It seemed to be somewhat more pronounced at higher temperatures. Atropine (10^{-6}) blocked it effectively. Graham (1949) reported that a low potassium concentration in the medium increased and prolonged this initial stimulation of rabbit auricles by acetylcholine. Although this point was not specifically investigated here, in going back over the tracings I find that in the present work neither high nor low concentrations of potassium had any marked effect on this phenomenon. Changes in the calcium concentration also had no demonstrable effect. In fact, no modifications of note were observed with any of the substances used to alter the response to acetylcholine (see Table X for a list of these substances), although in some instances this stimulation was not marked enough to be measured quantitatively. This stimulation in the amplitude usually amounted to 3–4 per cent and the duration was only 1–3 seconds or 2–5 beats. Such a stimulation was seen with other cardiac depressants. It occurred particularly clearly with potassium itself, the stimulation being more marked and more lasting than with acetylcholine. Pilocarpine and carbachol also produced initial stimulations that were in nature much like those produced by acetylcholine. This stimulation occurs only in the amplitude response and an increase in rate was never observed. It is doubtful whether this initial stimulation is related in any way to the stimulation of the auricles by acetylcholine in the presence of atropine, or to the stimulation of the exhausted auricles by acetylcholine.

The maximal depression of the auricles was usually reached between 20–40 seconds after addition of the acetylcholine and there followed immediately the initiation of spontaneous recovery in the presence of acetylcholine. It was observed that the recovery in the rate of beating was much slower than the recovery of the amplitude, this phenomenon being clearly shown in the curves for acetylcholine at 10^{-7} in Fig. 6. This spontaneous recovery occurred at a rate dependent on the con-

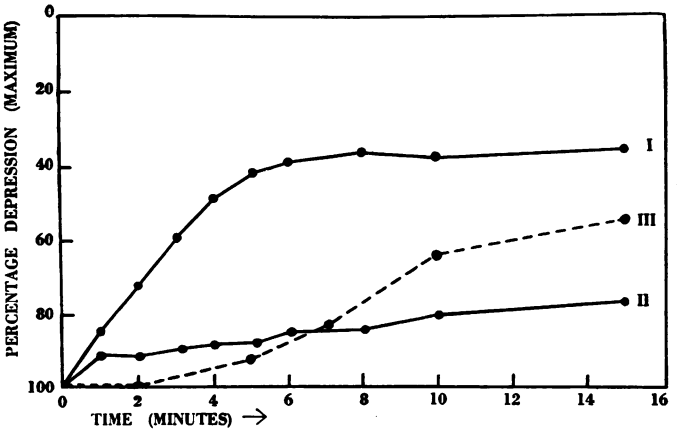

FIG. 6.—Recovery of auricles in the presence of acetylcholine, the maximum depression obtained being represented by 100. Curves for acetylcholine alone are average curves from ten auricles; physostigmine curve is an average of three experiments. I, Amplitude - acetylcholine (10^{-7}). II, Rate-acetylcholine (10^{-7}). III, Amplitude-acetylcholine (5×10^{-9}), physostigmine (10^{-6}).

centration of acetylcholine present in the medium as shown in Fig. 7. Such recovery processes must be of importance in the spontaneous recovery of hearts subjected to continuous vagal stimulation, since perfusion experiments have made it unlikely that there would be any immediate depletion of acetylcholine at the vagal nerve endings.

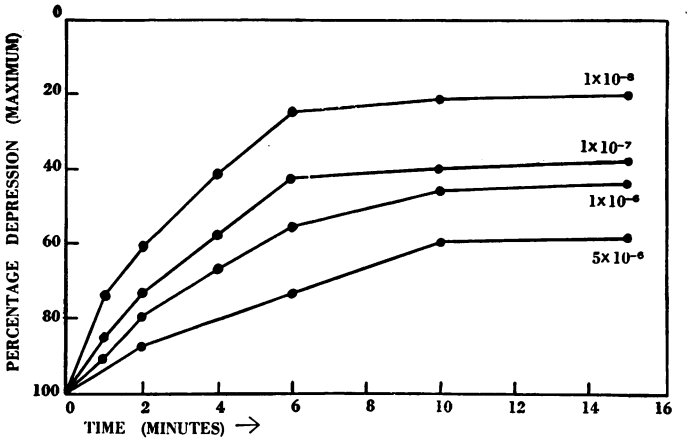

FIG. 7.—Recovery of the auricular amplitude in the presence of different concentrations of acetylcholine, the maximum depression produced being represented by 100 in each case. All curves from the same auricle.

Recovery of Aplysia hearts in the presence of muscarine was the initial observation that resulted in the formulation of the " potential theory " by Straub (1907) and such behaviour has been repeatedly observed with substances of this group (de Espanes and Martinez, 1939 ; Lendle, 1941), although the " potential theory " has been generally abandoned as the correct explanation. Needless to say, in the present work it was found that there was marked variation in the rates of recovery in different auricles and all comparative results, such as those shown in Fig. 7, were obtained from the same auricles. That the ratio A/R changes with time is obvious from an examination of Fig. 6, the ratio progressively becoming smaller after the maximum depression owing to the marked increase in amplitude with little recovery in the rate.

It was observed that recovery of the auricles in the presence of acetylcholine was not complete, the recovery curves approaching asymptotically some constant value. The final level reached was dependent on the concentration of acetylcholine ; the adjustment or adaptation made was characteristic for each concentration. In other words, an equilibrium was eventually established ; if more acetylcholine was added there was recovery to a new level. This behaviour was observed in the frog heart by Clark (1927). This is one of the phenomena that is difficult to explain by means of the " potential theory." The shapes of these recovery curves would also argue against the theory that destruction of acetylcholine by cholinesterase was the primary factor involved. However, it is easy to test the importance of the cholinesterase by determining the degree of recovery from acetylcholine depression in the presence of physostigmine. In Fig. 6 has been included such a curve averaged from several experiments and plotted on the same scale whereby the maximum depression produced is represented by 100. The concentrations of acetylcholine used with physostigmine were, of course, low, but the depressions in the amplitude were quite comparable to those produced by 10^{-7} acetylcholine alone. We see two things from a comparison of these curves. When the cholinesterase was inhibited the recovery

was slower than in the normal auricle, but the final levels reached were not greatly different. In other words, the final equilibrium level of activity was not markedly dependent on the cholinesterase, but this enzyme may well play a role in accelerating the rate of recovery. A possible mechanism by which this might be accomplished will be presented in the discussion.

It is also of interest to compare the rates of recovery from the depressions produced by other, similarly acting depressants. With choline, carbachol, and pilocarpine, definite recoveries were observed, but the rates of these recoveries were slower than with acetylcholine and, in fact, were roughly comparable to the rate of recovery of the auricles from acetylcholine depression in the presence of physostigmine. These results are averaged and collected in Fig. 8. It is doubtful whether there was any

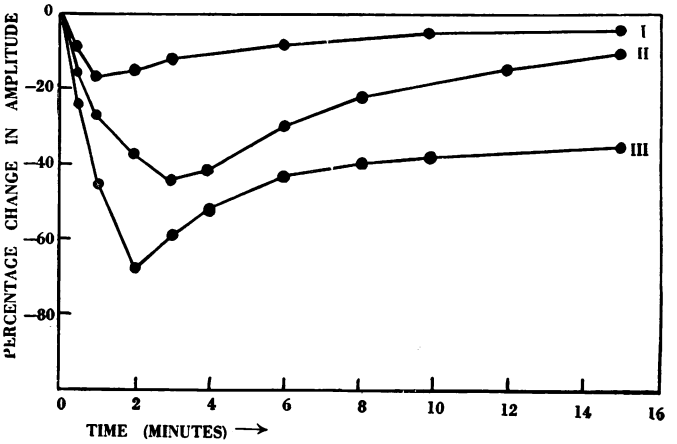

FIG. 8.—Depression and recovery of auricles in the presence of drugs acting in an acetylcholine-like manner. I, Choline (10^{-4}). II, Pilocarpine (10^{-6}). III, Carbachol (10^{-8}).

recovery in the rate of beating, either in the presence of these three substances or in the presence of physostigmine. It may well be that recovery in the rate in the presence of acetylcholine alone, although not marked, was due entirely to the cholinesterase. These results further confirm the fact that spontaneous recovery can occur in the auricles independently of the enzyme cholinesterase. In the presence of potassium, however, very little, if any recovery was observed, except occasionally at very low concentrations (below 0.0067 M) and then it was very slow and slight. This indicates a fundamental difference in action between potassium and these other depressants, and further demonstrates that this recovery is not merely a general phenomenon seen with any depression of the auricles.

There would seem to be no immediate effect on this rate of recovery by any of the metabolic substrates used in the present work (see Table X). Some metabolic inhibitors, e.g., fluoride, azide, and cyanide, may inhibit its development, but such effects were very difficult to measure when the activity of the auricles was changing under the effects of the inhibitors. One can only say that if there were any modifications in the recovery rate by these substances, the changes were not marked.

The development of tolerance to acetylcholine

The partial recovery of the auricles in the presence of acetylcholine would imply the development of a certain degree of tolerance to the action of acetylcholine,

inasmuch as the concentration of acetylcholine in the medium could not have been reduced sufficiently by destruction or binding of the acetylcholine by the tissue. In order to test for an increased resistance of the auricles to acetylcholine, further additions of acetylcholine were made to auricles already depressed by this substance. In a typical experiment the effect of acetylcholine at 10^{-7} was determined, the auricles allowed to recover to a constant level of activity, and then a second addition of acetylcholine was made. In some experiments the effects of several successive additions of acetylcholine were determined. Subsequent doses of acetylcholine were

TABLE V

EFFECTS OF SUCCESSIVE ADDITIONS OF ACETYLCHOLINE

Added concentration	Total concentration	Time min.	% change in rate	% change in ampl.	Auricles	Tests
10^{-7}	1×10^{-7}	0	−18.3	−63.8	8	17
10^{-7}	2×10^{-7}	4	−9.8	−24.7		
10^{-7}	1×10^{-7}	0	−25.5	−62.1	4	6
10^{-7}	2×10^{-7}	4	−18.0	−25.5		
10^{-7}	3×10^{-7}	10	−11.8	−15.3		
10^{-7}	1×10^{-7}	0	−18.8	−59.6	5	8
10^{-7}	2×10^{-7}	4	−9.6	−22.8		
10^{-6}	12×10^{-7}	10	−46.6	−66.6		
10^{-7}	1×10^{-7}	0	−7.5	−50.0	2	2
10^{-7}	2×10^{-7}	4	−4.0	−12.5		
10^{-6}	12×10^{-7}	7	−15.0	−41.0		
10^{-6}	22×10^{-7}	10	−9.0	−14.0		
10^{-5}	122×10^{-7}	15	−30.0	−38.5		
10^{-7}	1×10^{-7}	0	−7.0	−31.0	1	1
10^{-7}	2×10^{-7}	4	−7.0	−16.0		
10^{-7}	3×10^{-7}	8	0.0	−9.0		
10^{-6}	13×10^{-7}	12	−25.0	−28.0		
10^{-7}	1×10^{-7}	0	−23.0	−59.0	1	1
10^{-7}	2×10^{-7}	4	−19.0	−28.0		
10^{-7}	3×10^{-7}	7	−13.0	−12.0		
10^{-6}	13×10^{-7}	10	−14.0	−37.0		
10^{-5}	113×10^{-7}	14	−30.0	−69.0		
10^{-7}	1×10^{-7}	0	−10.5	−68.0	2	2
10^{-7}	2×10^{-7}	4	−5.0	−33.5		
10^{-7}	3×10^{-7}	7	−6.5	−22.0		
5×10^{-7}	8×10^{-7}	10	−7.0	−35.0		
10^{-6}	18×10^{-7}	14	−15.0	−28.5		
10^{-6}	28×10^{-7}	18	−8.5	−16.0		
10^{-5}	128×10^{-7}	23	−70.0	−57.0		
10^{-7}	1×10^{-7}	0	−7.0	−65.0	1	1
10^{-7}	2×10^{-7}	4	0.0	−26.0		
10^{-6}	12×10^{-7}	7	−27.0	−56.0		
10^{-6}	22×10^{-7}	10	−25.0	−23.0		
10^{-6}	32×10^{-7}	13	−20.0	−14.0		
10^{-6}	42×10^{-7}	16	−12.0	−11.0		
5×10^{-6}	92×10^{-7}	19	−75.0	−22.0		

always less effective than the earlier ones, and eventually a marked degree of tolerance could be developed, so that the auricles would continue to beat in concentrations of acetylcholine that initially would have stopped them immediately. These results are recorded in Table V, where the effects of subsequent doses of acetylcholine may be compared with the action on normal auricles. The interval of time between additions is not very important. In three experiments on one auricle with acetylcholine always at a concentration of 10^{-7}, the second additions were made at intervals of two, four, and ten minutes after the first addition and the results were essentially the same in each case.

Before correct evaluation of the degree of tolerance can be determined, we must consult the concentration-action curves of acetylcholine in order to calculate the results that would be expected from successive additions assuming that no tolerance is developed. Examination of these curves shows that subsequent additions of acetylcholine must of necessity produce less and less effect, merely owing to the fact that the curves are not linear. Let us assume that we add 10^{-7} acetylcholine and that the rate has been depressed 23 per cent and the amplitude 57 per cent (calculated from Figs. 2 and 3). If we take the normal, original values of the rate and amplitude to be 100, the rate is now 77 and the amplitude 43. If we now add a second 10^{-7} acetylcholine, we have made the total concentration 2×10^{-7}. The addition of 2×10^{-7} acetylcholine all at once to the normal auricles would have depressed the rate 30 per cent and the amplitude 68 per cent, reducing them respectively to 70 and 32 on the scale of 100. The change from adding the additional 10^{-7} acetylcholine would then be calculated to be a decrease of 9.1 per cent in the rate and 23.2 per cent in the amplitude, figures which agree quite well with those given in Table V. If we now add 10^{-6} acetylcholine, in the presence of 2×10^{-7} acetylcholine, we increase the total concentration to 12×10^{-7} and this amount, added all at once, would have depressed the rate by 54 per cent and the amplitude by 88 per cent, the values on the scale of 100 becoming 46 and 12 respectively. Thus, this third addition of acetylcholine should have produced a 35 per cent depression of rate and a 63 per cent decrease in amplitude, figures which are at least comparable with those given in the Table. Accurate comparisons cannot be made because the curves in Figs. 2 and 3 were obtained from different auricles. In a like manner we may calculate roughly the expected effects of subsequent doses. The phenomenon of decreasing effectiveness of successive doses of acetylcholine thus depends primarily on the nature of the concentration-action curves. The slope of the curves in the particular region investigated would determine the magnitude of the effect and thus, since we have seen that this slope is largely dependent on the activity of cholinesterase, we may conclude that indirectly the esterase activity is of importance in determining the effects of repeated additions of acetylcholine and, in all probability, the behaviour of the heart *in vivo* during repetitive stimulation of the vagus nerve.

However, there is in addition to this artificial effect a true development of tolerance to acetylcholine, for the auricles eventually became able to beat in the presence of concentrations of acetylcholine that would have stopped them initially. Furthermore, there is the recovery that is observed in the presence of acetylcholine and similar depressants, indicating that some manner of resistance appears in the cardiac tissue. In the longer experiments, where many successive additions of acetylcholine

were made, the deviations from the expected behaviour, as calculated from the concentration-action curves, were due to the development of this tolerance. In such comparisons, rough as they are, it is seen that more tolerance is developed with respect to amplitude changes than with respect to rate, just as we should have predicted from the recovery curves.

Successive doses of acetylcholine in the presence of physostigmine produced less effect than originally and similar calculations to those above were carried out using the concentration-action curves obtained in the presence of physostigmine. Here the rate effects of repeated doses followed very closely the calculations based on the curve because there was no disturbing development of tolerance with regard to the rate. However, in several experiments, it was shown that subsequent additions of acetylcholine produced definitely less effect on the amplitude than would have been predicted from the curve alone. In a typical experiment the auricles were in contact with 5×10^{-9} acetylcholine for 20 minutes in the presence of physostigmine (10^{-5}); the second addition of the same concentration of acetylcholine depressed the amplitude by 39 per cent, whereas the calculated depression would have been 70 per cent. Pilocarpine and choline produced in successive doses decreasing effects much like those of acetylcholine under similar circumstances. Furthermore, after contact of the auricles with pilocarpine or choline for various periods of time, the response to acetylcholine was much reduced. In one experiment the initial effect of acetylcholine was a 7 per cent reduction in rate and a 55 per cent reduction in amplitude; after 20 minutes' exposure to pilocarpine (10^{-6}), during which time a definite recovery in amplitude had taken place, the action of acetylcholine was an 8 per cent depression of the rate and a 20 per cent depression of the amplitude. Pilocarpine presumably acts on the auricles in the same manner as acetylcholine and this reduction in acetylcholine action by pilocarpine is probably explicable on the same basis as above for successive doses of acetylcholine. It is thus not quite correct, in such a case, to speak of the pilocarpine as blocking the receptor groups to acetylcholine. The effect is simply due to a combination of two factors: the necessary reduction in response because the auricles have been brought to a new level of functioning by the pilocarpine and the development of a certain amount of tolerance to the presence of substances that act in a similar manner to acetylcholine. In the past, several investigators have explained the inability of a drug to act strongly in the presence of a high concentration of the same drug as being due to the drug blocking itself from the receptor groups. Such a terminology, I believe, is misleading and it is better to speak of such a reduction in response as having its origin in the dependence of the drug response on the slope of the concentration-action curve in the investigated region and the possible development of tolerance by the tissues to the presence of the drug. It is likely that the same explanation is applicable to the experiments which have shown that a vagal stimulation to the heart is less effective than normally if it occurs during the depression produced by a previous stimulation (Gilson, 1932).

Stimulation observed after washing the acetylcholine from the medium

If acetylcholine was allowed to act upon the auricles and was then washed out by replacing the medium, an immediate recovery was observed. However, before the auricles return to their original and normal level of activity, they passed through

a phase of stimulation during which the amplitude was greater than the original value before the acetylcholine was added (Fig. 5). Such behaviour is here, for convenience, designated as a " post-wash " effect. The average results obtained with 10^{-7} and 10^{-6} acetylcholine are given in Table VI. The results at these two concentrations are not to be compared because the experiments were performed on different

TABLE VI

AVERAGES OF RESULTS ON POST-WASH STIMULATION

	Acetylcholine concentration	
	10^{-7}	10^{-6}
% Change in rate due to acetylcholine	−15.8	−70.2
% Change in amplitude due to acetylcholine	−48.4	−91.5
% Stimulation in post-wash rate	+0.2	−1.3
% Stimulation in post-wash amplitude	+32.1	+25.4
Exposure to acetylcholine (minutes)	4	4
Number of auricles	14	9

auricles. We see that simple exposure of the auricles to acetylcholine will induce, after removing the acetylcholine, a stimulation of 25–30 per cent in the amplitude without any post-wash stimulation of the rate. Occasionally a slight stimulation of the rate was observed, but more frequently it was still somewhat depressed below the normal value at the time the amplitude was stimulated. The maximal post-wash stimulation occurred approximately one minute after the acetylcholine was washed out.

The concentration of acetylcholine applied to the auricles was not of importance. In one experiment, where different concentrations of acetylcholine were used on the same auricle, the exposure time being four minutes in each instance, the post-wash stimulation of amplitude was found to be essentially the same for each concentration as shown in Table VII. The negative values for the post-wash rate simply mean

TABLE VII

RELATION OF CONCENTRATION OF ACETYLCHOLINE TO POST-WASH STIMULATION

Concentration	Exposure min.	Effect of acetylcholine		Post-wash	
		% change in rate	% change in ampl.	% change in rate	% change in ampl.
1×10^{-7}	4	−20	−61	−7	+28
5×10^{-7}	4	−57	−91	−7	+30
1×10^{-6}	4	−100	−100	−7	+28

that at the time of maximal amplitude stimulation the rate had not yet returned to its normal value. Such lack of dependence of post-wash stimulation on the concentration of acetylcholine was confirmed in other experiments. The degree of post-wash stimulation was found, however, to depend on the duration of exposure to acetylcholine. This may be seen in Table VIII where the results are given for

TABLE VIII

RELATION OF EXPOSURE TIME TO POST-WASH STIMULATION

Acetylcholine concentration	Exposure min.	% stimulation in post-wash rate	% stimulation in post-wash amplitude
10^{-7}	2	0	+18
10^{-7}	4	−7	+28
10^{-7}	6	0	+32
10^{-7}	10	−7	+35
10^{-6}	0.5	−7	+12
10^{-6}	4	−7	+28
10^{-6}	10	+21	+37

two concentrations. These experiments would indicate that some change is proceeding in the tissue during the action and presence of acetylcholine and that the extent of this change increases with the exposure time. It is possible that this process is related to the development of tolerance. If this is so, we should expect the greatest post-wash effects to be observed in auricles in which the tolerance had been developed to the greatest extent. The most marked post-wash effects were indeed seen when the auricles were subjected to several successive additions of acetylcholine and their tolerance thereby developed to a maximum. Table IX records the results obtained

TABLE IX

POST-WASH STIMULATION AFTER SUCCESSIVE ADDITIONS OF ACETYLCHOLINE

Final acetylcholine concentration	Additions	Total exposure min.	Final effect of acetylcholine		Post-wash	
			% change in rate	% change in ampl.	% change in rate	% change in ampl.
2×10^{-7}	2	5	−17	−54	+8	+115
2×10^{-7}	2	6	−40	−53	0	+89
2×10^{-7}	2	11	−11	−47	+4	+93
3×10^{-7}	3	11	−43	−31	0	+55
11×10^{-7}	3	9	−100	−100	+50	+79
12×10^{-7}	3	14	−60	−71	+7	+36
21×10^{-7}	3	7	−47	−65	−7	+49
110×10^{-7}	2	2	−40	−57	+11	+55
121×10^{-7}	4	13	−100	−100	+10	+83

on such auricles and it is evident that the observed stimulations were greater than after the simple exposure to one dose of acetylcholine and, furthermore, that now even the rate was frequently stimulated. The total exposure refers to the entire length of time that the auricles were exposed to acetylcholine, the concentration being slowly built up by successive additions, the number of these additions being given in the second column. The final percentage changes in rate and amplitude refer to the ultimate depressions produced by the final concentration of the acetylcholine and the state of the auricles immediately preceding the washing out of the acetylcholine. It is impossible to compare quantitatively one experiment with another here because of the variation in the post-wash effect between different auricles.

The results are not contradictory to the conception that the post-wash stimulation is a phenomenon connected with the development of tolerance to acetylcholine. The time relations of both are roughly similar. There is little tolerance developed to the rate depression and likewise less evidence of post-wash stimulation of the rate. Furthermore, no tolerance is developed to potassium and no post-wash stimulation was ever observed, despite the fact that recovery after potassium is quite rapid. It is possible that under the action of acetylcholine, the auricles change in some manner so that they become more resistant to acetylcholine. This change is not immediately reversible when the acetylcholine is removed and manifests itself as a hyperactivity of the cardiac tissue for a brief space of time. It is unlikely that this hyperactivity is due to the accumulation of high-energy substances such as adenosinetriphosphate, during the depression, because it has been shown that the respiration of the cardiac muscle decreases quite proportionately during the action of acetylcholine (Bohnenkamp and Friedmann, 1927; Garrey and Boykin, 1934; Loeper, Lemaire, Figarès, and de Soria, 1935; Gremels, 1936, 1937; Gollwitzer-Meier and Krüger, 1938; Kanda, 1938).

One possible explanation for the post-wash stimulation would be the release of adrenaline, or a similar substance, in the cardiac tissue after the period of depression produced by acetylcholine. However, there are definite differences between the response to adrenaline and the post-wash stimulation. In the first place, the action of adrenaline is much more prolonged when sufficient of it is given to produce the same degree of stimulation that is observed post-wash. In the second place, adrenaline has a marked tendency to increase the rate of beating and this was rarely seen to such a degree after removal of the acetylcholine.

The actions of several metabolic inhibitors on this post-wash stimulation were investigated. It was very difficult to determine such effects quantitatively because of the changes due to the inhibitors themselves, and the following results must be considered only as general observations. The customary post-wash stimulation was observed in the presence of fluoride (0.005 M, exposure time 47 minutes; 0.01 M, exposure time 10 minutes), malonate (0.005 M, exposure time 28 minutes), and iodoacetate (0.0002 M, exposure time 17 minutes). However, no such stimulation was seen in the presence of cyanide (0.0005 M, exposure time 4 minutes; 0.001 M, exposure time 4 minutes; 0.001 M, exposure time 8 minutes), azide (0.001 M, exposure time 8 minutes), or fluoroacetate (0.001 M, exposure time 31 minutes), and it is likely that these three inhibitors prevent either the changes occurring during the action of acetylcholine or the development of the stimulation when the acetylcholine is removed. We may temporarily conclude that this post-wash stimulation utilizes energy derived from those metabolic systems that involve the cytochrome system but that glycolysis is not important for its manifestation.

A quantitative determination of the post-wash stimulation by merely washing the acetylcholine from the bath was very difficult to make. The rate at which the acetylcholine is removed from the tissues may limit the full expression of such a phenomenon. We have two opposing factors operating after replacement of the medium: the decreasing depression of the acetylcholine and the development of the stimulation. If the former is slow it may mask the latter. This is perhaps the reason why such post-wash stimulation was rarely observed after the action of

pilocarpine or of carbachol. A more accurate method of determining the stimulation following acetylcholine depression is to add atropine in sufficient quantity to block all action of the acetylcholine. When this was done, a much greater stimulation was observed than when the medium was changed. Atropine had a very rapid action on auricles that were depressed by acetylcholine, recovery to the original levels of rate and amplitude occurring within 30 seconds. The maximal stimulation was observed between 40–100 seconds. This was particularly well seen when the auricles had been treated with several successive additions of acetylcholine and the activity had been brought to a very low level by the resulting high concentrations (around 10^{-5}). In five such experiments, the rate was increased over the normal value by 39, 18, 34, 45, and 27 per cent (average 33 per cent), and the amplitude was stimulated 204, 65, 76, 203, and 60 per cent (average 122 per cent). The atropine in these experiments was at a concentration of 10^{-5}, but it was noted that very low concentrations of atropine produced marked effects under such conditions. Atropine at 10^{-8} induced marked recovery of the acetylcholine depressed auricles and a concentration of 10^{-9} was also definitely effective. It is possible that such a technique would be applicable to the assay of very small quantities of atropine and related substances.

Modifications of the action of acetylcholine on the auricle

The effects of altering various factors on the acetylcholine response of the auricles were determined in order to gain some insight into the mechanism of action of acetylcholine. If the action of acetylcholine is in any way related to the metabolic processes in the cardiac tissue, this action should be quantitatively modified by altering the metabolic state of the auricular muscle. Therefore, the metabolic substrates and inhibitors used in a previous study (Webb, 1950) were investigated to determine whether they would modify the action of acetylcholine, and, in addition, the effects of anoxia and temperature changes were observed. Although the effect of changing the ionic concentrations of the medium on the acetylcholine response in other tissues has been studied, it was thought advisable to investigate these relationships on the rabbit auricle in order to compare the results with those obtained in other parts of the work. These results are presented in Table X, where the responses of the auricles to 10^{-7} acetylcholine, both in normal medium and in the presence of the substances, are given. The exposure refers to the time during which the auricles were in contact with the substance before the acetylcholine response was determined; zero exposure means that the results were obtained in ordinary medium and are to be taken as control readings.

Acetylcholine response determinations are quite accurate, and a sequence of such determinations on one auricle, if not spread out over too long a time, showed only minor deviations between the individual readings (within 1–3 per cent). Wherever possible, two control determinations were made, one before and one after the test determination, and in several instances alternate controls and test readings were made to increase the accuracy of the observations. However, it was felt that more reliable results could be obtained if different auricles were used, and thus in the Table each test generally refers to a different animal. Great care must be exercised in determinations of this type, since the substances that are being tested may be changing

TABLE X

MODIFICATIONS IN THE AURICULAR RESPONSE TO ACETYLCHOLINE

Substance	Molar concn.	Exposure (min.)	% change in rate	% change in ampl.	Tests
Pyruvate	0.001	0	−14	−45	2
		3	−14	−48	1
	0.005	0	−25.3	−62.6	10
		3	−22.5	−65.6	5
	0.005	0	−14.5	−41.5	2
		15	−11	−44	1
	0.01	0	−6.8	−48.0	6
		3	−4.7	−53.7	3
	0.01	0	0	−58.5	2
		15	0	−61	1
	0.02	0	−23	−46	2
		3	−23	−61	1
Malate	0.005	0	−14.0	−51.0	4
		3	−10.5	−43.5	2
	0.01	0	−11.5	−41.5	2
		15	−6	−48	1
Acetate	0.0005	0	−14.0	−51.3	6
		3	−11.7	−47.1	4
	0.001	0	−17.0	−49.3	4
		3	−14.5	−41.0	2
	0.001	0	−12	−54.5	2
		6	−12	−56	1
	0.01	0	−7	−50.5	2
		3	0	−49	1
	0.01	0	−3.5	−52.3	2
		10	−6	−56	1
Citrate	0.0001	0	−23	−47	2
		3	−23	−43	1
	0.001	0	−18	−46.5	2
		3	−17	−50	1
Glutamate	0.005	0	−5	−49.5	2
		3	−10	−50	1
	0.01	0	−10	−57	2
		3	−10	−54	1
Fumarate	0.005	0	−9	−48.5	2
		3	−11	−49	1
	0.01	0	−6.5	−56.5	4
		3	−3.0	−61.0	2
Succinate	0.005	0	−4.8	−43.2	5
		3	−7.7	−42.7	3
	0.01	0	−3.3	−52.5	4
		3	−3.5	−55.0	2
Oxalacetate ..	0.002	0	0	−46	2
		3	0	−47	1
	0.01	0	0	−48.5	2
		3	−6	−49	1

TABLE X—*Continued.*

Substance	Molar concn.	Exposure (min.)	% change in rate	% change in ampl.	Tests
β-hydroxybutyrate ..	0.001	0	−3.5	−50	2
		3	−6	−51	1
	0.005	0	−7	−49.5	2
		3	0	−47	1
Butyrate	0.001	0	−9	−60	2
		3	−6	−59	1
Caprylate	0.001	0	0	−44.5	2
		3	0	−52	1
Cyanide	0.0001	0	−21.2	−56.7	6
		5	−19.0	−50.7	3
	0.0005	0	−17	−46.5	2
		3	−29	−52	1
	0.001	0	−23.4	−56.2	6
		3	−22.7	−58.3	3
	0.001	0	−13	−53.5	2
		8	−22	−68	1
Azide	0.001	0	−20.5	−66.5	2
		1	−15	−65	1
	0.001	0	−20.5	−70.5	2
		2	−20	−82	1
	0.001	0	−14.3	−61.3	2
		5	−14	−72	1
	0.001	0	−23	−68.7	2
		10	−21	−84	1
Fluoride	0.005	0	−12.8	−41.4	5
		4	−34.3	−64.7	3
	0.005	0	−11.6	−49.5	3
		12	−24.5	−33.0	2
	0.01	0	−14.4	−47.0	7
		1	−47.0	−82.5	4
	0.01	0	−10	−37.5	2
		2	−33	−76	1
		13	−22	−38	1
		40	−10	−22	1
Iodoacetate ..	0.0002	0	−35.5	−75.5	2
		3	−33	−81	1
		8	−33	−79.5	1
		15	−60	−84	1
	0.0002	0	−7	−50	1
		4	−10	−51	1
		11	−7	−57	1
		17	−13	−69	1
	0.001	0	0	−43	1
		5	0	−56	1
		11	−7	−62	1
Fluoroacetate ..	0.001	0	−29	−79	1
		20	−27	−37	1
		30	−37	−28	1
	0.001	0	−23	−69	1
		12	−17	−61	1
		36	−33	−34	1
		50	−100	−100	1
	0.001	0	−17	−46	2
		20	−4	−17	1

TABLE X—*Continued.*

Substance	Molar concn.	Exposure (min.)	% change in rate	% change in ampl.	Tests
Malonate	0.005	0	−8.5	−55.7	3
		5	−6.0	−54.5	2
	0.01	0	−11	−54	2
		7	−11	−59	1
	0.02	0	−5.3	−51.0	4
		10	−1.0	−51.5	2
		20	0	−50.5	2
Pyrophosphate ..	0.0025	0	0	−46.5	2
		15	0	−50	1
Phloridzin	0.0001	0	−27	−60	2
		6	−33	−61	1
	0.001	0	−27	−60	2
		11	−38	−68	1
Magnesium ..	0.00061	0	0	−34	2
		3	−8	−32	1
	0.0021	0	0	−35	2
		3	−7	−34	1
	0.0041	0	−18	−63	2
		3	−18	−64	1
Lithium	0.0091	0	−33	−59	2
		3	−29	−66	1
Calcium	0.00036	0	−6	−43.5	2
		3	0	−60	1
	0.00072	0	−6	−44.3	2
		3	−6	−50	1
	0.00108	0	−4.5	−40.3	4
		3	−6	−47.5	2
	0.00144	0	−6	−44.8	2
		3	−6	−43	1
	0.0027	0	−24.7	−50.7	6
		3	−28.0	−50.9	3
	0.0036	0	−9.5	−45.7	4
		3	−7.5	−44.5	2
	0.0054	0	−6.3	−40.4	5
		3	−8.8	−33.3	3
	0.0072	0	−21.2	−53.3	5
		3	−27.3	−46.5	3
	0.009	0	−15.5	−58.3	4
		3	−26.0	−48.0	2
	0.0108	0	−24.5	−54.2	4
		3	−55.5	−69.2	3
Potassium	0	0	−22	−59.7	2
		3	−36	−77	1
	0	0	−23	−63.2	2
		7	−74	−91	1
	0.004	0	−7.5	−31.5	2
		5	−7	−31	1
	0.0054	0	−18.2	−51.8	8
		3	−15.8	−48.0	4
	0.0094	0	−23.3	−55.0	14
		3	−16.6	−47.3	7
	0.0094	0	−22.7	−46.3	4
		10	−15.5	−38.0	2
	0.0127	0	−34	−62	2
		3	−22	−49	1

TABLE X—*Continued.*

Substance	Molar concn.	Exposure (min.)	% change in rate	% change in ampl.	Tests
Adrenaline	$1:10^7$	0	−18.1	−55.7	5
		3	−15.5	−56.5	2
	$5:10^7$	0	−15.5	−57.5	2
		3	−19	−53	1
Nicotine	$1:10^5$	0	−15	−57	2
		5	−14	−51	2
Oxygen	Partial anoxia	0	−27	−67	2
		15	−23	−74	1
	Complete anoxia	0	−14.5	−57.5	2
		4	−10	−59	1
	Complete anoxia	0	−13	−59.5	2
		7	−8	−62	1

the rate or amplitude during the time that the action of acetylcholine is being observed and modifying the action in an artificial manner. For this reason, as far as possible, the acetylcholine was added only when the auricles had reached a steady functional state after the addition of the substance whose action was being tested. It was also important that complete removal of the test substance was achieved before the second control reading was taken.

One might suppose *a priori* that there would be possibly a general relationship between the absolute magnitude of the rate and amplitude and the quantitative response of the auricles to acetylcholine. In other words, the response of the auricles to acetylcholine might depend on the functional level of the tissue independently of factors that increase or decrease this level. If a depressant slowed the rate and decreased the amplitude, the action of acetylcholine on such an auricle would automatically be increased or decreased as a result of the depression, this change being independent of the fundamental alterations brought about by the depressant substance. However, there is no evidence that this is so, and the observations in the present investigation definitely lend support to the view that there is no necessary relationship between acetylcholine response and functional level. Careful inspection of the various results where the amplitude or rate has been raised or lowered by various substances, change in temperature, or other factors, reveals no correlation between these changes and any modifications in response to acetylcholine. As an example, the action of acetylcholine on the auricular rate was quite constant between 21° and 34°, during which time the absolute rate had changed from 33 to 132. Although adrenaline increased both rate and amplitude markedly, it was found that the response to acetylcholine was not changed. Some depressants of auricular activity increased the sensitivity to acetylcholine and some decreased it. It is likely that the observed modifications in the responses to acetylcholine are real changes brought about by the altered states of the cardiac muscle cells and are not mere artefacts. It must always be remembered in such work that if a substance potentiates the action of acetylcholine, it may be doing this by an inhibition of the cholinesterase in the

cardiac tissue; conversely, a depression of the acetylcholine response may be due to an activation of the cholinesterase. This factor is very difficult to eliminate because experiments made when the cholinesterase was completely inhibited by physostigmine (10^{-5}) were inaccurate owing to the slowly increasing depression brought about by accumulation of endogenous acetylcholine.

Action of metabolic substrates on the acetylcholine response

The substrates that had no definite effect on the acetylcholine response were succinate, fumarate, citrate, oxalacetate, glutamate, butyrate, β-hydroxy-butyrate, and caprylate. Pyruvate, malate, and acetate showed some ability to modify the acetylcholine action but these effects were never striking. No remarkable change in sensitivity to acetylcholine was brought about by the presence of any of these substrates and consequently it is unlikely that acetylcholine depresses the auricular tissue by inhibiting metabolic reactions providing energy for the functioning of the cardiac tissue. When the auricles were markedly depressed (rate −50 to −60 per cent, amplitude −70 to −80 per cent) by high concentrations of acetylcholine which had been built up by several successive additions, the final concentration of acetylcholine reaching 5×10^{-6} to 10^{-5}, the addition of any of the substrates used had no effect and never produced any recovery of the auricles or in any way altered the level of activity. The action of acetylcholine on the auricle is also quantitatively unlike that of any metabolic inhibitor used in the previous study (Webb, 1950) and this lends support to the conception that acetylcholine is not acting by simply depressing or blocking some metabolic reaction. The observation that acetylcholine decreases the respiration of beating cardiac tissue is probably to be explained on the basis of a fall in oxygen consumption accompanying the decreased activity, rather than as a direct effect of acetylcholine on the metabolism.

Although the effect of pyruvate on the acetylcholine response was not marked it was quite constant. At all concentrations and time intervals used there was a potentiation of the action on the amplitude (an average of +7.9 per cent with properly weighted observations) and either no action on the rate response or a decrease (an average of −12 per cent). Neither the concentration of the pyruvate nor the time of exposure to it was found to be of importance in this action. Lower concentrations of acetate were found to decrease the action of acetylcholine (average of −15.8 per cent for rate and −11 per cent for amplitude; 0.0005 − 0.001 M, exposure of 3 minutes), but higher concentrations and longer exposures had the reverse effect (average of +84 per cent for rate and +10 per cent for amplitude; 0.01–0.03 M, exposures of 10–15 minutes). Malate, in general, seemed to behave much like acetate, antagonizing the acetylcholine effect on amplitude at low concentrations and potentiating it at higher concentrations and longer exposures, although the relative scarcity of data here does not allow definite conclusions to be drawn.

No previous work of this type has been done on the heart, but one may recall the report that high concentrations of butyrate and β-hydroxy-butyrate decrease the sensitivity of skeletal muscle to acetylcholine (Torda and Wolff, 1946) while lactate has been shown to increase the vasodilator action of acetylcholine (Densham, 1927).

Action of metabolic inhibitors on the acetylcholine response

Cyanide at a low concentration (0.0001 M) antagonized the action of acetylcholine on the auricles slightly (average of − 9.5 per cent for rate and − 10.5 per cent for amplitude), but at higher concentrations, when its action was more pronounced, there was potentiation of the acetylcholine (average of +20.3 per cent for rate and +9.8 per cent for amplitude; 0.0005–0.001 M). Azide behaved very much like cyanide, the potentiation of the action of acetylcholine proceeding as the degree of azide depression increased, although little effect was seen on the rate response in these experiments. During marked azide depression there was approximately a 19 per cent potentiation of the amplitude response. It was also noted that whenever azide was used, the sensitivity of the auricles to acetylcholine remained high long after the azide had been thoroughly washed out and its effect had disappeared.

Iodoacetate definitely increased the response of the auricles to acetylcholine and this effect became more marked as the iodoacetate inhibition proceeded. Such an increase in potentiation may be seen in each of the three time series given in Table X. Torda and Wolff (1946) reported that iodoacetate had no influence on the reaction of skeletal muscle to acetylcholine and thus concluded that SH-groups were not involved in the action of acetylcholine. Despite the fact that in the rabbit auricle a definite effect of iodoacetate on the acetylcholine response was observed, it is not inferred that SH-groups are directly involved in this change of sensitivity. The action of iodoacetate is in marked contrast to that of fluoroacetate, for this inhibitor only antagonized the action of acetylcholine on the amplitude. This effect was quite striking when the depressant action of fluoroacetate was well advanced, the action of acetylcholine on the amplitude being reduced to less than half its normal value.

Fluoride was found to have a complex effect on the response of the auricles to acetylcholine. At first, for approximately the first five minutes after addition of the fluoride, there was a marked potentiation of the acetylcholine effects on both rate and amplitude (the optimal effect was at one minute), but this augmentation gradually disappeared and was replaced by an antagonism to the actions of acetylcholine. Fluoride has a biphasic effect on the normal auricle (Webb, 1950) but, although the time relations are similar, it is not likely that there is a correlation between this and the modifications of acetylcholine action. The primary acetylcholine potentiation by fluoride is probably related to the known inhibition of cholinesterase by fluoride. No previous work has been done on the heart, but Kahlson and Uvnäs (1935) found that fluoride increased strongly the sensitivity of frog skeletal muscle to acetylcholine, the potentiation occurring at concentrations of fluoride too low to inhibit cholinesterase *in vitro* and, furthermore, occurring when cholinesterase was inhibited by physostigmine. Torda and Wolff (1946) found the same thing but assumed that the action was through the cholinesterase although without any evidence for this. Crivetz (1946) reported that fluoride did not prevent the stimulating action of acetylcholine on frog skeletal muscle but did not mention any potentiation.

The initial depression of normal auricles by fluoride (0.01 M) was not rapid and required four to five minutes for its full development. However, if fluoride was added to auricles that were partially depressed by acetylcholine, the auricles were very rapidly stopped, usually within one to two beats. This action paralleled very

closely that of physostigmine under similar conditions. One way of testing the possibility that the potentiating action of fluoride is by inhibition of the cholinesterase is to determine whether there is any augmentation by the fluoride of the effects of substances that act similarly to acetylcholine, e.g., pilocarpine and carbachol. This procedure was somewhat difficult because these substances act more slowly than acetylcholine and one must separate their actions from those of the fluoride itself. The results are given in Table XI. Although in some instances a slightly greater

TABLE XI

MODIFICATIONS OF THE ACTIONS OF PILOCARPINE AND CARBACHOL BY PHYSOSTIGMINE AND FLUORIDE

Substance	Molar concn.	Exposure (min.)	% change in rate	% change in ampl.	Tests
		PILOCARPINE			
Fluoride	0.01	0	−21	−30	2
		1	−29	−46	1
	0.005	0	−7	−32	1
		4	−18	−28	1
	0.0025	0	−18	−24	1
		1	−22	−27	1
Physostigmine ..	3.1×10^{6}	0	−14	−22	2
		5	−27	−27	1
		CARBACHOL			
Fluoride	0.01	0	−25	−46	2
		2	−30	−34	1

effect of pilocarpine was observed when fluoride was present, there was not the marked effect seen in the acetylcholine response. These minor changes could be explained by mere summation of the effects of pilocarpine and fluoride. It is interesting to note that physostigmine slightly potentiated the action of pilocarpine, for this might help to explain part of the fluoride effect. This potentiation may be related to the ganglionic action of pilocarpine which, although normally small, might be greatly magnified when the auricles were sensitized by physostigmine, although it was found that nicotine (10^{-5}, exposure time of 10 minutes) did not block any of the actions of pilocarpine (10^{-5}). No potentiation of carbachol action by fluoride was observed. From these experiments we may conclude that the potentiating action of fluoride on the acetylcholine response is primarily due to the inhibition of cholinesterase, but that later some further alteration takes place whereby the action of acetylcholine is inhibited or antagonized.

Malonate had no observable effect on the acetylcholine response of the auricles and no marked effects were observed with either pyrophosphate or phloridzin.

Effect of glucose deficiency on the acetylcholine response

If the auricles were allowed to beat in glucose-free medium, there was no obvious change in their response to acetylcholine for one to two hours, but it was then observed that the action was quantitatively altered so that there was slightly more depression of the rate and slightly less depression of the amplitude. The following

results were obtained in experiments with auricles from three animals involving 68 determinations of the acetylcholine response: With glucose present, - 11.2 per cent change in rate and - 59.9 per cent in amplitude; with glucose absent, - 15.1 per cent change in rate and - 51.0 per cent in amplitude. It is likely that these differences are significant because the response to acetylcholine soon reverted to its original value when glucose was restored to the glucose-depleted auricles. It thus seems likely that the response of the auricles to acetylcholine can be modified by the type of metabolism proceeding within the cardiac tissue, confirming generally the results obtained above with the metabolic inhibitors.

Effect of anoxia on the acetylcholine response

Auricles made partially or completely anoxic invariably showed a slightly greater amplitude response to acetylcholine and a slightly smaller rate response, but the effects were not marked and one may question their significance. The auricles were made partially anoxic by stopping the oxygenation of the medium; they were made completely anoxic by bubbling nitrogen through the medium. The behaviour of the auricles under anoxic conditions is shown in Fig. 9. It will be seen that at the times

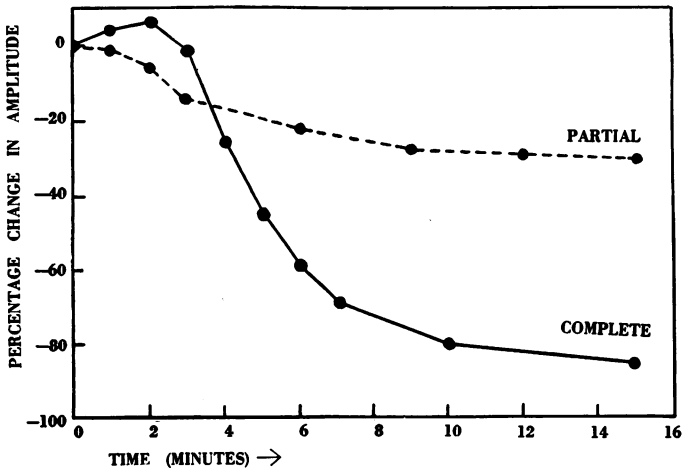

FIG. 9.—Effect of partial and complete anoxia on rabbit auricles. Average curves from five experiments.

of testing the acetylcholine response the auricles were definitely depressed. It is interesting to note that anoxia initially induces a stimulation of both rate and amplitude before the depression begins. This may be related to a similar temporary stimulation observed with cyanide. In two experiments it was noted that auricles under the influence of acetylcholine and physostigmine, which had developed some tolerance during the treatment, were more resistant to anoxic conditions, and that the above-mentioned initial stimulation was both more marked and more prolonged.

Effect of altering the ionic composition of the medium on the acetylcholine response

The actions of high and low concentrations of calcium and potassium on the rabbit auricle are shown in Figs. 10 and 11. Altering the concentration of calcium

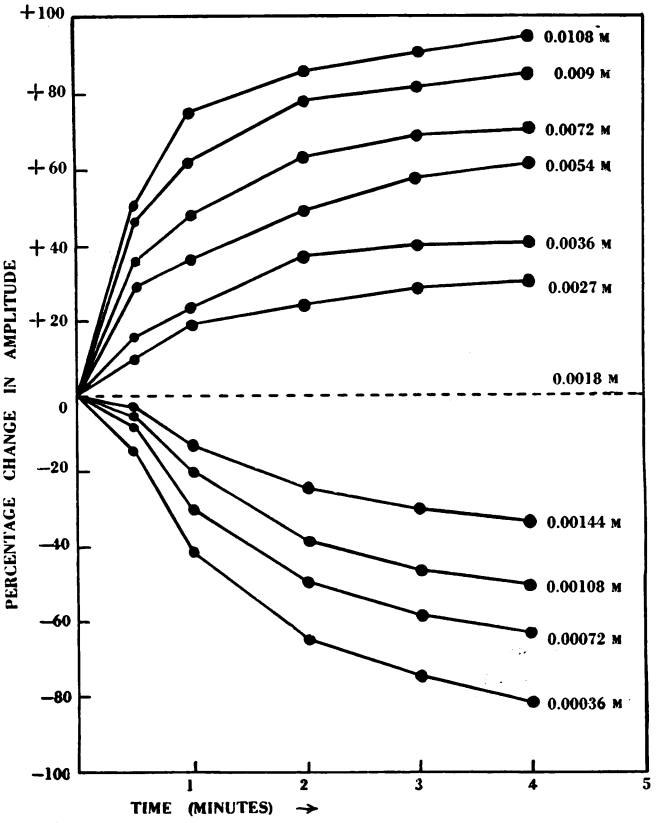

FIG. 10.—Effect of calcium concentration on the auricular amplitude.

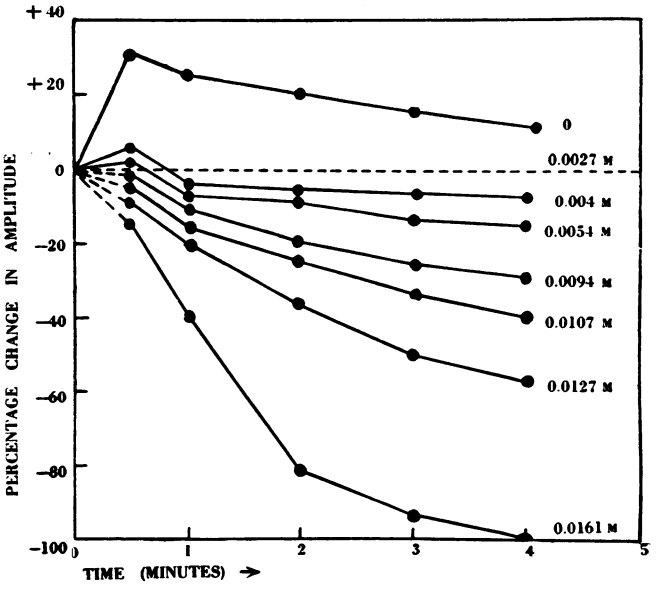

FIG. 11.—Effect of potassium concentration on the auricular amplitude.

or potassium within reasonable limits had very little effect on the auricular rate despite large changes in the amplitude. The opposing actions of these two ions are well demonstrated on the rabbit auricle, although the functional level is not dependent merely on the ratio of the concentrations of these ions. Magnesium in concentrations between 0.0006–0.004 M (normal concentration in the medium is 0.0001 M) had no observable effect on the rate and only a slight depressant action on the amplitude. Lithium (0.0009–0.009 M) had essentially no effect on the rate and amplitude of normal auricles.

The earlier work dealing with the actions of ionic changes on the cardiac response to acetylcholine and related drugs was done almost exclusively on frog hearts. Loewi (1912) reported that neither high nor low calcium concentrations influenced the actions of pilocarpine and muscarine, but Zondek (1920) found that increased calcium concentration depressed the action of muscarine on the frog heart, an observation soon confirmed by Kolm and Pick (1921) for acetylcholine as well as muscarine. Clark (1927) by more thorough work established that a high calcium concentration antagonized the action of acetylcholine, but stated that low concentrations had no influence on the response. With regard to the action of potassium, Bouchaert (1921) observed that frog hearts responded less readily to pilocarpine in low potassium concentrations and Davis (1931) found that frog hearts were less sensitive to acetylcholine when the potassium concentration was low. However, Clark (1927) found the reverse to be true, namely that low potassium increased the action of acetylcholine while high potassium decreased it. Recently Graham (1949), working with rabbit auricles, has confirmed the finding of Clark, the action of acetylcholine being more pronounced in low potassium concentrations.

The results obtained in the present work are graphically represented in Figs. 12 and 13 and recorded in Table X. It is evident that the response of the auricles to acetylcholine was definitely altered by changes in the ionic composition of the medium. With regard to the action of acetylcholine on the amplitude, low concentrations of both potassium and calcium augmented it whereas high concentrations reduced it. In this respect the rabbit auricle behaved quite similarly to the frog heart. With regard to the action of acetylcholine on the rate, the effect of changing the potassium concentration ran parallel with the changes in amplitude response, but with calcium we find a very marked difference. Low concentrations of calcium had very little effect on the rate response to acetylcholine, but high concentrations definitely potentiated it. This was not only manifested in the degree of depression produced, but also in the rate of the acetylcholine action, the slowing of the rate being almost immediate in the higher calcium concentrations whereas in the normal medium the maximal effect on the rate was observed only after 30–60 seconds. Concentrations of acetylcholine (10^{-7}) that would not stop the auricles in normal medium often did this in high calcium concentrations, the cessation of the beat being due to a failure in the discharge of impulses from the pacemaker cells. When the auricles did beat, they beat with very large amplitude. We have here an instance where the rate mechanisms were selectively depressed while the amplitude of contraction was much greater than normal, the opposite condition of the normal auricle which is stopped by acetylcholine while the pacemaker cells are still discharging. The behaviour of the auricles to acetylcholine under these high calcium concentra-

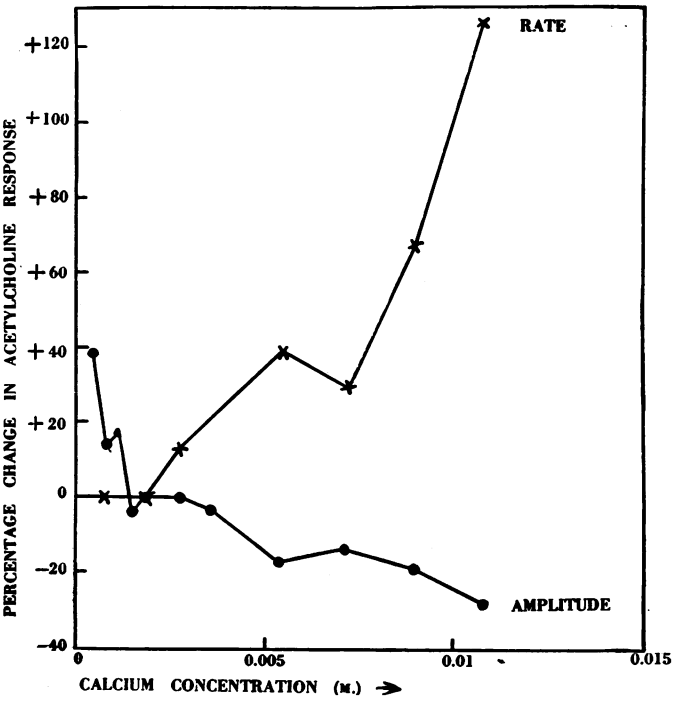

FIG. 12.—Effect of calcium concentration on the auricular response to acetylcholine.

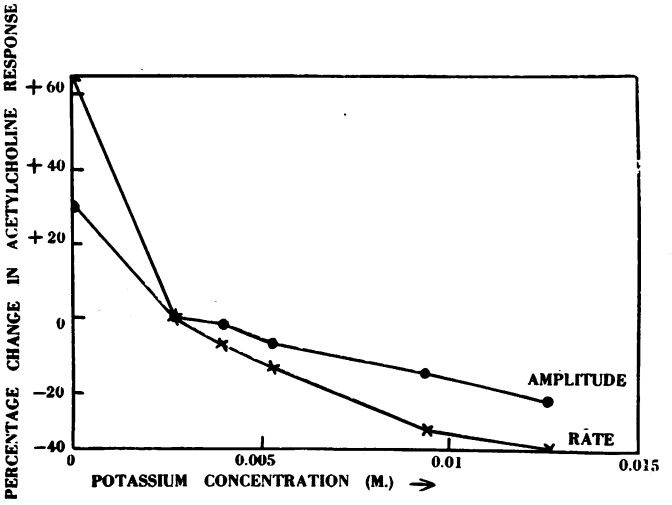

FIG. 13.—Effect of potassium concentration on the auricular response to acetylcholine.

tions was often erratic. Occasionally a stimulation of the amplitude was observed although the rate was markedly slowed. In general one may conclude that a high calcium concentration has the tendency to potentiate the effect of acetylcholine on the rate, at the same time blocking the depressant action on the amplitude while leaving the stimulant component of the acetylcholine action untouched.

It is obvious that these changes in sensitivity to acetylcholine were not simply due to changes in the rate and amplitude brought about by the ionic changes in the medium, for we found, for example, calcium and potassium to produce the same modification in the acetylcholine response and yet one increased and one decreased the amplitude. It is also unlikely that potassium reduced the action of acetylcholine by competing with it for some receptor group since it is quite certain that potassium does not produce its depression of cardiac tissue by acting at the same location as acetylcholine does. In the rabbit auricle this is definitely seen by the fact that concentrations of atropine between 5×10^{-6} and 5×10^{-4}, concentrations capable of blocking all acetylcholine action, had absolutely no effect on the depressant action of potassium. It is probable that we must look for the relationship between acetylcholine and these ions in the changes of state induced in the cellular membranes and the contractile proteins of the muscle.

Effect of temperature on the acetylcholine response

There has been no quantitative work on the effect of temperature on the cardiac response to acetylcholine. Clark (1926) reported that there was no definite effect in the frog heart, while Rost (1923) had stated earlier that the lower the temperature the less the action of muscarine on the frog heart. The effects of changing the temperature on the activity of the rabbit auricle are shown in Fig. 14 and the modi-

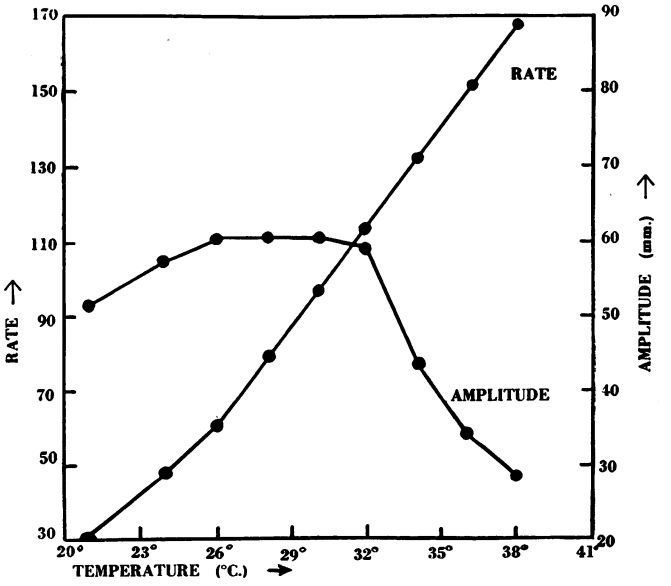

FIG. 14.—Effect of temperature on the normal auricular rate and amplitude.

fications induced in the response to acetylcholine are given in Fig. 15. The curves are averages from two series of experiments, each experiment involving two to three determinations of acetylcholine response at each temperature. The auricular rate showed a marked and regular dependence on the temperature, the Q_{10} being almost exactly 2.5 over the range investigated. The amplitude changed relatively little

below 34° but above this temperature it steadily fell. This decrease may be due to the rapid rate energetically restricting the contractile mechanism. That it is not merely a mechanical restriction imposed by the rapid rate is shown by the fact that auricles beat with even greater amplitude at the very rapid rates that may be induced by adrenaline.

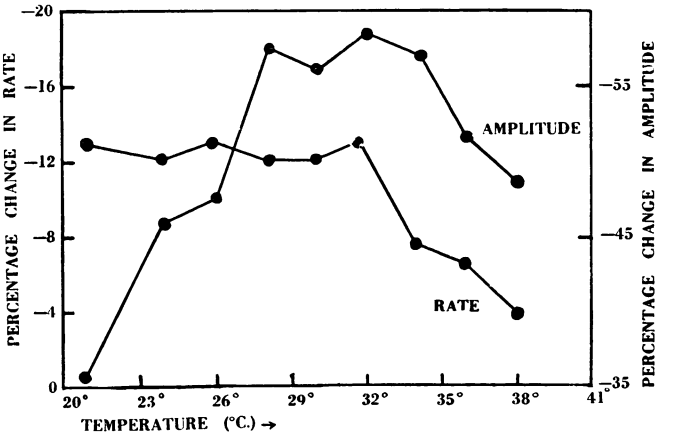

FIG. 15.—Effect of temperature on the auricular response to acetylcholine.

The effect of acetylcholine on the auricular rate was relatively constant from 21° to 33° and then began to decrease. At the highest temperatures the action on the rate may almost entirely disappear. The amplitude response rose to a maximum around 32°–33° and then fell off at higher temperatures. The general picture leads one to conclude that there are several factors involved in these changes. The decrease in amplitude response to acetylcholine above 33° paralleled quite closely the decrease in the amplitude at these temperatures and one is tempted to assume a relationship. One factor that must be of some importance is the activity of the cholinesterase at various temperatures, since it has been shown that the quantitative response of the auricles to acetylcholine is largely governed by the activity of this enzyme. Like most enzymes, cholinesterase increases in activity with increasing temperature. This has been demonstrated in intact rabbit heart muscle by Genuit and Labenz (1941) through perfusion experiments, although their results were complicated by possible changes in the coronary flow. An increase in cholinesterase activity as the temperature rises should reduce the effectiveness of acetylcholine and this may contribute to the falls in rate and amplitude response above 33°, but it is quite certain that there are other important factors, the nature of which is at present unknown.

Action of adrenaline on the acetylcholine response

It has been claimed recently that the presence of acetylcholine modifies the action of adrenaline on the frog heart (Loewi, 1949). Low concentrations of acetylcholine, which were almost without depressant effect, were able to antagonize the stimulant action of adrenaline, while higher concentrations almost abolished the adrenaline response. If acetylcholine antagonizes the actions of adrenaline, it might be expected that the reverse would hold true, namely that the presence of adrenaline would inhibit

the response to acetylcholine. However, Kolm and Pick (1920) have shown that adrenaline potentiates the action of the vagus on the frog heart and recently Middleton and Talesnik (1949) have demonstrated that perfusion of cat or guinea-pig hearts with adrenaline increases their sensitivity to acetylcholine. In the present investigation on the rabbit auricle it was found that there was no particular alteration in the response to acetylcholine while the auricles were markedly stimulated in the presence of adrenaline (10^{-7}: rate +24 per cent, amplitude +51 per cent; 5×10^{-7}: rate +50 per cent, amplitude +76 per cent). These results would indicate that acetylcholine and adrenaline are antagonistic substances on the rabbit auricle, but merely exert their actions in opposite directions, there being no necessary interference of one with the other.

Endogenous formation of acetylcholine

There are ganglion cells and post-ganglionic fibres in the isolated auricle and it is possible that these slowly and continuously release acetylcholine. It is also possible that some acetylcholine is formed and released by the cardiac muscle itself independently of the nerves that are present. If the normally beating auricles are being depressed to any extent by endogenously formed acetylcholine, it is reasonable to suppose that addition of atropine would induce a release from this inhibition and result in a certain stimulation of the auricles. In ten experiments when atropine (10^{-5}) was added to normal auricles, there was no alteration of activity in half of these and a slight stimulation in the other half (average of +3.7 per cent for rate and +7.1 per cent for amplitude). This shows that occasionally there was sufficient acetylcholine present in the auricular tissue to exert a slight depressant action, but in other cases the acetylcholine formed and released was being destroyed or rebound at a rate sufficient to maintain the concentration below the active level.

Addition of physostigmine or neostigmine to normally beating auricles should also indicate the presence of acetylcholine formation since now the acetylcholine will continue to accumulate and exert an increasing depressant action. Physostigmine and neostigmine in concentrations between 10^{-6} and 10^{-5} invariably caused some depression of the auricles and the extent of this depression varied greatly between the auricles from different animals, indicating different abilities to form the acetylcholine. In Table XII are given the average results obtained from seven auricles upon addition of 10^{-6} to 5×10^{-6} physostigmine. It is probable that at this concentration level of physostigmine, the cholinesterase is not completely inhibited, but

TABLE XII

AVERAGE VALUES FOR THE ACTION OF PHYSOSTIGMINE ON THE AURICLES

Time (min.)	% change in rate	% change in ampl.
1	−2	−6
2	−7	−12
3	−9	−17
5	−12	−21
10	−20	−25
20	−33	−36

if the concentration is increased there is always the danger that the drug is exerting a direct action of its own. These results may not be a true measure of the rate of formation of acetylcholine because it is possible that some of the acetylcholine was rebound or otherwise disposed of. Furthermore, there is the development of tolerance to the acetylcholine that is formed and this would to a certain extent counteract the depression. It was noted that the rate continues to decrease for a longer period of time than the amplitude, presumably because the rate mechanisms do not become rapidly tolerant to the acetylcholine. That these effects were due to the accumulation of acetylcholine was indicated by the rapid and effective stimulation of such auricles by atropine.

Discussion

The results of the present investigation have brought out clearly the great importance of cholinesterase in the quantitative responses of the auricle to acetylcholine. The activity of cholinesterase is involved in the large variation between different hearts of the same species in the sensitivity to acetylcholine and probably plays a role in the differences between the hearts of different species. The marked difference between the responses in rate and amplitude to acetylcholine is partly based on different cholinesterase activities in the contractile cells and the pacemaker cells. The presence of cholinesterase influences the shape of the concentration-action curve for acetylcholine, making it flatter and producing a very wide range of effective acetylcholine concentrations. This important role of cholinesterase in the action of acetylcholine on the heart is equally reflected in the response of the heart to vagal stimulation in the intact animal. It is possible that the altered responses of the heart in disease are partly due to variations in the cholinesterase activity. There were auricles in the present work that gave very poor rate responses to acetylcholine, despite the fact that the amplitude was markedly depressed, and this was certainly due to a particularly high cholinesterase activity in the pacemaker cells, for by the application of physostigmine to such auricles they were made to respond to acetylcholine by a typical slowing of the rate. It may be that in human hearts that are abnormally slow or fast the vagal activity is not the only important factor but also the response of the heart to the released acetylcholine and thus to the cholinesterase activity and the distribution of the enzyme throughout the heart.

It is likely that by means of some structural arrangement cholinesterase reduces the effective concentration of acetylcholine in those regions surrounding the receptor groups with which acetylcholine reacts. Various indications point to both the cholinesterase and these receptor groups being at or near the cellular surface. We have seen here that neostigmine is more potent than physostigmine on the auricle, and, furthermore, acts just as rapidly or even more rapidly. Since neostigmine has a quaternary nitrogen atom and is permanently charged, whereas physostigmine is present to a certain extent in the uncharged form, it would appear that penetration of such substances is not of importance in their actions upon the auricle. The simplest conception of the manner in which cholinesterase reduces the concentration of the acetylcholine is to assume a screen of these enzyme molecules at the surface of the cell. If both cholinesterase and receptor groups lie on the same plane at the cellular surface, as in Fig. 16A, the concentration of acetylcholine would be very

little reduced unless the enzyme molecules were quite close to the receptors, perhaps intimately connected with them, because the bombardment of the receptor groups by the acetylcholine molecules would proceed from the medium and the probability of reaction with a receptor group would not be greatly influenced by the cholinesterase. It is difficult to understand how the effective concentrations are

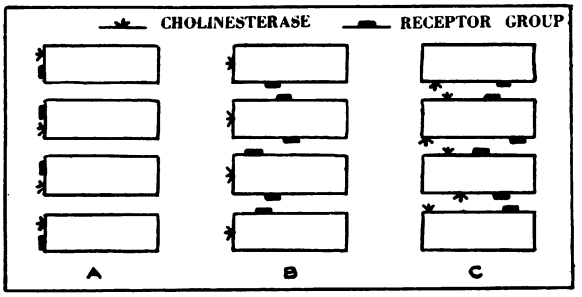

FIG. 16.—Diagrams of the possible relationships between cholinesterase and acetylcholine receptor groups in the oriented micellar cell membrane.

often reduced to less than 1/100th of the external concentration by such an arrangement. For the same reasons, an arrangement as in Fig. 16B, where the enzyme molecules are arranged at the surface while the receptors lie within the membrane, is unlikely, for the cholinesterase would not interfere greatly with the entrance of the acetylcholine into the intermicellar spaces. It is more probable that by some means acetylcholine molecules are partially destroyed as they are diffusing to the receptor regions in such a manner as is roughly illustrated in Fig. 16C. These diagrams are, of course, not meant to represent reality closely but are presented only as a crude basis on which to picture the results of the present work. It is very difficult to work out quantitatively the kinetic relations involved here because the actual structural arrangement is all-important. We do know that the activity of cholinesterase rises with the concentration of acetylcholine to a maximum and then decreases, this decrease occurring at concentrations much higher than those employed in work on the heart; such might contribute to the increasing effectiveness of the cholinesterase as the acetylcholine concentration increases and the greater difference between the internal and external concentrations when the acetylcholine is present at the higher concentrations.

In considering the influence of cholinesterase on the acetylcholine response of the heart we must bear in mind that three factors may alter: (*a*) the concentration of the cholinesterase, (*b*) the enzymatic activity of the cholinesterase, and (*c*) the structural arrangement between the cholinesterase and the acetylcholine receptor groups. The first factor would be of importance in determining differences between different hearts and between different regions of a single heart, but in short time experiments with a particular tissue would be constant. The second factor would be of importance in determining the actions of substances that modify the response to acetylcholine. However, the third factor would be of general importance in all work of such nature. It is conceivable that two hearts might have the same concentrations of cholinesterase, of the same activity in the two hearts, but if the distribution of the cholinesterase relative to the receptor groups varied the two tissues might behave differently. Such structural variations may possibly give rise to the difference

in response of rate and amplitude ; it is not necessary that the enzymatic concentrations be different. But of even more importance is the question whether such spatial arrangements may alter in response to changes in the functional activity of the cardiac tissue or as the result of the administration of various substances. It is not likely that the cellular membrane is a completely static and rigid structure. There is evidence, through changes in permeability and electrical properties, that the cellular membrane undergoes structural changes during the passage of an impulse, perhaps in response to the marked alterations in the electrical field across the membrane. It would be expected that a micellar membrane (these micelles being held together by weak bonds) containing many charged groups would be of necessity spatially altered by the great changes in the electrical field brought about by the passage of an impulse. Similarly, it is likely that reactions of these charged groups with drugs would produce some alteration in the membrane structure, such as an increase or decrease in the micellar distances. Such changes would not need to be great in order to modify the response to acetylcholine.

It is highly probable that acetylcholine produces a change in the electrical properties of the cardiac cellular membranes, but the exact nature of this change is unfortunately still obscure (Gaskell, 1887 ; Einthoven, 1908 ; Meek and Eyster, 1912 ; Einthoven and Rademaker, 1922 ; Samojloff, 1923 ; Bohnenkamp, 1923 ; Bertha, 1928 ; Bogue and Mendez, 1930 ; Gilson, 1932 ; Monnier and Dubuisson, 1934a, b ; Bacq and Monnier, 1935 ; Hönger, 1936 ; Lueken and Schütz, 1938, 1939 ; and others). The consensus of opinion is that the vagus and acetylcholine increase the resting potential and decrease the action potential. It seems quite certain that in nerve and muscle acetylcholine produces a depolarization of the membrane. Such alterations in the potential could be brought about either by a direct effect of acetylcholine on the membrane potential by combination with charged groups in the membrane or indirectly through modification of the processes responsible for the development of polarization or depolarization. On the former supposition the exact positions of the acetylcholine receptor groups in the membrane would be of the utmost importance. If these groups were near the outer positively charged surface, combination with acetylcholine would lead to an increase in the potential, but if they were nearer the inner edge of the membrane the reverse effect would be obtained. Whether acetylcholine stimulates or depresses a tissue might depend on whether there is an increase or a decrease of the potential and this would be controlled by the particular locations of the receptor groups in the membrane. On the latter supposition, acetylcholine, by stimulating polarizing processes in the cardiac cell membranes, would both increase the resting potential and decrease the development of the action potential depolarization. Such a mechanism could account for the observed decrease in the refractory period induced by acetylcholine or the vagus (Raaflaub, 1914 ; Lewis, Drury, and Bulger, 1921 ; Andrus and Carter, 1929 ; Gilson, 1932 ; Wedd, 1934 ; Cohn and Macleod, 1939 ; Wedd and Blair, 1945 ; de Elío, 1947 ; and many others), the slowing of conduction rate (Dale and Mines, 1913 ; Noth, Essex, and Barnes, 1939 ; Wedd and Blair, 1945), and the shortening of the action potential or excitation time (Hofmann, 1905, 1920 ; Samojloff, 1914 ; Bertha, 1928 ; Bogue and Mendez, 1930 ; Lueken and Schütz, 1938). Owing to the decreased degree and time of depolarization brought about

by the action of the acetylcholine, the excitation process would be decreased and fewer myofibrils would contract in response to the passage of the impulse. Such changes would also slow the rate of discharge from the pacemaker cells, for it would require a longer time for the development of a greater area of depolarization in order that the impulse might be propagated.

Although over a period of time there would be a general and homogeneous depression of the excitation process, at any specific instant there would probably be certain regions where acetylcholine was producing its effect and certain regions free from its action. If we imagine acetylcholine molecules at low concentration combining with micellar receptor groups here and there in a mosaic of such micelles, some regions would be inactive or " dead," the impulse either not passing over these regions or affecting them only to a minor extent. As the concentration of acetylcholine rises there would be some overlapping of these inactive areas and more and more interference with both the passage of the impulse and the excitatory state by which the myofibrils are stimulated to contract.

Let us consider briefly the two mechanisms given above: the direct action of acetylcholine on the membrane potential by combination with charged groups and the indirect effect on the potential through stimulation of certain processes concerned with polarization of the membrane. The evidence at hand would seem to favour the latter. In the first place it is doubtful if static charges on the membrane micelles contribute to the electrical potential across the cell surface. Furthermore, it is difficult to understand why charged molecules like atropine or physostigmine do not produce similar effects since they also combine with groups on or in the surface. In the present investigation it was shown that the action of acetylcholine could be modified by changing the metabolic state of the cardiac tissue (presence of certain substrates, presence or absence of glucose, anoxia, action of most metabolic inhibitors) and this relationship can better be explained or visualized if we assume that acetylcholine is acting upon a process involving, directly or indirectly, metabolic reactions. The electrical potential across the membrane of a cardiac cell is maintained with the energy derived from these metabolic reactions; such reactions most probably proceed within the membrane, otherwise long-range transference of energy would have to be invoked. Since the membrane potential is primarily due to an asymmetrical distribution of ions, it is likely that in some manner these metabolic reactions are connected with ion movements within the membrane. If this is so, it is logical to assume that these enzymatic reactions are spatially arranged in a definite way. These polarizing reactions are continuously active and any disturbance or inhibition of them would result in a gradual slowing down of the rate of polarization, this in turn increasing the action potential, increasing the refractory period, and speeding the impulse conduction. Depression of the polarizing reactions could result from either a direct inhibition of one of the steps or a diversion of the substrates and intermediates involved so that their energy could not be properly utilized. It is not likely that the acetylcholine molecule acts as an active prosthetic group in any enzyme complex and yet it acts catalytically in the sense that the effect produced is out of proportion to the amount of acetylcholine involved. However, such behaviour could be explained on the basis that acetylcholine acts as a structural link between certain enzymatic reactions, facilitating an energetic coupling and

altering the path of energy flow. Through the action of acetylcholine, two enzymes in a sequence of metabolic reactions may be brought closer together and the production of utilizable energy by this metabolic system thus increased. Such an action would explain the fact that both ends of the acetylcholine molecule are of importance in its effect and also the observation that the distance between the "active centres" in the acetylcholine molecule must lie within a certain range for maximal activity (Ing, 1949). We may further assume that the polarizing reaction consists metabolically of two general parts: a low-energy phase which is instrumental in maintaining the resting potential and a high-energy phase responsible for the rapid repolarization after the passage of an impulse. The former would operate continuously and the latter cyclically. These two phases may correspond to the "resting" and "activity" metabolisms observed in many tissues. If so, it is probable that the high-energy phase, which is accelerated by acetylcholine, involves the cytochrome system. Whether stimulation of such a reaction would increase the resting potential or not is unimportant; the modification of the action potential would be the real factor in determining the response of the contractile units. Most changes in cardiac function are probably not brought about by a direct action on the contractile units, the activity of which is principally controlled by the level of ATP; it is usually only when the ATP drops to a sufficiently low concentration that there is an interference with the functioning of such units.

Let us consider inhibition of the polarization process and the resulting disturbances in cardiac contraction. A substance that specifically inhibited the high-energy or "active" phase would alter the resting potential very little but would retard the repolarization process following the passage of an impulse, or during the passage of the impulse, and thereby increase the degree and duration of the excitation state. Such an action would produce stimulation of the heart. On the other hand, a substance that predominantly inhibited the "resting" phase would progressively lower the resting potential and this would soon limit the development of the action potential and depress the heart. A substance such as adrenaline might act in the former capacity and an inhibitor like iodoacetate in the latter. Cyanide, acting primarily on the cytochrome system, would first inhibit the "active" phase and cause some stimulation; however, this would be soon followed by depression owing to the decrease in the resting potential. If the resting potential of the cardiac tissue had dropped to a value that did not allow the conduction of effective impulses, the addition of acetylcholine might stimulate the heart by increasing the potential to the level where activity could be resumed. This would not occur, of course, if the metabolic systems in which acetylcholine acted were inhibited (e.g., by cyanide, iodoacetate, and other inhibitors), but it might well account for the stimulation of day-old, exhausted auricles by acetylcholine. In the latter, there is sufficient substrate but the enzyme complexes may have become disorganized so that insufficient energy is available to maintain the resting potential; acetylcholine may temporarily stimulate these systems by facilitating the coupling discussed above.

Assuming such general mechanisms of action as outlined above, how can we explain the spontaneous recovery of the auricles in the presence of acetylcholine and the temporary stimulation that was observed after the acetylcholine was washed from the bath? We shall consider first the recovery that occurs in the presence

of physostigmine and thus does not involve the cholinesterase. In the normal auricle there is probably a certain level of intermediate substrate which may be utilized by the "active" phase of the polarization process. When this "active" phase is accelerated by the addition of acetylcholine the initial effect is maximal but it is gradually slowed as it depletes this level and becomes adjusted eventually to the equilibrium conditions wherein its rate is limited by the rate of formation of such substrates. This would explain why the auricles under acetylcholine influence finally reached certain levels of activity and did not exhibit complete recovery. When the acetylcholine is removed (or suddenly inhibited in its action by atropine), the "active" phase would operate for a short time more slowly than in normal auricles, owing to the readjustment that must be made between it and the "resting" metabolism, and during this time the auricles would be stimulated as the result of a decreased repolarization rate. From the fact that there is no recovery in the presence of potassium and no stimulation after its removal, we may conclude that potassium does not inhibit the cardiac tissue in the same manner as acetylcholine. With regard to that minor portion of the recovery that is dependent on the cholinesterase, we may postulate that it is due to a slight spatial readjustment between the cholinesterase molecules and the acetylcholine receptor groups, inasmuch as it is improbable that the enzymatic activity of the cholinesterase can change under such conditions. Such a small structural change in the cellular membrane is not at all unlikely during the altered functional state of the cardiac muscle.

It is very difficult to discuss the possible mechanisms by which various factors may modify the action of acetylcholine on the auricles. The situation is too complex to allow an accurate analysis at the present time. The addition of some substance, for example, might alter the action of acetylcholine by (*a*) changing the affinity between the receptor groups and acetylcholine, (*b*) changing the activity of cholinesterase, (*c*) altering the spatial relations between cholinesterase and the acetylcholine receptors, (*d*) modifying any of the enzyme systems which are involved in the polarization process, or (*e*) changing quantitatively the response of the contractile units to the excitation state. That pyruvate potentiates the action of acetylcholine might be taken as evidence that pyruvate can serve as a substrate for the "active" phase of the polarization process, but such generalizations are dangerous when other mechanisms are possible. It is interesting to note that most metabolic substrates were found to depress the auricles (Webb, 1950) and some of these may do this by increasing the rate of the repolarization reaction. The action of the metabolic inhibitors on the acetylcholine response is even more complex because none of the inhibitors are truly specific and, furthermore, even a specific action would lead to many secondary changes in the general metabolic pattern. It is also impossible to predict what a simple lowering of the resting potential would do to the acetylcholine response. The metabolic inhibitors were shown to have definite and distinctive effects upon the response of the auricles to acetylcholine, but the elucidation of the mechanisms involved must await the time when we shall have more knowledge of the behaviour of the systems involved. We might expect that cyanide would depress the acetylcholine response by reason of its inhibition of the cytochrome system and indeed it does this at low concentrations of cyanide, but at higher concentrations, when the resting potential is probably strongly reduced, it has the opposite action.

It is not so surprising that iodoacetate should increase the acetylcholine action because it would decrease the resting potential without interfering markedly with the "active" phase or the formation of substrates upon which this phase may act (e.g., fat metabolism may proceed to some extent in the presence of iodoacetate). It is also not surprising that fluoroacetate should antagonize the acetylcholine response, if it interferes with the functioning of the tricarboxylic acid cycle, for it might effectively reduce the formation of substrates upon which the "active" phase depends. However, these are only tentative suggestions. The effects of high and low concentrations of calcium and potassium on the response to acetylcholine are also clear and distinctive, but even less is known about how these ions act than about the changes induced by metabolic inhibitors. These simple ions, by virtue of their ability to alter the charges on micelles and enzymes, might easily influence the ability of acetylcholine to accelerate the coupling of the "active" phase of the polarization process. Excess calcium stimulates the auricular amplitude and it could do this by interference with the normal initiation of the repolarization reaction during the passage of the impulse and by the same mechanism make it more difficult for the acetylcholine to stimulate this reaction. Lack of calcium would then produce the reverse effect as observed. Although potassium acts by a mechanism different from that of acetylcholine, it is likely that it influences the polarization process in some manner, since it temporarily stimulates before it inhibits and also stimulates somewhat when it is present in a concentration lower than normal. Simply by acting in the same direction as acetylcholine, potassium might reduce the effectiveness of acetylcholine despite the fact that the two substances are acting in different ways.

Throughout this discussion the effects of acetylcholine on the amplitude of the auricles have been emphasized because so little is known concerning the processes controlling the rate of discharge from the pacemaker cells. The pacemaker cells evidently possess cyclical depolarization reactions in addition to the processes which restore the electrical potential and this introduces further complexity. It is true that effects upon the rate and amplitude usually run parallel, but there are instances where distinct differences are seen, for example in the potentiation of the rate response to acetylcholine by excess calcium while the amplitude response is decreased. Previous work with metabolic substrates and inhibitors showed that the processes controlling rate and amplitude differ metabolically and it is not surprising that these differences should manifest themselves in an investigation of the response of the auricle to acetylcholine and of the modifications of this response.

CONCLUSIONS

1. A quantitative investigation of the action of acetylcholine on the rabbit auricle was made in the effort to obtain evidence that will be of use in the elucidation of the mechanism of action of acetylcholine.

2. It was shown that the response of the auricle to acetylcholine is dependent on the activity of cholinesterase in the cardiac tissue and the distribution of this enzyme within the tissues. Variations between different auricles with regard to the response to acetylcholine, the difference between the rate and amplitude responses, and the position and shape of the concentration-action curves for acetylcholine were all demonstrated to be dependent on cholinesterase activity.

3. A general picture of how cholinesterase might reduce the effective acetylcholine concentration in the region of the acetylcholine receptor groups is presented. If such a situation exists, it is evident that not only the concentration and enzymatic activity of the cholinesterase is of importance, but also the spatial relations between the cholinesterase and the acetylcholine receptor groups.

4. The spontaneous recovery of the auricles in the presence of acetylcholine and the simultaneous development of tolerance to acetylcholine were investigated, as well as the stimulation observed after the acetylcholine is removed from the bath or its action prevented by atropine.

5. The influence of various factors upon the acetylcholine response was determined. These factors included the presence of metabolic substrates and inhibitors, glucose deficiency, anoxia, changes in the temperature, changes in the ionic composition of the medium, and adrenaline stimulation.

6. Some experiments were made to exhibit the extent of the formation of endogenous acetylcholine in the auricular tissue.

7. A general theory of the action of acetylcholine is presented as a working basis and the results of the present work are discussed relative to this theory. Acetylcholine is pictured, in this theory, as able to stimulate certain repolarization processes in the cellular membranes through acceleration of the coupling of a high-energy or " active " phase on to a low-energy or " resting " metabolism, thereby reducing the extent and duration of the excitatory state.

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