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ACTION OF ACETYLCHOLINE ON RABBIT AURICLES IN RELATION TO ACETYLCHOLINE SYNTHESIS

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In a recent paper (Burn & Vane, 1949) the hypothesis has been discussed that the action of acetylcholine on the heart is not restricted to that of a humoral transmitter of vagal impulses, but that acetylcholine is present in heart muscle playing an important part in the processes responsible for its spontaneous contractions. This discussion arose from observations showing that when the isolated auricles of the rabbit heart are exposed to the action of proguanil (paludrine) for a sufficient time, their spontaneous contractions cease, and can then be restarted by the addition of acetylcholine to the bath. Further additions of acetylcholine serve to increase the amplitude and rate of the contractions. When the contractions have continued for some time, the normal inhibitory effect of acetylcholine returns.

To test the hypothesis by a method which avoids the use of the substance paludrine, experiments have now been performed on isolated auricles allowed to beat for many hours until they stopped. The addition of acetylcholine under these circumstances has been found to restore spontaneous activity. This phenomenon has been investigated in detail and in its relation to the synthesis of acetylcholine which takes place in auricular tissue.

Experiments on the living tissue

METHOD

Freshly dissected auricles were suspended in an isolated organ bath containing Tyrode solution at 28° C., aerated by a mixture of oxygen and 5% CO_2. When left overnight, the temperature was reduced to that of the room (usually 20–22°) and raised again in the morning to 28° C. During the day, the Tyrode solution was changed about once an hour. Acetylcholine bromide was used for the observations and the doses were expressed in terms of the weight of this salt.

RESULTS

Isolated rabbit auricles were found to beat spontaneously for about 24 hr. The beat continued throughout the day, but sometimes stopped during the night

when the temperature was low. If it had stopped, the raising of the temperature next morning often started it again. Some preparations continued to beat for as long as 48 hr.

The arrest of the beat occurred in different ways. Sometimes the beat became gradually smaller and finally disappeared. Sometimes the beat stopped abruptly. Sometimes the beat became irregular, and periods of arrest alternated with periods of regular activity before the final arrest.

The addition of acetylcholine to the bath was found to restart the contractions in fourteen out of twenty-two experiments. The effect is shown in Fig. 1. In this experiment the beat had ceased for 1 hr.; when acetylcholine was added, it began after a latent period of 2 min. At first the beat was small and infrequent; it gained in rate and amplitude until it reached its maximum in 15 min.; during the next 45 min. it declined to about half the amplitude, at which point the auricles were removed from the bath.

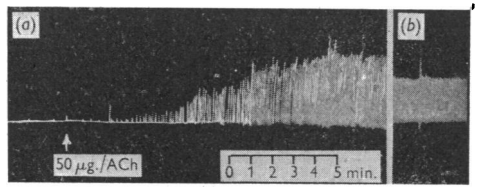

Fig. 1. Isolated auricles of rabbit. Tyrode bath 75 c.c. Temp. 28° C. Auricles suspended in bath on previous day; record begins 1 hr. after the beat stopped. (*a*) Addition of 50 μg. ACh starts the beat again; (*b*) 1 hr. later.

The concentration of acetylcholine required to start the beat varied in different experiments from 1 in 100 million to 1 in 400,000. No connexion was observed between the concentration required and the length of time which had elapsed since the beat stopped. The character of the restarted beat, however, appeared to be related to the duration of the arrest. If the period of arrest was short, 5–15 min., the auricle resumed a regular beat at once; if the period was long, e.g. 1 hr. or more, the auricle began by beating irregularly.

When the acetylcholine was removed by replacing the fluid in the bath with fresh Tyrode, the beat stopped again. An example is given in Fig. 2. At the beginning (Fig. 2*a*) the auricles were still beating spontaneously though with a small amplitude. The addition of 4 μg. ACh depressed and slowed the beat, and the arrest of the beat first occurred on washing out. Two minutes later 4 μg. ACh was again added; there was one beat, and after 30 sec. regular activity started. The acetylcholine was left in the bath for 9 min. and then removed. This produced a short burst of improved activity, followed by arrest.

A repetition of the addition of 4 μg. ACh (Fig. 2*b*) now had much less effect than before; it elicited a series of small contractions which stopped again after

1 min. The addition of 20 μg. started slow but regular activity, and the further addition of 100 μg., after an inhibitory effect during 45 sec., increased both the amplitude and the rate of the contractions. Seven minutes later, double this amount of acetylcholine depressed the beat.

Fig. 2*c* (which follows directly on Fig. 2*b*) demonstrates the removal of the depression by washing, and then the opposed effect of smaller and larger amounts of acetylcholine. The addition of 50 μg. caused stimulation, and the subsequent addition of 100 μg. caused inhibition. At this stage of the experiment the contractions of the auricles were bigger than at the beginning of Fig. 2*a*, and it was difficult to stop them again. However, by changing the fluid in the bath four times during the next 20 min. the beat was arrested, and Fig. 2*d* shows that the dose of 100 μg., which last depressed the beat, now restored it. Activity being resumed, the further addition of 100 μg. caused depression again.

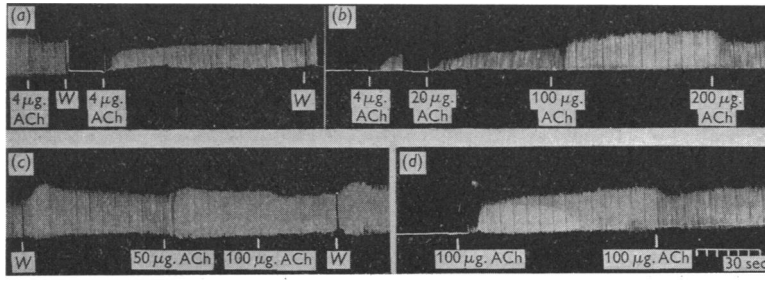

Fig. 2. Rabbit auricles as Fig. 1. (*a*), (*b*) and (*d*) show further examples of the beat restarted by acetylcholine. Note that the same dose of acetylcholine (100 μg.) can stimulate as well as inhibit. See text.

The observation was repeatedly made that a dose of acetylcholine, which had caused inhibition of the normal beat, would start the beat when it had stopped, and that when the beat was resumed the same dose would cause inhibition once more. Further, when first the beat was started it could be stopped by washing out fairly soon, but if activity had been resumed and allowed to continue for some time, then it was difficult to arrest it by washing out.

Before the auricles had stopped their normal beat, no matter how feeble this was, the addition of acetylcholine, however small the dose, was never observed to cause stimulation. When the auricles had been restarted, a further addition of acetylcholine in several experiments caused increase of rate and amplitude (as in Fig. 2*b*, *c*), but as a general rule its effect was again inhibitory. There was, however, a difference. Whereas before the auricles had stopped, the inhibitory effect of small doses of acetylcholine was usually much greater than in freshly prepared auricles; in the restarted auricles, on the other hand, the inhibitory effect was much less.

In Fig. 3, three experiments are illustrated, in the first of which the beat was started by 0·5 μg. ACh and in the second by 100 μg. ACh (added to a bath of 75 c.c.). In each experiment the effect was prolonged, and the results suggested to us that the duration of the effect was not dependent on the size of the dose, but on some process initiated in the muscle which, once initiated, was self-maintained. At a time when the resumed contractions of the auricle were fully developed they did not stop when the bath fluid containing acetylcholine was replaced by fresh Tyrode. In some other observations the addition of acetyl-

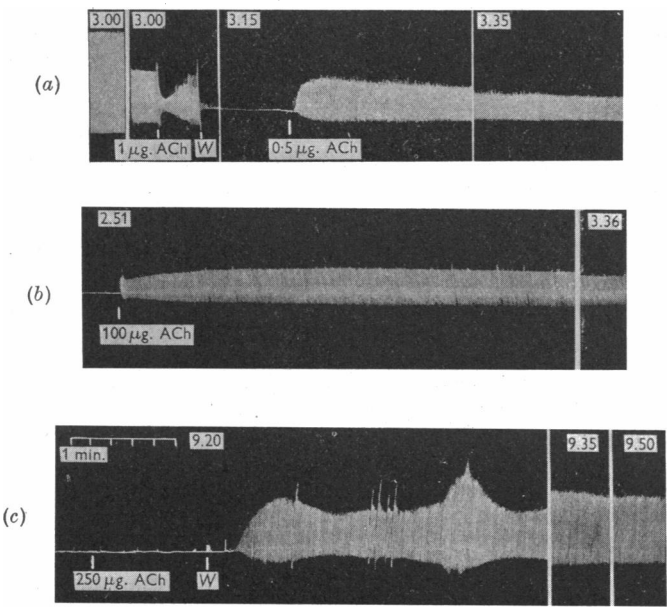

Fig. 3. Rabbit auricles. (*a*) shows (1) initial beat when freshly put up; (2) beat 24 hr. later inhibited by acetylcholine and stopped on washing out the bath; (3) the restart by 0·5 μg. ACh; (4) 20 min. later. (*b*) shows another auricle isolated for 26 hr. Stopped for 10 min. before record begins. Restarted by 100 μg. ACh; beat 45 min. later. (*c*) shows a third auricle isolated for 31 hr. Stopped 40 min. before record begins. Four previous doses of acetylcholine failed to start the beat, but on washing out the bath the beat began. See text.

choline at first appeared to have no effect, but when it was removed from the bath contractions started. Such an example is shown in Fig. 3*c* in which five successive doses of acetylcholine (1, 5, 25, 125, finally 250 μg.) administered during 20 min. did not start the beat. But, on washing out, regular activity began; this we believe was the result of the previous application of acetylcholine.

The experiments so far suggested that when the auricle was exhausted some metabolic process ceased. If the addition of acetylcholine was necessary to initiate this process on which the contractions of the auricle depended, and if

this process continued after the added acetylcholine had been removed, it seemed possible that acetylcholine was synthesized in the heart muscle in order to enable it to contract.

Acetylcholine synthesis

METHOD

We have applied to rabbit auricles the method described by Feldberg & Mann (1946) for estimating acetylcholine synthesis in brain tissue. For this purpose the auricles were converted into an acetone-dried powder, and the amounts of acetylcholine synthesized during 75 min. incubation were expressed in μg. per g. acetone powder. Comline (1946) has recorded that auricles may synthesize up to 90 μg./g./hr. In studying synthesis by brain tissue, Feldberg & Mann found that three factors greatly increased the activity, adenosine triphosphate, citric acid, and a third substance prepared by extracting the acetone powder of brain with boiling saline. This third substance was called 'activator'. The importance of adenosine triphosphate was first pointed out by Nachmansohn & Machado (1943). We incubated our preparations accordingly in the following way for 75 min. To 50 mg. acetone-dried auricle powder suspended in 2 c.c. activator were added:

	c.c.
KCl, 6%	0·1
NaF, 2%	0·1
Choline chloride, 3%	0·1
Eserine sulphate, 0·5%	0·1
Phosphate buffer, M/15	0·3
$MgCl_2$, 4%	0·1
Sodium citrate, 15%	0·1
ATP-P, 0·4 mg. in	0·4
Cysteine, 4·5 mg. in	0·2
Saline	1·0
Total	4·5

Alongside each preparation a control was incubated containing everything except the auricle powder. The estimations of acetylcholine were carried out on the frog rectus muscle, using as standard solutions varying concentrations of acetylcholine made up in the activator control which was diluted to the same extent as the preparation with which it was compared. A detailed example is given later. A few of the later experiments were carried out without converting the auricle tissue into an acetone-dried powder, but grinding the fresh tissue with sand in activator, and after 5 min. centrifugation taking the supernatant fluid for incubation. The amounts of activator and of the other ingredients were calculated after it had been established that one part of acetone-dried powder represented seven parts of fresh tissue. The results obtained with acetone-dried powder and with supernatant fluid from fresh tissue were alike.

RESULTS

Synthesis in relation to activity. Estimations were first made of the synthesizing power of powders from: (*a*) freshly excised auricles, (*b*) auricles which had stopped beating at the end of 20–25 hr., (*c*) auricles which, after stopping, had been started by the addition of acetylcholine.

We made thirty-two observations on fresh auricles and found that the amount synthesized varied from 20 to 75 μg. ACh/g./powder/75 min., the mean figure being $40{\cdot}2 \pm 12{\cdot}7$. The mean figure for stopped auricles was $14{\cdot}9 \pm 10{\cdot}6$. We believe that the wide variation is partly due to variation in the activity of the

ATP preparations used. An investigation of the reason for this variation is in progress, jointly with Dr L. A. Stocken of the Department of Biochemistry, Oxford, to whom we wish to express our thanks for supplying us most generously with the ATP used in this research. In comparing auricles of different kinds, fresh, stopped and restarted, we therefore relied on comparisons between the synthesizing power of each kind on the same day, employing the same reagents. Such results are given in Table 1, in which it is seen that in each experiment the value

TABLE 1. Acetylcholine synthesis by rabbit auricles during 75 min. incubation

			Restarted		Failed to restart	
Exp.	Fresh, synthesized (μg./g.)	Stopped, synthesized (μg./g.)	Synthesized (μg./g.)	ACh added to 75 c.c. bath (μg.)	Synthesized (μg./g.)	ACh added to 75 c.c. bath (μg.)
1	75	<5	40*	50	—	—
2	40	—	35†	100	—	—
3	55	8	—	—	5	100
4	40	8	32	10	5	100
					<5	100
					8	100
5	47	12	40	30	—	—
		18	38	30	—	—

* Determination made on auricle used in Fig. 1.

† Determination made on auricle used in Fig. 3*b*.

for the stopped auricle was much less than that for the fresh auricle, while the value for the restarted auricle approached that of the fresh auricle. The restarted auricles were taken for the determination of synthesizing power 45–60 min. after restarting. The mean figures in the five experiments were, for the fresh auricles 51·4, for the stopped auricles 10·2, for the restarted auricles 37·0. In the first experiment shown in Table 1, the stopped and the restarted auricle had both stopped for 60 min. when the former was taken out of the bath for acetylcholine synthesis, while the beat of the latter was started by the addition of 50 μg. ACh and its synthesizing power was determined 1 hr. later. In the fourth experiment the period of arrest was only 8 min. at the end of which the synthesizing power of the stopped auricle was 8 μg./g. while that of the restarted auricle 45 min. later was 32 μg./g. There appeared to be a clear correspondence between the synthesizing power and the functional state of the auricle.

This conclusion was supported by observations on auricles which had stopped but did not start on the addition of acetylcholine to the bath. On the day of the third experiment in Table 1 two auricles had stopped for 45 min. and the synthesizing power of one was then determined and found to be 8 μg./g. To the other, acetylcholine (100 μg./75 c.c.) was added, but it failed to start. One hour later its synthesizing power was determined and found to be 5 μg./g. The fourth experiment, in which the period of arrest was only 8 min., shows the same point.

This evidence eliminates the possibility that the acetylcholine determined after incubation of the powder might not actually be synthesized but might be the acetylcholine added to the bath.

Further, an experiment was carried out in which two auricles, which had stopped after 25 and 26 hr. respectively, were restarted by the addition of acetylcholine (100 μg./75 c.c.). After 45 min. both were converted to powders and the usual ingredients were added to each. At this point, before incubation, the acetylcholine content of one was determined, while the other was incubated as usual. The non-incubated sample contained no acetylcholine, while the other contained an amount indicating a synthesis of 35 μg./g./75 min.

Effect of acetylcholine on synthesis. Two points had now emerged. The first was that auricles which had stopped could be started again by the addition of acetylcholine. The second was that auricles which had stopped had lost most of their power of synthesis, but regained it when the contractions were restarted. These facts suggested that acetylcholine might influence the power of synthesis *in vitro*.

An experiment was therefore performed, part of which is already recorded as the last experiment in Table 1. The powder from the fresh auricle was divided into two equal parts, and to one of these, before incubation, 0·25 μg. ACh was added per 50 mg. powder. The powder from each of the two stopped auricles was also divided into two equal parts, and the same proportion of acetylcholine (0·25 μg./50 mg. powder) was added to one of them. The results are given in Table 2. The figures in Table 2 show that the addition of acetylcholine before

TABLE 2. Acetylcholine synthesis in rabbit auricles (μg./g./75 min.)

	Incubated without ACh	0·25 μg./50 mg. ACh added before incubation
Fresh	47	33
Stopped	12	20
Stopped	18	30

incubation diminished the synthesis of acetylcholine by the preparation from the fresh auricle, while it augmented the synthesis by the preparation from the stopped auricle.

The estimation was carried out in the following way. For each sample of powder incubated with acetylcholine a control sample containing the same amount of acetylcholine was incubated alongside. By estimation on the frog rectus, the amount of acetylcholine synthesized was determined in comparison with known amounts of acetylcholine added to this control sample. Having thus obtained one estimate of the amount of acetylcholine synthesized, a second estimate was obtained by comparing the solution containing the powder incubated with acetylcholine with the solution containing the powder incubated without acetylcholine after adding the difference found by the first estimate. Good agreement was obtained.

An example is shown in Fig. 4. *A* is the solution containing the powder incubated without acetylcholine. Fig. 4*a* shows that:

2 c.c. *A* is less than 0·2 μg. ACh in 2 c.c. activator control;

2 c.c. *A* is less than 0·18 μg. ACh in 2 c.c. activator control;

2 c.c. *A* is greater than 0·16 μg. ACh in 2 c.c. activator control.

Therefore 2 c.c. *A* = 0·17 μg. ACh.

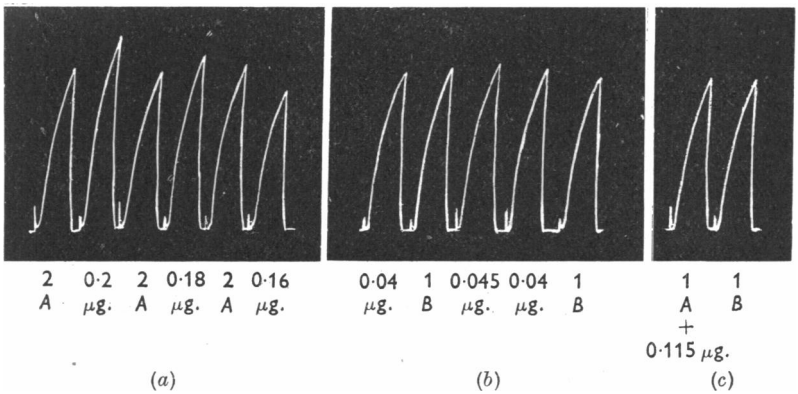

Fig. 4. Part of an acetylcholine assay on the frog rectus described in detail in the text.

As 2 c.c. *A* contained 4 mg. powder, the amount synthesized per g. powder was

$$\frac{0{\cdot}17}{4} \times 1000 = 43\ \mu\text{g./g./75 min.} \qquad (1)$$

B is the solution containing the powder incubated with acetylcholine. The solution contained 2 mg. powder/c.c., and acetylcholine had been added before incubation in the amount of 80 μg./g., or 0·16 μg./2 mg. Fig. 4*b* shows that: 1 c.c. *B* (incubated with 0·16 μg. ACh) was almost equal to 0·04 μg. ACh in 1 c.c. activator control (also incubated with 0·16 μg. ACh per c.c). As 1 c.c. *B* contained 2 mg. powder, the amount synthesized was

$$\frac{0{\cdot}04}{2} \times 1000 = 20\ \mu\text{g./g./75 min.} \qquad (2)$$

The total amount of acetylcholine in 1 c.c. *B* was thus

$$0{\cdot}04 + 0{\cdot}16 = 0{\cdot}2\ \mu\text{g. ACh.}$$

Now 2 c.c. *A* contained 0·17 μg. ACh.,

1 c.c. *A* contained 0·085 μg. ACh.

Hence the amount of acetylcholine to be added to 1 c.c. *A* so that 1 c.c. *A* should produce the same effect as 1 c.c. *B* is

$$0{\cdot}2\ \mu\text{g.} - 0{\cdot}085\ \mu\text{g.} = 0{\cdot}115\ \mu\text{g.}$$

This calculation was confirmed as shown in Fig. 4*c*, in which 1 c.c. *A* + 0·115 μg. is equivalent to 1 c.c. *B*.

If both portions of the same auricle powder had synthesized the same amount of acetylcholine, i.e. 43 μg./g., then the difference between *A* and *B* would have been 80 μg./g., since this was the amount added to *B* before incubation. But actually to match the contraction of the frog rectus produced by 1 c.c. *B* only 0·115 μg. ACh had to be added to 1 c.c. *A*. An addition of 0·115 μg. to 2 mg. corresponds to an addition of 57 μg./g. Since 80 − 57 = 23 μg., this confirms the conclusions in (1) and (2) that sample *B* synthesized 23 μg./g. less than sample *A*.

The rate of synthesis. In order to study the effect of acetylcholine upon the rate of synthesis, as well as on the total amount synthesized, two experiments, one on six auricles and one on seven, were carried out. In both the fresh tissue was ground in activator, and, after centrifuging, the supernatant fluid was used for incubation. In each experiment six aliquot portions were taken, three of which were incubated with 1 μg. acetylcholine, and three without. At varying intervals, a pair of samples (one with ACh and one without) was removed for estimation. The results are shown in Fig. 5 and it is seen that the synthesis at

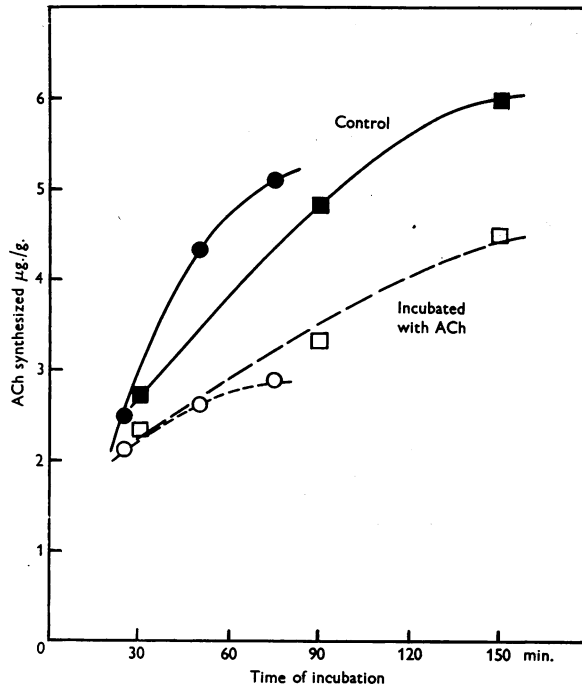

Fig. 5. Two experiments showing the rate of synthesis of acetylcholine by fresh auricle extract (continuous lines), and the slowing of the rate by the addition of acetylcholine (1 μg. to extract of 350 mg. auricle) (broken lines). Ordinates: acetylcholine synthesized (μg. per g. fresh weight). Abscissae: minutes. First experiment shown by squares; second experiment shown by circles.

all points of both experiments is depressed by the addition of acetylcholine. The addition of acetylcholine does not act by allowing synthesis to proceed at a normal rate until a certain total amount is reached, at which point the synthesis stops; but the addition of acetylcholine slows the rate of synthesis at an early stage.

Effect of varying amounts of acetylcholine on fresh auricles. The inhibition by acetylcholine of the synthesis in preparations from fresh auricles shown in Table 2 and Fig. 5 was studied in a series of experiments in which different

amounts of acetylcholine were added to portions of the same powder. For this purpose a sufficient quantity of acetone-dried powder was prepared from the auricles of two, three, or four rabbits for each experiment. The results of fifteen experiments are given in Table 3. In each of these experiments the

TABLE 3. Acetylcholine synthesized by fresh auricles

(μg./g./75 min.)

Incubated without ACh	ACh added per g. acetone powder				
	5 μg.	10 μg.	20 μg.	40 μg.	80 μg.
20	6	5	3	2	—
22	16	12	9	—	—
25	18	19	—	—	0
25	20	20	—	—	6·5
32	—	—	23	—	—
33	—	—	22	—	—
33	—	27	—	22	—
36	—	—	—	18	—
36	—	—	21	—	—
38	—	37	—	—	—
40	—	—	—	25	20
40	—	35	33	31	—
43	—	—	20	—	—
43	—	—	24	27	20
45	33	—	—	—	—
45	40	—	—	—	—
47	33	—	—	—	—

addition of acetylcholine diminished the amount synthesized. The diminution was greater the greater the amount added. In one experiment the synthesis was entirely suppressed by adding 80 μg./g. powder. The mean course of the results is shown in Fig. 6, curve *A*, in which the percentage depression is expressed. The points were obtained by calculating each figure in Table 3 as a percentage of the control figure in the same experiment, and then determining the mean percentage for each amount of acetylcholine added. The curve *A* in Fig. 6 falls to 30% of the initial synthesis; the fall is steepest at first, the addition of 5 μg./g. reducing the synthesis to 70%.

Effect of varying amounts of acetylcholine on stopped auricles. Similar experiments were carried out with preparations made by pooling auricles which had been set up in isolated organ baths the day before and taken down at the end of 21–26 hr. Some of them had stopped beating, but others had not. We have divided the results accordingly. Table 4 gives results of stopped auricles only, while Table 5 gives results of auricles which were taken out of the bath simultaneously but of which only a proportion had stopped.

In Table 4 it is seen that in each experiment the addition of acetylcholine increased the synthesis. In the first experiment the synthesis was doubled with the addition of 5 μg./g., and trebled with the addition of 10 μg./g. With further additions, the increase became less, but the synthesis did not fall below that of the control sample.

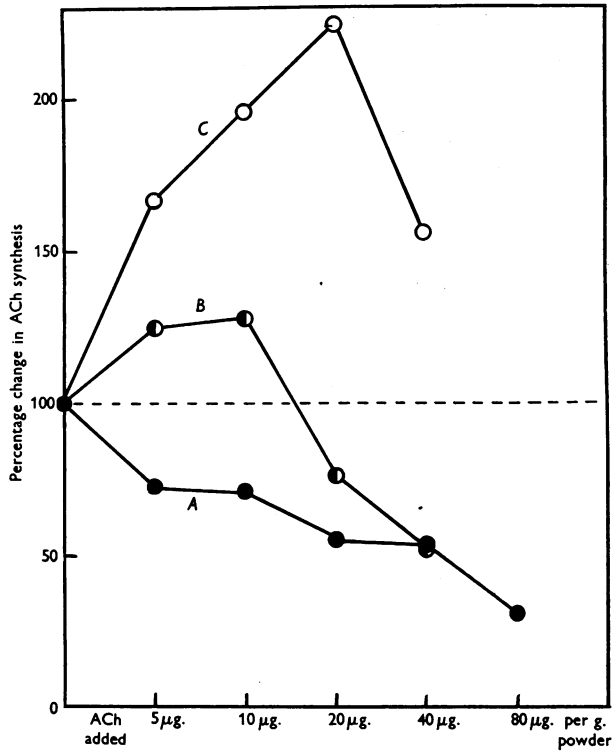

Fig. 6. Effect of acetylcholine on synthesis of acetylcholine by acetone powder prepared from fresh auricles (*A*), stopped auricles (*C*) and auricles not all stopped (*B*). The synthesis in the absence of acetylcholine is taken as 100. The points are calculated from the values in Tables 3–5. Ordinates: percentage change in synthesis. Abscissae: amounts of acetylcholine added per g. powder.

TABLE 4. Acetylcholine synthesized by stopped auricles (μg./g./75 min.)

Incubated without ACh	ACh added per g. acetone powder			
	5 μg.	10 μg.	20 μg.	40 μ
4·5	9	15	13	7
5	—	—	8	—
12	20	—	—	—
18	30	—	—	—
19	—	29	—	—
27	37	—	—	—
29	—	50	—	—
32	—	40	—	—

TABLE 5. Acetylcholine synthesized by auricles not all stopped (μg./g./75 min.)

Proportion of auricles which stopped	Incubated without ACh	ACh added per g. acetone powder			
		5 μg.	10 μg.	20 μg.	40 μg.
0/3	12	12	13·5	9	8·5
1/2	9	11	14	5	3
2/4	20	25	25	21·5	—
3/4	12	19	15	8	—

In Table 5, which gives results obtained with auricles of which only a proportion had stopped, three points are evident. First, that the addition of small amounts of acetylcholine caused an increase in synthesis. Secondly, that the percentage increase was less than that produced by the same additions of acetylcholine in Table 4. Thirdly, that the addition of larger doses of acetylcholine caused a diminution in synthesis below the original value.

Each result in Table 4 and 5 was calculated as a percentage change from the original figure (taken as 100). The mean figure for each addition of acetylcholine was then plotted as shown in Fig. 6, curve *C*. Additions of acetylcholine up to 20 μg./g. produced effects in opposite directions on the synthesis by fresh auricles and on that by stopped auricles. While the synthesis in fresh auricles was depressed, that in stopped auricles was increased; with the larger addition of 40 μg./g., however, the curve for stopped auricles turned round, and synthesis was less than with half this dose. The curve for those auricles of which only a proportion had stopped was found to lie intermediate (curve *B*). With additions of acetylcholine up to 10 μg./g., the synthesis of these auricles increased; with further additions this synthesis was diminished to a similar degree as was that of the fresh auricles.

Some observations (which are being extended) have been made on the synthesizing power of auricles which were still beating at the end of 24 hr. These suggest that there is no gradual fall of synthesizing power, but that it remains within the normal limits of fresh auricles until the beat actually stops, after which it falls rapidly. The effect of acetylcholine on the synthesis by those powders from auricles, of which only a proportion had stopped, must therefore have been the algebraic sum of the increase of synthesis in the stopped auricles and the decrease of synthesis in the auricles still beating.

Effect of potassium. Since acetylcholine has the property of starting a stopped auricle, then, because of the similarity of its pharmacological action, KCl might also start it. Three experiments have been performed to test this. Amounts from 1 to 25 mg. KCl were added to a bath of 75 c.c., and failed to start the beat of the auricle. In one experiment the subsequent addition of 10 μg. acetylcholine started the auricle, and in a second, the subsequent addition of 100 μg. ACh started the auricle, in the third the subsequent addition of acetylcholine did not start the auricle, though it had started it before KCl was tried.

Experiments were also carried out to determine the effect of adding KCl to material prepared for incubation. Acetone powder from fresh auricles was used in two of the experiments, and the auricles freshly ground (after beating for 30 hr.) were used in the third. The results are shown in Table 6, in which amounts from 6 to 48 mg. KCl were added before incubation. The amount of 6 mg. is the normal addition of KCl. In the first experiment there was no inhibition of synthesis by KCl such as was always produced by acetylcholine, and indeed there was a slight increase. Because the amount of potassium

present in the activator (boiled brain extract) was not known, a second experiment was performed omitting the activator. In this experiment the biggest increase (33%) was observed, but again there was no depression. The third experiment was carried out on auricles which were freshly ground with sand and activator, centrifuged, and aliquot amounts of the supernatant incubated. The figures for synthesis are therefore smaller, being calculated per g. fresh weight. A slight depression of synthesis was seen in this experiment in the sample to which 24 mg. KCl was added. We do not regard this single observation, which differs by not more than 12%, as significant. We conclude that the action of KCl does not resemble the action of acetylcholine on synthesizing power.

TABLE 6. Effect of potassium chloride on acetylcholine synthesis (μg./g./75 min.)

	Amount of KCl added per 50 mg. powder or 0·35 g. fresh weight				
Preparation	0	6 mg.	12 mg.	24 mg.	48 mg.
Acetone powder + activator	17·5	22·5	25	26	—
Acetone powder + saline	15	15	17	20	16
Freshly ground + activator	—	6	6·15	5·25	—

Mann, Tennenbaum & Quastel (1939*a*) observed that KCl (0·027 M) increased the synthesis of acetylcholine when intact brain slices were used. Higher concentrations of KCl inhibited the synthesis. In brain brei, however, these authors (1939*b*) found KCl to have much less effect.

Action of adrenaline. The auricle which has stopped at the end of 24–30 hr. can often be started by the addition of adrenaline to the bath, and stops again soon after the adrenaline is removed. In some experiments adrenaline failed to start the auricle when subsequently acetylcholine was successful in doing so. In other experiments adrenaline started the auricle, and after washing out the bath, acetylcholine failed to start it. Torda & Wolff (1944) found that adrenaline increased acetylcholine synthesis in frog brain using the early method of Quastel, Tennenbaum & Wheatley (1936). We found that the synthesis of acetylcholine by a powder prepared from fresh auricle was not modified by the addition of adrenaline in amounts from 0·1 to 10 μg./50 mg. powder.

DISCUSSION

The observation has been made that when the auricles of the rabbit heart have ceased to beat after isolation in Tyrode solution for a period of 24–30 hr., the addition of acetylcholine starts the beat again, and further additions may increase it in rate and amplitude. Thus two effects of acetylcholine can be observed in the same auricle: (1) the usual inhibition which is observed throughout the period in which the auricle is beating, and (2) a stimulation of the stopped auricle shown by the resumption of contractions which gradually increase and may be augmented by a further addition of acetylcholine.

We have observed two other effects of acetylcholine, in parallel to these, in the course of investigating enzyme activity. In studying the power of synthesizing acetylcholine which the rabbit auricle possesses, we have found it to be high in the freshly beating auricle, and to be depressed by acetylcholine. On the other hand, we have found the synthesizing power of the stopped auricle to be low, and to be augmented by acetylcholine. In these observations we therefore have found a parallelism between the pharmacological effect of acetylcholine recorded on the drum and its biochemical effect exerted in the test-tube. Our experiments suggest that the enzymic activity in question, the synthesis of acetylcholine, has a close relation to the spontaneous activity of cardiac muscle. First, the synthesizing power is high in the freshly excised auricle which beats well, and in addition the action of acetylcholine which depresses this beat also depresses the synthesis in the fresh auricle. Secondly, the synthesizing power is low in the auricle which has ceased to contract, and the action of acetylcholine which starts the beat again augments the synthesis also. Thirdly, when the contractions have been restarted by acetylcholine, the synthesizing power approaches the value for fresh auricle.

The conception that acetylcholine may have another function in the body in addition to that of a transmitter of a nervous impulse has arisen for several reasons. Acetylcholine is present in the placenta (Chang & Gaddum, 1933) and synthesized there (Feldberg, 1945) though no transmission of nerve impulses is known to occur.

Evidence that acetylcholine plays a part in cardiac metabolism is suggested also by the experiments of Gremels (1936), who found that the continuous infusion of very small amounts into the heart-lung preparation caused a diminution in the oxygen consumption without any decline in the work done and therefore increased the efficiency.

A further indication is given by the pharmacological action of various substances on muscular tissue. Quinine and quinidine have long been known to prolong the refractory period of cardiac muscle, and in 1946 Dawes, using the isolated rabbit's auricle, found that this property is shared by procaine and many other substances having local anaesthetic and spasmolytic action. He drew attention to the fact that quinine and procaine antagonize the effect of acetylcholine in all forms of muscle. These observations suggested that the power of prolonging the refractory period might also be due to an antagonism to acetylcholine. In 1920, Drury, Lewis & Bulger showed that vagal stimulation reduced the refractory period, and Dawes observed that acetylcholine had the same effect.

The conception that acetylcholine plays a part in some process in cardiac muscle on which the excitability depends, and that this process is antagonized by substances like quinine, required the demonstration that acetylcholine possessed not only an inhibitory action on the heart, but also a stimulant

action. That such an action exists has been shown by Spadolini & Domini (1940), who perfused guinea-pig hearts with Ringer-Locke solution and found that low concentrations of acetylcholine stimulated the heart. The effect was abolished by atropine. McDowall (1946) has made similar observations in cats, rabbits and rats. Burn & Vane (1949) observed that auricles exposed to proguanil (paludrine) could be stimulated by acetylcholine, and when they had stopped, could actually be started by acetylcholine. What action proguanil may have in heart muscle is uncertain, and thus we were led to examine the effect of acetylcholine in auricles which had ceased to beat after many hours in Tyrode solution when no drug had been applied. Having found that acetylcholine started the beat, we proceeded to investigate its effect on acetylcholine synthesis by auricular tissue.

The view that acetylcholine plays a part in the contraction of cardiac muscle has been put forward by Koshtojanz (1938), on the ground that sodium fluoride applied to the frog heart reverses the effect of vagal stimulation. The view has also been put forward by Abdon (1945) who found, together with Hammarskjöld (1944), that rabbit hearts contain a precursor of acetylcholine which they extracted in a pharmacologically inactive form; on heating in acid solution, acetylcholine was liberated. In a later paper (Abdon & Borglin, 1945) on the effect of vagal stimulation no change in precursor content was found during the period in which the heart was arrested, but at the end of stimulation, when the heart beat was presumably good, the precursor content fell. Thus they were unable to demonstrate any diminution in the amount of precursor until vagal stimulation has been applied for at least 10 min. and they did not find any corrleation between this diminution and the heart's action. Later Abdon (1945) described experiments showing that in the isolated perfused rabbit heart the precursor content changed in parallel with the amplitude of the beat. The author does not explain how the amplitude of beat, plotted in his charts as percentage, was determined. However, assuming that a relation is established between precursor content and activity, it would remain to show that it was the precursor store which governed the activity, for the converse might equally be true. The conclusion that the precursor is continually broken down and resynthesized is a deduction since the experimental evidence is only concerned with precursor.

Our experiments have not been concerned with precursor store, but with synthesis of acetylcholine. Beznák (1934) was the first to observe that synthesis of acetylcholine occurred in cardiac tissue; he used the press juice of frog hearts which he incubated in the presence of eserine. He discussed the possibility that acetylcholine is present in the heart as a labile inactive precursor which gives off acetylcholine on vagal stimulation. Our experiments suggest that the normal rhythmic contractions of the heart depend on a synthesis of acetylcholine, which proceeds at a rate which is depressed by an addition of

acetylcholine from without (as when the vagus is stimulated). This might fit with the hypothesis of Brown & Eccles (1934) on the action of acetylcholine on the rhythmic mechanism of the pacemaker. They say 'The rhythmic mechanism of the pacemaker sets up a beat when its excitement reaches a certain threshold intensity. A.C. substance inhibits by acting as a quantitative antagonist to this excitement, the setting up of a beat being delayed until the excitement is built up to such an intensity that the uninhibited excitement attains a threshold value.' If in the foregoing quotation 'the synthesis of acetylcholine' is substituted for 'the building up of excitement', an approximation to our own conception is reached.

In considering the purely biochemical aspects of this work, it is not surprising to find that acetylcholine depresses its own synthesis in freshly excised tissue. It is common to find that addition of an end-product slows a chemical reaction. If, however, we turn to the increase of synthesis which acetylcholine brings about in the stopped auricle, we are faced by a phenomenon which so far as we know has no parallel, and for which we can offer no explanation.

The facts suggest, however, that in these biochemical observations we have a clue to the relation between motor and inhibitor effects. In this auricle these are not due to two distinct mechanisms, but are produced by one mechanism. Thus acetylcholine stimulates contraction when it is applied to auricles in which acetylcholine synthesis is proceeding at a low rate; acetylcholine inhibits contractions in auricles in which the synthesis is proceeding at a higher rate.

When the auricle is left to beat for 24–36 hr. in Tyrode solution, the beat eventually fails. We suppose that in this period some substances are diffusing out of the auricle into the bath, or else some store of material which supplies energy is gradually becoming exhausted. However, until the failure occurs there is no loss of synthesizing power, and any addition of acetylcholine still produces depression. When the beat stops and the synthesizing power rapidly declines, the addition of acetylcholine restores the beat and augments the synthesizing power. The effect of this addition is remarkable because it appears to start a process which then maintains itself, continuing after removal of the added acetylcholine. This strongly supports the view that the activity of the heart and the synthesis are inseparably linked, and that the activity is perhaps responsible for maintaining the synthesizing power.

SUMMARY

1. The auricles of the rabbit heart when freshly excised and placed in a bath of Tyrode solution will beat for periods of 24–36 hr. During this time the beat is depressed by the addition of acetylcholine. When the beat stops, it can be restarted by acetylcholine, and then continues for a long period. A further addition of acetylcholine may increase rate and amplitude.

2. The acetone-dried powder of freshly excised rabbit auricles incubated

according to the method of Feldberg & Mann (1946) synthesizes acetylcholine in varying amounts, the mean figure being 40 μg./g. powder/75 min.

3. The powder from auricles, which have stopped beating after being kept in a bath of Tyrode for 24 hr., synthesizes less acetylcholine, the mean figure being 15 μg./g. powder/75 min.

4. The addition of acetylcholine at the beginning of incubation inhibits the synthesis of acetylcholine by a powder made from fresh auricles. On the other hand, the addition of acetylcholine stimulates the synthesis of acetylcholine by a powder made from stopped auricles.

5. The view is put forward that the activity of the auricular muscle and the synthesis of acetylcholine are inseparably linked.

REFERENCES

Abdon, N.-O. (1945). *Acta Pharmacol. Toxicol.* **1**, 169.
Abdon, N.-O. & Borglin, N. E. (1945). *Acta Pharmacol. Toxicol.* **1**, 162.
Abdon, N.-O. & Hammarskjöld, S. O. (1944). *Acta physiol. Scand.* **8**, 75.
Beznák, A. B. L. (1934). *J. Physiol.* **82**, 129.
Brown, G. L. & Eccles, J. C. (1934). *J. Physiol.* **82**, 242.
Burn, J. H. & Vane, J. R. (1949). *J. Physiol.* **108**, 104.
Chang, H. C. & Gaddum, J. H. (1933). *J. Physiol.* **79**, 255.
Comline, R. S. (1946). *J. Physiol.* **105**, 6*P*.
Dawes, G. S. (1946). *Brit. J. Pharmacol.* **1**, 90.
Drury, A. N., Lewis, T. & Bulger, H. A. (1920). *J. Physiol.* **54**, 97*P*.
Feldberg, W. (1945). *J. Physiol.* **103**, 397.
Feldberg, W. & Mann, T. (1946). *J. Physiol.* **104**, 411.
Gremels, H. (1936). *Arch. exp. Path. Pharmak.* **182**, 1.
Koshtojanz, C. S. (1938). *C.R. Acad. Sci. U.R.S.S.* **19**, 315.
McDowall, R. J. S. (1946). *J. Physiol.* **104**, 392.
Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1939*a*). *Biochem. J.* **33**, 822.
Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1939*b*). *Biochem. J.* **33**, 1506.
Nachmansohn, D. & Machado, A. L. (1943). *J. Neurophysiol.* **6**, 397.
Quastel, J. H., Tennenbaum, M. & Wheatley, A. H. M. (1936). *Biochem. J.* **30**, 1668.
Spadolini, I. & Domini, G. (1940). *Arch. Fisiol.* **40**, 147.
Torda, C. & Wolff, H. G. (1944). *Proc. Soc. Exp. Biol., N.Y.*, **56**, 86.