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A CONTRIBUTION TO THE PHARMACOLOGY OF MOVEMENT IN THE LIVER FLUKE

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Baldwin and Moyle (1949) found that nerve-muscle preparations from ascaria responded in a different manner from those of annelids. The present study has been undertaken to extend this type of comparison to include a representative of the platyhelminthes (trematoda), *Fasciola hepatica*, the liver fluke.

METHODS

The method of collecting and keeping liver flukes for the study of their movement has been described in our previous paper (Chance and Mansour, 1949).

An attempt was first made to isolate a neuro-muscular preparation from the parasite consisting of one layer of muscle, thus exposing a maximum amount of tissue to the effects of the different drugs. It was found that preparations made from only one side of the muscular layer did not show any rhythmical movements on the kymograph.

An alternative preparation which was made by cutting off the oral end provided a means of eliminating the effects of the circum-oesophageal ganglia. This preparation was attached to the kymograph lever in the same way as the whole preparation. When fully relaxed, the muscle between the two attachments was stretched to about 2½ cm., and contracted to a minimum length of about 1 cm. The magnification of the lever was 1½. Fresh, active flukes of a moderate size were chosen. The preparations were completed as quickly as possible in a glass vessel containing medium at 37° C. They were then mounted in a 50-ml. organ-bath. For the composition of the medium see Chance and Mansour (1949). Drugs were added to the organ-bath, and each observation was repeated four times. Initially, movements were rhythmical, very weak, and slow, but they were soon stimulated by the weight of the lever. Addition of weights, or moving the lever up and down to induce tension stimulation, was a good method of initiating rhythmical activity. After this treatment, the majority of the preparations began to show movement of fairly constant frequency and tone for at least two hours.

For the chemical studies the parasites were dissected from the bile ducts of infested cattle, transferred to Locke's solution (maintained at a temperature of 37° C.), and quickly taken to the laboratory. There the flukes were washed in warm Locke's solution, dried between filter papers, and weighed. They remain fully active under these conditions (Chance and Mansour, 1949).

Enzyme extracts were prepared by grinding the whole parasite to a fine suspension in Krebs' Ringer. Warburg's manometric technique was employed to determine the carbon dioxide evolved on incubation with two different substrates; 0.18 M acetyl-β-methylcholine chloride and 0.36 M benzoylcholine chloride.

RESULTS

The undulating movement of the posterior part of the body is one of the characteristic movements of the normal parasite. This movement results in shortening of the body, but it is soon followed by extension. It was found that the undulatory movements of preparations from the degangliated parasites were similar to those of the whole parasite.

Acetylcholine chloride was tested on both preparations at concentrations varying from 10^{-4}–10^{-3}. The whole preparation was unaffected. Only at a concentration of 10^{-3} was a response obtained from the degangliated preparations (Fig. 1). This was a contraction, followed by cessation of normal rhythmical movement in a relaxed position, for 15 minutes, and then followed by gradual recovery. The period of depression varied from one preparation to another in intensity and duration, but was not less than 12 minutes.

Carbaminoylcholine chloride. — Carbachol, which is not readily destroyed by choline esterase, produced complete paralysis in both preparations in concentrations varying from 10^{-4} to 2×10^{-4}; the stronger solution was more dependable in causing paralysis. The effect produced a marked contraction, followed by paralysis in the relaxed position.

Eserine.—The whole and the degangliated preparations were very sensitive to the action of eserine. The response was characterized by either

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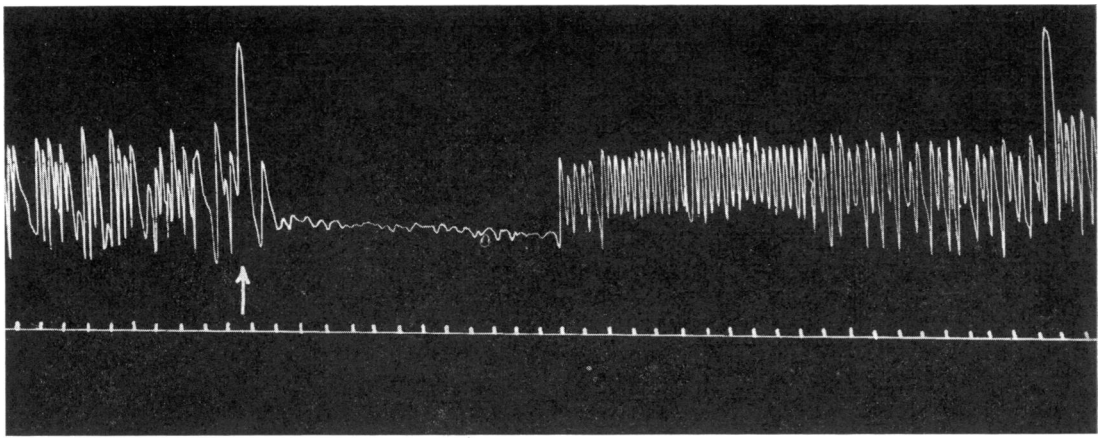

FIG. 1.—Degangliated liver fluke preparation. At the arrow acetylcholine was added, to give a concentration of 10^{-3}. Time marks minutes.

complete cessation of movement or by marked depression of amplitude and rate of rhythmical movement whilst the preparation was relaxed Preparations from the whole or degangliated parasite showed these responses at concentrations above 10^{-5}. The preparations which were not immediately paralysed by eserine were greatly affected, sometimes to the point of paralysis, if left in contact with the drug for about 90 minutes. This shows that the action of eserine is not reversible like that of acetylcholine. It also shows that eserine, like carbachol, lowers the tone and obliterates the rhythmical movement, but it does so more slowly. The two actions differ only in the rate of induction.

Eserinization at concentrations which did not paralyse the preparation was continued for 30–40 minutes, and after this time the acetylcholine was added in appropriate amounts.

The results with the degangliated preparations were as follows: In the degangliated preparation acetylcholine had no effect in the absence of eserine, even in a concentration of 500 μg./ml. (4 exps.). High concentrations of eserine (20 μg./ml. and over) had a direct depressant effect. A concentration of 10 μg./ml. had no effect by itself (13 exps.), but sensitized the preparation to acetylcholine so that a concentration of 100 μg./ml. paralysed one preparation out of 2 and 200 μg./ml. paralysed 4 preparations and depressed 1, and a concentration of 500 μg./ml. paralysed all of 5 preparations. In the presence of eserine (5 μg./ml.) one preparation was depressed and another paralysed by acetylcholine (100 μg./ml.).

In the whole preparation 1,000 μg./ml. of acetylcholine had no effect in the absence of eserine, but 200 μg./ml. depressed preparations sensitized by eserine (2–5 μg./ml.).

Higher concentrations of eserine (10–100 μg./ml.) had some depressant effect and also sensitized the muscle to acetylcholine (100–200 μg./ml.).

Cholinesterase and an Acetylcholine-like Substance.—The results of the estimations of cholinesterase activity as recorded by the evolution of carbon dioxide from the ground material of the liver fluke in the presence of different substrates is seen in Table I. Koelle and Gilman (1949) classify this enzyme in two parts: "true cholinesterase," which will hydrolyse acetyl-β-methylcholine chloride but not benzoylcholine chloride, and "pseudo-cholinesterase," which will hydrolyse benzoylcholine chloride but not acetyl-β-methylcholine chloride. The results have shown that the material from whole parasites could hydrolyse

TABLE I

HYDROLYSING CAPACITY OF THE WHOLE PARASITE TISSUE TO DIFFERENT CHOLINE ESTERS AND ITS ABILITY TO ABSORB CO_2

Substrate	Concentration in 0·5 ml.	No. of Manometers	μl. CO_2 Evolved or Absorbed/g. (Wet Weight)/hr.	Mean
Acetyl-β-methylcholine chloride	0·18 M	3	752	1,008
		3	1,024	
		5	1,256	
		5	1,000	
Benzoylcholine chloride	0·36 M	2	−80	−118
		2	−160	
		2	−64	
		3	−168	
Krebs' Ringer solution		4	−64	−136
		6	−248	
		6	−104	
		3	−96	
		2	−168	

acetyl-β-methylcholine chloride but not benzoylcholine. This fact is evidence for the presence of true cholinesterase in the liver fluke.

The presence of an acetylcholine-like substance and the synthesis of it in the material of the liver fluke was demonstrated with eserinized material from four different samples of mixed and ground fresh liver flukes. It was assayed on the guinea-pig ileum against acetylcholine as a standard. The values for the separate samples were 0.33, 0.2, 0.19, and 1.7 μg./g. wet weight. The remainder of the material was then incubated for one hour and reassayed. The following values were then obtained: 10.8, 4.0, 4.3, and 12.7 μg./g. wet weight. The activity both before and after incubation was blocked by atropine, and a sample of the material was in each instance inactivated after boiling with N/3 NaOH. It may be concluded that an acetylcholine-like substance is present and that it is synthesized by the tissue.

Nicotine.—The responses in whole and in degangliated preparations were characterized by a rapid contraction, which very gradually relaxed to half tone, and continued for a considerable time at this level. A point of interest should be noted here. Nicotine did not reproduce the action of acetylcholine because acetylcholine produced the same strong contraction which was soon followed by a marked relaxation.

Curare and Atropine.—*d*-Tubocurarine chloride and atropine sulphate were found to be without effect on the whole and on the degangliated preparations up to a concentration of 10^{-3} on the whole preparation, and five times this concentration on the degangliated parasite. The above substances were then tested for their antagonism to acetylcholine and carbaminoylcholine chloride.

Atropine sulphate or *d*-tubocurarine chloride at concentrations of 5×10^{-2} were left in contact with the preparations for 1 hour, and the drug to be tested was then added to the organ bath at a high concentration. On the whole and degangliated preparations, atropine and curare were found to cause at least partial block to the effect of acetylcholine and carbachol. The marked contraction which immediately follows the addition of acetylcholine was also abolished.

Eserine, after the usual atropinization or curarization, was tested at concentrations of 10^{-5} to 5×10^{-4}, but at all concentrations its usual effect was undiminished.

Responses to Sympathomimetic and Other Neuromuscular Drugs.—In our previous investigation amphetamine, phenylethylamine, ephedrine, and tyramine were all found to have a stimulant effect on whole parasite preparations (Chance and Mansour, 1949). The present study revealed that phenylalkylamines possess this stimulant effect, and the minimum effective concentration for each amine is enumerated below.

Stimulants: *d*-Amphetamine SO_4, 1 in 80,000; *r*-amphetamine SO_4, 1 in 20,000; *l*-amphetamine SO_4, 1 in 10,000; β-phenylethylamine HCl, 1 in 5,000; propadrine HCl, 1 in 2,000; ephedrine HCl, 1 in 2,000; tyramine HCl, 1 in 1,000; kephrine HCl, 1 in 5,000, and neosynephrine HCl, 1 in 1,000, depressed movement, and adrenaline HCl, 1 in 2,000, had no effect. All these substances constitute Group A referred to below.

Nikethamide, 1 in 1,000, and metrazol, 1 in 1,000, were without effect, but the following substances were all stimulants: Strychnine HCl, cocaine, procaine HCl, amethocaine HCl, quinidine SO_4—referred to as Group B.

Group A has been reported as resistant to the oxidative action of amine oxidase, but able to form stable complexes with this enzyme. On the other hand, catechol compounds, adrenaline, kephrine, and those with a similar pharmacological action (e.g. neosynephrine) did not show any stimulant effect. Adrenaline hydrochloride at concentrations below 2×10^{-3} did not interfere with rhythmical movement, while kephrine and neosynephrine had a depressant action.

Adrenaline is highly sensitive to destruction by amine oxidase, while ephedrine blocks its effect; it was therefore thought worth while to test the effect of adrenaline on preparations treated with ephedrine 10^{-4} for different lengths of time; it was found that, even in the presence of ephedrine, adrenaline hydrochloride had no effect.

In Group B, three local anaesthetics showed a stimulant action; cocaine was the most potent of this group. Strychnine, a convulsant to vertebrates, was a strong stimulant to the degangliated and whole parasite preparations. The type of stimulation due to strychnine was irregular.

Discussion

The rhythmical movement of the degangliated preparation was the same as that of the whole preparation, and the behaviour of the degangliated preparation in response to the drugs which have been tested was (except acetylcholine) also the same. We may conclude, therefore, that the peripheral neuromuscular mechanism possesses its own intrinsic rhythm and that the action of these drugs was on this mechanism, and was not mediated by any action on the anterior ganglia. The responses evoked by different types of

neuromuscular drugs seem to differ strikingly from those obtained with other types of helminths. It is already known that acetylcholine produces a sudden contraction, which is continuous for as long as it is in contact with annelid preparations (Führer, 1918). On exposed ascaris muscle preparations acetylcholine produces a stimulant effect marked by a sharp rise in tone and corresponding fall of amplitude (Baldwin and Moyle, 1949). In contrast, acetylcholine relaxes the fluke and depresses its rhythmical movement after a brief contraction, but recovery is spontaneous in the presence of the drug. Moreover, eserine produces a similar effect and intensifies the action of acetylcholine on the fluke. Baldwin and Moyle (1949) were unable to demonstrate a potentiating action of eserine on the effect of acetylcholine on ascaris. The liver fluke synthesizes an acetylcholine-like substance, and is able to destroy it through the action of true cholinesterase. It seems likely, therefore, that the action of eserine is due to the presence of a cholinergic mechanism, similar in some respects to that found in mammals, which plays a part in the mechanism controlling movement.

The responses of the preparation to the different sympathomimetic amines are surprisingly in line with their stimulant action on the mammalian central nervous system, as found by Schulte *et al.* (1941), with mice in "jiggle" cages. These authors found *d*-amphetamine the most potent amine. In their tables, the substances are arranged in descending order of potency, assessed by the dose required to produce maximum activity at the peak period. The order was *d*-amphetamine, *l*-amphetamine, propadrine, phenylethylamine, ephedrine, and adrenaline. Tyramine and neosynephrine did not show any stimulant effect in any dose tolerated by mice. The order here is not very different from the action on flukes. The three amphetamine derivatives had the same order, while phenylethylamine was superior to propadrine. Tyramine showed a definite stimulant effect on the liver fluke, but it did not on the activity of mice, while neosynephrine and kephrine depressed the rhythmical movement.

Morton and Tainter (1940) and Lawrence, Morton, and Tainter (1942) made use of the hypothesis put forward by Gaddum and Kwiatkowski (1938), on the mode of action of ephedrine, to explain the mechanism of other amine oxidase inhibitors. This enzyme is responsible for the deamination of some of the sympathomimetic amines, including adrenaline. Tainter and his colleagues put tyramine, propadrine, phenylethylamine, and cocaine among the ephedrine group; while neosynephrine was put in the adrenaline group, which owe their effect to a direct action on the effector cells. From the results reported here, it can be seen that all the amines of the ephedrine group, and also cocaine, possessed a stimulant action. Yet amines of the adrenaline group were either ineffective or had a depressant action.

Amphetamine, as a representative of these sympathomimetic amines, produced its stimulant effect in the presence of nicotine and carbachol. Conversely, however, when the fluke was under the influence of any stimulant drug, the preparation was insensitive to the inhibiting action of carbachol.

Degangliated and whole preparations were highly sensitive to the stimulant action of strychnine. Nachmansohn (1941) demonstrated *in vitro* that strychnine inhibits the hydrolysis of acetylcholine by cholinesterase. He then attributed the effect of strychnine on the central nervous system to the fact that this stimulant causes an increase in the acetylcholine, and this in turn causes a marked condition of excitement. This cannot be so in the case of the fluke, since an increase of acetylcholine intrinsically produced by eserine, or extrinsically by the addition of acetylcholine itself, causes depression to the rhythmical movement, if not complete relaxation.

Most of these stimulant compounds were tried by Baldwin and Moyle (1949) on nerve-muscle preparations from ascaris. They found that cocaine possesses a paralysing action, while strychnine, ephedrine, and tyramine are innocuous to rhythmical movement of a preparation. This difference provides further evidence of the existence of important physiological and pharmacological differences between the liver fluke and ascaris.

Acetylcholine is well known to be closely connected with the motor activity of invertebrates and vertebrates (Bacq, 1949). A recent and convincing contribution in this field of study has been the work by Bülbring, Lourie, and Pardoe (1949). They demonstrated the manner in which *Trypanosoma rhodesiense* (motile protozoa) contains and synthesizes acetylcholine. It was also proved that the non-motile protozoa *Plasmodium gallinaceum* neither contained nor synthesized acetylcholine. Bülbring and Burn (1949) provided evidence to suggest that even in mammalian heart muscle, where acetylcholine is supposed to be inhibitory, the activity of atrial muscle is closely linked with the synthesis of acetylcholine.

Fasciola hepatica is a parasite well equipped for a high degree of motility. Motor activity of neuromuscular preparations has been recorded on the kymograph, even in the absence of the central ganglia in the proboscis of the parasite (Chance and Mansour, 1949). Acetylcholine and its allied compounds were found to be inhibitory to the muscular activity recorded from these preparations. In important respects, therefore, the musculature of the liver fluke resembles the heart muscle of mammals, not only in the rhythmical nature of the movement, but also in the possession of cholinergic components and in its response to acetylcholine.

The presence of cholinesterase in the fluke provides an explanation of the effects of the cholinergic drugs, the action of which was the same on the whole and degangliated preparations, and makes it likely that the absence of the effect of acetylcholine at 10^{-3} was due to slow penetration resulting in the destruction of the substance before it could reach the receptors, rather than that it has an opposing action on the ganglia.

Summary

1. A technique for the removal of the central ganglia in the liver fluke has been described and the action of drugs on this preparation has been compared with the whole animal.

2. These preparations showed rhythmical movement when suspended under tension in Ringer solution. Carbaminoylcholine chloride, acetylcholine, and eserine affected the degangliated preparation in a similar manner to the normal, either producing paralysis of movement or reducing the amplitude of the contractions.

The relaxing action of acetylcholine was temporary, but that of carbaminoylcholine chloride was long-lasting.

Eserine sensitized the preparation to the effect of acetylcholine.

3. The effect of the two choline esters was blocked by atropine or curare.

4. Rhythmical activity of the preparations of the liver fluke was stimulated by some sympathomimetic amines. There is a close similarity between this action and the action of these substances as central nervous system stimulants in mammals.

5. These results, when compared with published work on ascaria, show that these two parasites differ markedly in their reaction to the same drugs.

6. Liver flukes have been found to contain an acetylcholine-like substance in amounts equivalent to 0.19–1.7 μg./g. (wet weight) of acetylcholine.

The formation of an acetylcholine-like substance has been demonstrated *in vitro* in amounts equivalent to 4.0–12.7 μg./g. (wet weight) of acetylcholine in 1 hr. at 37° C.

The presence of true cholinesterase and the absence of pseudo-cholinesterase in the liver fluke has also been demonstrated.

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