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THE POSSIBLE ROLE OF ACETYLCHOLINE IN *SCHISTOSOMA MANSONI*

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It has been demonstrated that extracts of *Schistosoma mansoni* contain high acetylcholinesterase and choline acetylase activities (Bueding, 1952). Subsequently, acetylcholine esterase and choline acetylase activities were found by Chance & Mansour (1953) in another trematode, the liver fluke *Fasciola hepatica*. Furthermore, it has been reported that *Fasciola hepatica* contains an acetylcholine-like substance and that a variety of cholinomimetic agents exert an inhibitory effect on the muscular activity of this parasite (Chance & Mansour, 1953). Similarly, inhibitory effects of some cholinomimetic agents on the motor activity of *Schistosoma mansoni* have been observed (Bueding, 1962). This report deals with motor responses of *Schistosoma mansoni* to cholinomimetic and other pharmacological agents and with experiments designed to determine the presence of acetylcholine in this parasite. Observations are reported indicating that cholinomimetic agents and cholinesterase inhibitors produce a paralysis of the worms, which is reversed by certain antagonists of acetylcholine-like drugs. Furthermore, *S. mansoni* contains a material the properties of which are similar to those of acetylcholine.

METHODS

Individual adult worm pairs of *Schistosoma mansoni* (Puerto Rican strain) were incubated in 75% horse serum in distilled water at 37° C in cylindrical glass vessels (17 mm inside diameter, 12 mm high). Motility of the worms was observed under a dissecting microscope at predetermined time intervals (5 min, 15 min, thereafter every 15 min, for 1 hr, and thereafter every hour for two to four hours). At least four worm pairs (i.e., four males and four females) were used at any given concentration of a tested compound. Normal motor activity consisted of waves of contraction along the entire body, with occasional whipping motion and of frequent contractions of the oral sucker and acetabulum. Activity of the acetabulum may also result in adhesion to the bottom of the vessel with occasional moving along the glass. Paralysis and stimulation are readily observable deviations from this motor behaviour. The activity of control worms in 75% horse serum did not change significantly during 5 hr, the maximum period of observation.

With two exceptions (see below) all compounds were added in an aqueous solution to 0.75 ml. of horse serum and enough distilled water was added to bring the total volume to 1.0 ml. The two water-insoluble compounds, 0,0-dimethyl-0-(2,4,5-trichlorophenyl) phosphorothioate (Ronnel) and its oxygen analogue (McCollister *et al.*, 1959) were dissolved in 95% ethanol and 0.02 ml. of this solution were added to a mixture of 0.75 ml. of horse serum and 0.23 ml. of water. The motor activity of schistosomes was not affected in 2% ethanol.

For the identification of acetylcholine, schistosomes were homogenized in an ice-cold solution of perchloric acid (3% w/v), using an all-glass homogenizer surrounded with ice. Perchloric acid solution, 0.2 ml., was used per 100 worm pairs. The homogenate was centrifuged at 12,000 g for 20 min at 2° C. A measured volume of the resulting supernatant layer was neutralized with 2N KOH to *p*H 7.0–7.5 and stored in the frozen state. This procedure was repeated until a total of 2,060 worm pairs had been collected. The pooled neutralized extract was defrosted, centrifuged at 5,000 g for 15 min at 2° C and the resulting supernatant solution stored in the frozen state until its effect on the isolated ileum was tested. For this purpose, male albino guinea-pigs (Hartley strain) weighing 400–500 g were killed and exsanguinated from the jugular veins. The terminal ileum was located, and about six inches were removed from the animal. The intestinal contents were removed by washing with a Tyrode's solution of the following composition (g/l.) : NaCl 8.0, KCl 0.2, $MgCl_2$ 0.05, NaH_2PO_4 0.04, $NaHCO_3$ 1.0, glucose 1.0. The solution was gassed with pure oxygen, and contained 0.1 μg/ml. mepyramine maleate to eliminate the effects of histamine in the extracts. The tissue was placed in a beaker of oxygenated Tyrode's solution at room temperature. A 2 cm segment of tissue was set up in a 15 ml. isolated organ bath, which was maintained at 36.5 ± 0.5° C. Pure oxygen entered the bath from the bottom through a sintered glass dispersion disc. The tissue was attached to a frontal writing lever with a tension of 2 g and a magnification of $\times 6$.

Drugs were added to the bath from hypodermic syringes after removing an equivalent volume of fluid from the organ bath in order to maintain a constant bath volume. Spasmogens were added every 2 min and allowed to stay in contact with the tissue for exactly 10 sec. Drugs were washed from the bath by overflow. Where indicated, atropine sulphate was added 1.5 min before the addition of spasmogen.

Cholinesterase was prepared by centrifuging whole heparinized guinea-pig blood at 2,000 *g* for 15 min in a refrigerated centrifuge. The packed cells were suspended four times in three volumes of 0.9% saline, the cells being separated by centrifugation after each washing. The washed cells, lysed by addition of three volumes of distilled water, were used as the final enzyme preparation. Incubation with cholinesterase was carried out at 37° C in an Eberbach water bath with shaking. Acetylcholine 0.15 ml., (1.5 μg/ml.), or 0.2 ml. extract were incubated with 0.2 ml. enzyme in the presence of 0.2 ml. 0.05 M potassium phosphate buffer, *p*H 6.7. The final incubation volume was 0.6 ml., the balance consisting of 0.9% NaCl. A control, containing enzyme and buffer alone, was incubated for the same period of time. After 15 min the contents of the tubes were added to the organ bath. To establish that enzymatic solution of the spasmogens was due to an esterase, the same incubations were carried out following the addition of 1 μg of neostigmine bromide in 0.01 ml. saline to all tubes. The final concentration of neostigmine in the incubation mixture was 5.4×10^{-6} M.

RESULTS

Cholinomimetic and anticholinesterase drugs

Carbachol and arecoline, as well as several cholinesterase inhibitors, produced a flaccid paralysis of the worms ; onset of paralysis occurred within two min of exposure to these drugs. All of the compounds lifted in Table 1 produced the same qualitative effect. When paralysis was complete, the worm was entirely motionless, stretched out, and flaccid ; both the oral sucker and the acetabulum were immobile in a splayed-out fashion. At lower concentrations, all compounds produced a complete paralysis of the latter two organs, but little or no depression of body motility (Table 1). This higher sensitivity to these agents was also readily observable when the heads of the worms were cut off below the acetabulum. For a period of at least one hr, both the isolated head preparations and the headless bodies maintained the same motor activity as that seen in intact worms. Paralysis in these isolated preparations was produced by the same cholinomimetic agents.

TABLE 1

CHOLINOMIMETIC DRUGS AND ANTICHOLINESTERASES PRODUCING PARALYSIS OF *SCHISTOSOMA MANSONI*

Property	Compound	Minimal molar concentration producing paralysis of: Body musculature	Minimal molar concentration producing paralysis of: Oral sucker and acetabulum
Cholinomimetic agents	Arecoline	5×10^{-6}	2×10^{-7}
	Carbachol	1×10^{-4}	2×10^{-5}
	Acetylcholine*	1×10^{-2}	3×10^{-3}
Cholinesterase inhibitor	Neostigmine	1×10^{-3}	5×10^{-5}
	Physostigmine	1×10^{-5}	2×10^{-6}
	Dyflos	1×10^{-3}	1×10^{-4}
	Dipterex	1×10^{-4}	2×10^{-5}
	Oxygen analogue of Ronnel	5×10^{-4}	1×10^{-4}

* In glucose-containing buffered salt medium.

It should be noted that in a buffered glucose-containing salt solution, acetylcholine produced a paralysis of the body musculature and of the two suckers at molar concentrations of 1×10^{-2} and 3×10^{-3}, respectively. Acetylcholine was ineffective in 75% serum, probably because of its hydrolysis by serum cholinesterase. Some cholinomimetic agents, including muscarine and pilocarpine, failed to produce a paralysis of schistosomes (Table 2).

TABLE 2

CHOLINOMIMETIC DRUGS LACKING AN EFFECT ON THE MOTOR ACTIVITY OF *SCHISTOSOMA MANSONI*

Drug	Molar concentration range tested
Pilocarpine	1×10^{-7} to 1×10^{-2}
Muscarine	1×10^{-7} to 1×10^{-2}
Nicotine	1×10^{-7} to 1×10^{-3}
Methacholine	1×10^{-5} to 1×10^{-2}
Ronnel	1×10^{-5} to 1×10^{-3}

Worms exposed for one hour to paralyzing concentrations of two organic phosphorus compounds—i.e., dyflos (1×10^{-3} molar) or 0,0-dimethylhydroxy-2,2,2-trichlorethylphosphonate (Dipterex) (1×10^{-4} molar)—and after 3 transfers into 75% serum containing no cholinesterase inhibitor, were incubated in this medium; under these conditions, the motor depression persisted for at least one hour. However, under these conditions, the paralysis was reversed completely by 2-pyridine aldoxime methochloride (PAM) in a concentration of 1×10^{-3} molar. This compound has been shown to reactivate acetylcholinesterase inhibited by organic phosphorus compounds (Wilson and Ginsberg, 1955; Kewitz and Nachmansohn, 1957).

Reversal of paralysis

Paralysis of schistosomes produced by cholinomimetic agents was abolished by several acetylcholine antagonists (Table 3). These agents usually produced a stimulatory effect as well as a reversal of paralysis when added with any of the paralyzing agents. Furthermore, when added alone, they produced hyperactivity of the body musculature, of the

TABLE 3

ACETYLCHOLINE ANTAGONISTS WHICH STIMULATE THE MOTOR ACTIVITY OF *S. MANSONI* AND WHICH REVERSE CARBACHOL-INDUCED PARALYSIS OF THE PARASITE

Blocking agent	Minimal stimulatory molar concentration	Minimal molar concentration reversing carbachol-induced paralysis
Mecamylamine	2×10^{-4}	5×10^{-5}
Pempidine	2×10^{-4}	2×10^{-4}
Atropine	2×10^{-4}	5×10^{-5}
SU-1194 (7)	8×10^{-3}	8×10^{-3}

TABLE 4

NEUROMUSCULAR AND GANGLION BLOCKING AGENTS WHICH HAVE NO EFFECT ON THE MOTOR ACTIVITY OF *S. MANSONI* AND WHICH FAIL TO BLOCK THE CARBACHOL-INDUCED PARALYSIS OF THE WORM

Nature of blocking agent	Blocking agent	Molar concentration range tested
Ganglion	Tetramethylammonium	2×10^{-6} to 2×10^{-2}
	Hexamethonium	5×10^{-5} to 2×10^{-3}
	Pentolinium	2×10^{-4} to 2×10^{-2}
	Chlorisondamine	1×10^{-4} to 1×10^{-3}
Neuromuscular	d-Tubocurarine	5×10^{-5} to 1×10^{-3}
	Decamethonium	5×10^{-5} to 1×10^{-3}
	Succinylcholine	1×10^{-4} to 1×10^{-3}

TABLE 5

COMPOUNDS DEVOID OF ANTI-ACETYLCHOLINE ACTIVITY PRODUCING STIMULATION OF MUSCULAR ACTIVITY OF *S. MANSONI*

Compound	Onset of action	Minimal effective molar concentration
5-Hydroxytryptamine	Immediate	2×10^{-5}
Amphetamine	Immediate	1×10^{-4}
Reserpine	45 to 60 min	1×10^{-4}
Tyramine	50 to 60 min	1×10^{-4}
5-Hydroxytryptophane	45 to 60 min	1×10^{-3}

oral sucker, and of the acetabulum in the intact worm, as well as in the separated head and the headless body preparations. The latter action may be due to a blockade of endogenous acetylcholine in the worm. This stimulatory effect usually persisted for several hours, and eventually was followed by depression. SU-1194 (bis-diethylaminoethyldiethylaminoethylamine) (Plummer *et al.*, 1954), in addition, produced an opening of the male's gynecophoric canal, resulting in the release of the female. Table 4 lists those blocking agents which failed both to reverse carbachol paralysis or to stimulate the motor activity of the worms in the absence of a cholinomimetic agent.

Carbachol paralysis was also abolished by several agents which do not block mammalian cholinoceptive receptors (Table 5). These agents produced stimulation when added with or without cholinomimetic agents. The onset of stimulation was immediate with 5-hydroxytryptamine (5-HT) (Mansour, 1957) and with amphetamine, but occurred only after 45 to 60 min with reserpine, tyramine, and 5-hydroxytryptophane. The

minimal effective concentrations of these compounds are listed in Table 5. The stimulation produced by these agents was qualitatively similar to that produced by the blocking agents listed in Table 3.

Effect of an extract of Schistosoma mansoni *on the isolated guinea-pig ileum*

Fig. 1 shows that the neutralized perchloric acid extract of *S. mansoni* had spasmogenic activity. The contraction produced was rapid and not of the slowly developing type, as would be seen, for instance, with bradykinin. Some indication of a secondary contraction was observed when the extract was washed from the organ bath. This was probably due to the presence of a second spasmogenic factor in the extract, but no attempt has been made to identify it. The contraction produced by 0.2 ml. extract was between that produced by 0.01 μg and 0.02 μg acetylcholine/ml.

The spasmogenic activity of the extract and of an approximately equiactive amount of acetylcholine was destroyed by incubation with red cell cholinesterase. This is illustrated in Fig. 2. The cholinesterase preparation itself did not inhibit subsequent additions of acetylcholine or of the extract, so that the failure of acetylcholine or of the extract to stimulate the muscle after cholinesterase incubation was apparently not due to non-specific inhibition by some constituent of the enzyme preparation.

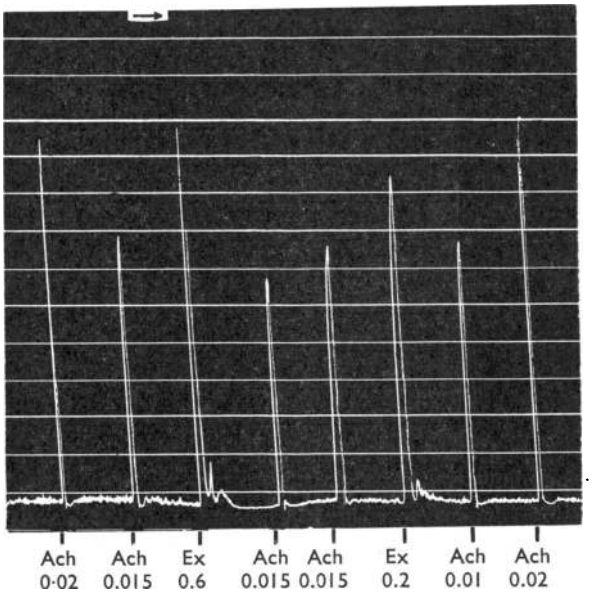

Fig. 1. Spasmogenic activity of *S. mansoni* extract (Ex). At Ach acetylcholine was injected into the bath. Figures indicate the final bath concentration in μg/ml. At Ex the indicated volume (in ml.) of neutralized perchloric acid extract of *S. mansoni* (see Methods) was added. 0.6 ml. caused a contraction which could not be recorded beyond the arrow (——→). Accordingly 0.2 ml. Ex was added giving a contraction in the acceptable range. Subsequent responses to acetylcholine were not affected by previous exposure of the preparation to the extract. Note that the contraction due to Ex was rapid in onset and was easily reversed by washing: a small secondary contraction was observed after washing.

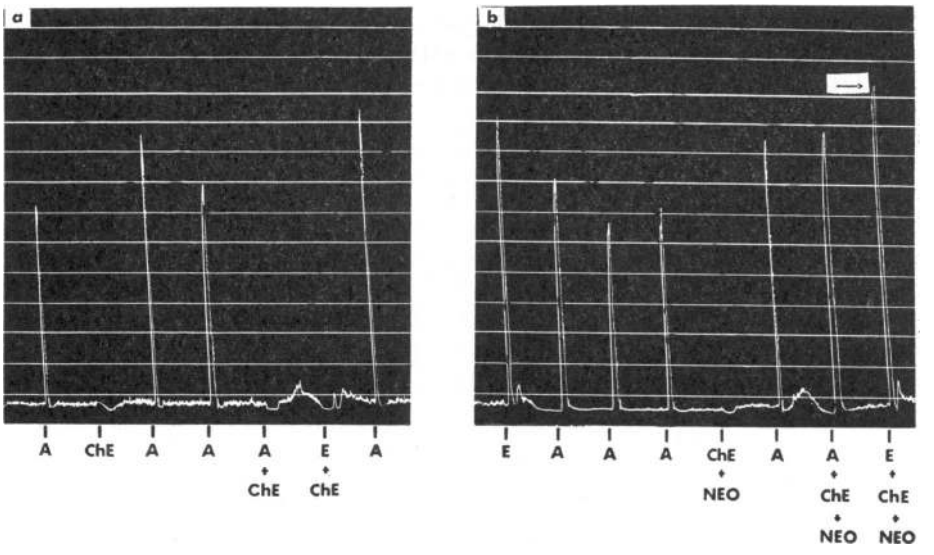

Fig. 2. Effect of cholinesterase on the spasmogenic activity on *S. mansoni* extracts. For details of incubations see Methods. Same preparations as in Fig. 1. At A Ach 0.015 μg/ml. E—*S. mansoni* extract 0.2 ml. ChE—Cholinesterase preparation. In panel a. contractions due to A and E were abolished by incubation with ChE. At ChE the control enzyme incubation (i.e., enzyme + buffer + saline) was added to insure that subsequent contractions due to Ach were not inhibited by any constituent of the incubation mixture. The spasmogenic effect was not lost when ChE incubations were carried out in the presence of neostigmine bromide 5.14×10^{-6} M (panel b).

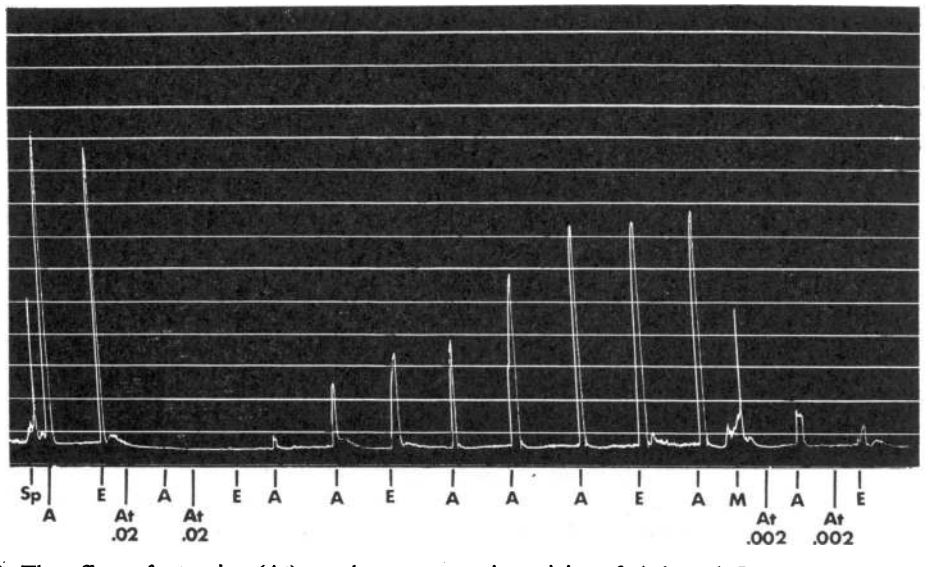

Fig. 3. The effect of atropine (At) on the spasmogenic activity of Ach and *S. mansoni* extract (E). Same experiment as in Figs 1 and 2. At A, ACh .015 μg/ml. At E—0.15 ml. extract. Sp—spontaneous contraction. M—release of mucus.

When the same incubations were carried out in the presence of 5.4×10^{-6} M-neostigmine bromide, the spasmogenic activities of the extract and of acetylcholine were no longer destroyed (Fig. 2, panel b), suggesting that the loss of activity in the absence of neostigmine was due to hydrolysis by an esterase.

The spasmogenic activity of both the extract and of acetylcholine could be inhibited completely by low concentrations of atropine. Fig. 3 illustrates that atropine (0.02 μg/ml.) inhibited both acetylcholine and the extract completely. Recovery from this inhibition occurred at the same rate with acetylcholine as with the extract. When recovery was apparently complete, 0.002 μg atropine/ml. was added to the bath. Both the extract and acetylcholine itself now produced very small contractions and these were of approximately the same magnitude. Thus the sensitivity of the extract to atropine was approximately the same as that of an equiactive amount of acetylcholine.

The evidence, therefore, suggested the presence in *S. mansoni* extracts of a spasmogenic substance with properties similar to those of acetylcholine. A dose-effect relationship for the extract and for acetylcholine suggested that 0.2 ml. extract contained activity equivalent to 0.23 μg acetylcholine. From this it was possible to make an approximate estimation that the parasites contained spasmogenic activity equivalent to 5.3 μg acetylcholine per g wet weight.

DISCUSSION

The evidence presented suggests the presence of cholinoceptive receptors in *S. mansoni*. However, these receptors are apparently not identical with any of the three well-defined types of cholinoceptive receptors of their mammalian host. Autonomic cholinergically innervated effector organs of vertebrates—i.e., those with "muscarinic" receptors—are stimulated by muscarine, pilocarpine, methacholine, and arecoline, whereas the worms are paralysed by arecoline, but not by the other three agents. The responses both of *S. mansoni* and of mammalian ganglionic receptors are blocked by mecamylamine, pempidine, SU-1194, and relatively high concentrations of atropine; these four agents are secondary and tertiary amines. On the other hand, in contrast to the liver fluke (Chance & Mansour, 1953), nicotine has no effect on the motor activity of schistosomes. Furthermore, none of the quaternary ammonium ganglion blocking agents and no neuro-muscular blocking agent reversed the carbachol-induced paralysis of the worm. It appears unlikely that this is due to a lack of permeability of schistosomes to quaternary ammonium compounds, because the worms respond to other compounds belonging to this category—e.g., acetylcholine, carbachol and arecoline. It is more probable that the cholinoceptive receptors of schistosomes have some similarities with receptors in the autonomic ganglia of their host, but are not identical with them nor with the other two pharmacologically defined cholinoceptive receptors of their host. Therefore, there is a possibility, at least theoretically, for designing cholinomimetic agents selective for the worm.

It has been reported that atropine and d-tubocurarine do not affect the motor activity of *Fasciola hepatica* but that they reverse, at least partially, paralysis by cholinomimetic agents in this parasite (Chance & Mansour, 1953). By contrast, in *Schistosoma mansoni* atropine both stimulates motor activity and blocks cholinomimetic effects, while d-tubocurarine has neither of these properties. Another difference between these two parasites is reflected in the relative lack of sensitivity of *Fasciola hepatica* to physostigmine (Chance

& Mansour, 1949). On the other hand, this trematode, like *Schistosoma mansoni,* is paralysed by low concentrations of arecoline, but its motor activity is not affected by pilocarpine or methacholine (Chance & Mansour, 1949).

It has been stated that the cholinesterase inhibitor Dipterex (Cerf *et al.,* 1962 ; Taläat *et al.,* 1963) is an antischistosomal agent *in vivo.* The concentration of this compound required to produce paralysis of the worms *in vitro* is rather high, when compared with the dosage claimed to be effective chemotherapeutically. Therefore, it is questionable whether cholinesterase inhibition can account for an antischistosomal action of this organic phosphorus derivative *in vivo.* The same applies to Ronnel and its oxygen analogue.

The paralysis of schistosomes produced by cholinergic agents may be analogous to the inhibitory action of acetylcholine on vertebrate heart and vascular smooth muscle. A similar effect of this neurohormone on the molluscan heart has been demonstrated by Welsh (1953) ; furthermore, cholinomimetic agents reduee the motor activity of the trematode liver fluke (Chance & Mansour, 1953).

A number of observations reported above suggest the occurrence of an endogenous inhibitory cholinergic transmitter in schistosomes. For example, cholinesterase inhibitors produce a paralysis of the worm indistinguishable from that induced by carbachol. Therefore, it appears that inhibition of acetylcholinesterase increases the concentration of endogenous acetylcholine, resulting in an inhibitory action on the motor activity of the worm. Furthermore, acetylcholine esterase and choline acetylase activities of schistosomes are of a high order and equal to those of mammalian cortical grey matter (Bueding, 1952). In addition, every compound which blocks the response of the worm to cholinomimetic agents also produces stimulation of its motor activity when added alone. This effect could be explained by a block of an interaction of the worm's cholinoceptive receptors with endogenous acetylcholine, resulting in the failure of this humoral transmitter to exert its physiological inhibitory effect on motor activity. In this connection, it should be noted also that administration of the antischistosomal drug p-rosaniline (Thompson *et al.,* 1962) results in a histochemically demonstrable inhibition of acetylcholinesterase in the acetabulum and the oral sucker (Schiller *et al.,* 1964). This inhibition is associated with a paralysis of these two organs, a loss of the attachment of the worms to the mesenteric veins and their shift towards the liver. The paralysis of the two sucker organs as a result of the administration of p-rosaniline to the host is reversed by mecamylamine and atropine *in vitro* (Schiller *et al.,* 1964). Finally, the evidence obtained in this study strongly suggests the presence of acetylcholine in a neutralized perchloric acid extract of *S. mansoni.* First, the extract produces a rapid contraction of the isolated ileum of the guinea-pig. Second, the spasmogenic activities of the extract and of an equiactive amount of acetylcholine were destroyed by incubation with a preparation of acetylcholinesterase from the guinea-pig's erythrocytes. Third, the destruction of both acetylcholine and of the spasmodic activity of the extract by cholinesterase are prevented by neostigmine. Fourth, atropine antagonizes the effects of both acetylcholine and of the extract to the same degree. The high sensitivity of the spasmogenic effect of the extract to atropine would strongly suggest that the activity is indeed due to acetylcholine or some closely related choline ester. This evidence, together with the presence of mepyramine, a specific antihistamine (Schild, 1947), in the bathing fluid, rules out

histamine as being responsible for the spasmogenic activity of the extract. The active material cannot be substance P because the activity of the extract is sensitive to cholinesterase and to low concentrations of atropine, whereas substance P has been shown to be resistant to both of these substances (Pernow, 1953). 5-Hydroxytryptamine is also ruled out because its spasmogenic activity is not completely antagonized by such small concentrations of atropine (Cambridge & Holgate, 1955; Timms, 1956). On the basis of this evidence, it is suggested that the spasmogenic activity of the schistosome extract is due to acetylcholine or some closely related substance and that its concentrations in these worms is approximately as high as in the grey matter of mammalian brain cortex (Giarman & Pepeu, 1964) and 3 to 30 times higher than in the liver fluke (Chance & Mansour, 1953). Definitive characterization of the material must await chemical identification.

Although 5-hydroxytryptamine (5-HT) and the other compounds listed in Table 5 do not belong to the category of acetylcholine antagonists, they produce similar stimulatory effects on *S. mansoni*. Welsh (1957) has shown that 5-HT is a potent stimulant of the heart of molluscs, and has obtained evidence that it may be a physiological transmitter in these organisms. 5-HT, amphetamine and tyramine also have been shown (Chance & Mansour, 1949 and 1953; Mansour, 1957) to stimulate the motor activity of the trematode *Fasciola hepatica*. On the basis of the available evidence, it cannot be ascertained whether the stimulatory effects of 5-HT on the motor activity of schistosomes are those of a physiological transmitter or of a pharmacological agent. The delayed stimulatory effects of 5-hydroxytryptophane could be due to a slow rate of its decarboxylation in the worm, resulting in the formation of 5-HT.

The presence in schistosomes of a physiological stimulatory transmitter substance is suggested by the motor hyperactivity of schistosomes by tyramine and by reserpine, two compounds known to release 5-HT and other amines in vertebrate tissues. However, it would be premature to ascribe this effect to 5-HT *per se*, since no positive identification of this amine has been made in *S. mansoni*.

SUMMARY

1. The motor activity of *Schistosoma mansoni* in response to cholinomimetic agents has been studied. Carbachol, arecoline, and a variety of cholinesterase inhibitors produced a paralysis of the worms. Considerably lower concentrations of these compounds were required to paralyse the oral sucker and the acetabulum than the remainder of the body musculature. Pilocarpine, muscarine, methacholine, and nicotine had no effect on the motor activity of the worms.

2. Paralysis of schistosomes produced by cholinomimetic agents and cholinesterase inhibitors was reversed by atropine and by secondary and tertiary amines, but not by quaternary ammonium ganglion blocking agents.

3. Evidence is reported suggesting the presence of acetylcholine in *S. mansoni* in a concentration equal to that in the grey matter of brain cortex.

4. The motor responses of schistosomes to various pharmacological agents suggest the presence in the worm of an inhibitory cholinergic system.

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