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ANTICHOLINERGIC PROPERTIES OF THE ANTISCHISTOSOMAL DRUG HYCANTHONE*

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Abstract. The effect of the antischistosomal drug hycanthone on the motor activity of *Schistosoma mansoni* was studied in vitro. Hycanthone stimulates motor activity at concentrations of 10^{-6} to 10^{-5} M, and partially blocks the paralytic effects of carbachol and physostigmine. Lucanthone, a closely related although less active congener of hycanthone, does not produce these same effects in vitro. Some blocking of acetylcholine can also be produced by atropine, although this drug is less active in this regard than is hycanthone. These findings suggest that the therapeutic efficacy of hycanthone may be related to interference with acetylcholine receptors in schistosomes. Hycanthone is an inhibitor of acetylcholinesterase (ACHE) from *S. mansoni*, but is less effective against ACHE of mammalian origin. In contrast, physostigmine inhibits the mammalian enzyme more effectively than it does the helminth enzyme. These observations suggest that schistosome ACHE differs from the mammalian enzyme with respect to the configuration of the active center, and that hycanthone may have a selective affinity for schistosomal cholinergic systems.

Hycanthone (Etrenol®) is a primary candidate for large scale use in the control of schistosomiasis mansoni and haematobium. Although the drug is known to be effective in most patients when given as a single intramuscular dose, it is possible that a small fraction of individuals may require more than one course of treatment. Unfortunately, hycanthone has several undesirable effects which may restrict its usefulness. Drug resistance,[1] mutagenicity,[2] hepatotoxicity,[3] and carcinogenicity[4] have been reported in various laboratory studies. However, these effects have not been widely encountered in the field where nearly a million patients have been treated with hycanthone. Other workers have reported studies showing no tumorigenic effect,[5] no drug resistance,[6] and no genetic effects[7] after hycanthone treatment. Some of these conflicting reports may reflect differences among strains of schistosomes. A recent study by Katz et al.[8] suggests that all schistosome strains are not equally sensitive to various antischistosomal drugs, including hycanthone, and that drug resistance could become a problem in some geographic regions.

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While it is not yet certain whether hycanthone has severe toxic effects when used therapeutically, there is no doubt that less dangerous forms of toxicity, such as nausea and vomiting, are seen quite often. There is considerable interest, therefore, in the development of analogs of hycanthone which might be more safely used in humans. In conducting such a search, it would be very useful to understand the mechanisms of action of hycanthone, so that congener compounds can be identified which will have the desired lethal effect on schistosomes, but which entail minimal human toxicity risks. This paper seeks to introduce information concerning the mechanism of action of hycanthone.

It is known that a low concentration (10^{-5} M–10^{-6} M) hycanthone stimulates motor activity in *Schistosoma mansoni*.[9] However, it is quite uncertain whether such stimulation is a direct effect of the drug on neuromuscular receptors or acts indirectly by mobilization of presynaptic 5-hydroxytryptamine (serotonin) (5HT). Chou et al.[10] have recently reported that hycanthone-exposed worms show a marked increase in uptake of exogenous 5HT when they are removed from a mouse host. However, since hycanthone stimulates schistosome activity in vitro in the absence of added serotonin, some other mode of action on the worms ought to be considered.

Acetylcholine is believed to be an inhibitory

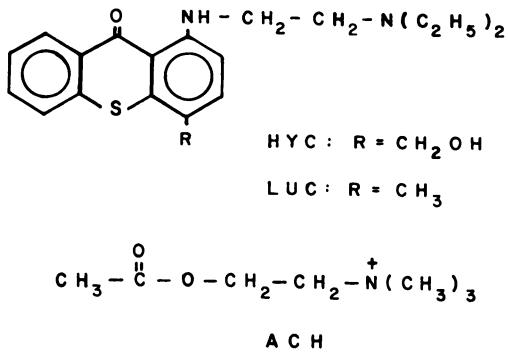

FIGURE 1. Structural formulas of hycanthone (HYC), lucanthone (LUC), and acetylcholine (ACH).

neurotransmitter in schistosomes.[11,12] Since hycanthone bears some structural resemblance to certain anticholinergic phenothiazines, we have conducted experiments which attempt to determine whether some of the actions of hycanthone in schistosomes could be explained on the basis of an acetylcholine antagonism.

To determine whether the observed actions of hycanthone are likely to be related to its antischistosomal effects, we also have studied the effects of lucanthone on some of the same responses. Lucanthone bears a close structural relationship to hycanthone, but it is believed that lucanthone is inactive against schistosomes in vivo unless it is hydroxylated to hycanthone by the action of bacteria within the gut of the host animal.[13,14]

The structural formulas of hycanthone, lucanthone, and acetylcholine are shown in Figure 1.

MATERIALS AND METHODS

Infected snails used to initiate our cycle were obtained courtesy of Dr. Monte Bowden (Harvard School of Public Health) from a stock originally sent from Dr. Henry van der Schalie's laboratory at the University of Michigan. Our strain is maintained in this laboratory by repeated passage through a mouse (CF^1)-snail (*Australorbis glabratus*, Puerto Rican origin) cycle. Young, female mice, of about 25 grams weight, are exposed percutaneously to about 200 cercariae by tail immersion. After 45 to 60 days, the mice are killed by cervical fracture after etherization and the worms are recovered from the portal or mesenteric veins by gentle hook dissection.

Hycanthone mesylate (S-W #R-015-FF) was obtained through the courtesy of Dr. Marvin Legator. This batch is reported to have an LD_{50} of 12.5 mg/kg body weight (intramuscularly) for schistosome worms maintained in mice in the Sterling-Winthrop laboratories. A routine test of its efficacy in our laboratories showed that *S. mansoni* worms were partially displaced into the liver and were dead or dying there 7 days after giving 80 mg/kg to host mice. We therefore considered our schistosome strain to be "hycanthone-sensitive." Lucanthone (S-W #R-013-CO) was obtained through the kindness of Dr. Allen Yarinsky of Sterling-Winthrop Research Laboratories.

The motor response of *S. mansoni* to drugs was determined by using a "micro-activity cage" apparatus. Worm pairs are incubated in glass bottomed cells which are placed over an array of CdSe photocells. Movement of the worms in the cells obscures a light beam. The photocells register the light intensity fluctuations due to these movements, and the resultant electronic changes are translated into numerical "counts" which are proportional to the total amount of movement. Worms are incubated in Fischer's Medium (Grand Island Biological Co., Grand Island, N. Y.) plus 10 mM tricine buffer, 15 μg/ml streptomycin and 15 U/ml penicillin. The apparatus allows four simultaneous experiments to be run concurrently. After each 2-minute wiggleometer measurement, new medium (with or without added drug) is flushed through the chambers. The apparatus and general techniques have been described elsewhere in detail.[9,15,16]

Data are accumulated automatically and are plotted by a Wang 700B computer to give a graph of overall movement rates and patterns. In experiments where statistical analysis seemed desirable, a digitizer (Numonics, Inc.) was used to enter information from the primary graphic motility records into a Wang 2200B computer, which then calculated mean motility rates and, by a linear regression procedure, determined the slope of the line over any desired region. Results under different experimental conditions could then be compared by t-test. In most cases, however, we have found that statistical calculations merely confirm what is apparent by inspection of the motility graphs, and therefore we have not

reported statistical analysis of experiments which show very large effects.

In the mammalian blood pressure experiments, a cannula was placed in the carotid artery of a rat and blood pressure was recorded on a Beckman Dynograph via a Statham pressure transducer. Acetylcholine (20 ng/kg) was injected via the cannula and a transient drop in blood pressure was recorded during the next minute. This procedure was carried out before and after the rat was treated with hycanthone, up to 32 mg/kg intravenously.

For smooth muscle studies, a guinea pig ileum was placed in a bath of Tyrode's solution (NaCl 8 g/l, KCl 0.2 g/l, $CaCl_2$ 0.2 g/l, $MgCl_2 \cdot H_2O$ 0.1 g/l, $NaH_2PO_4 \cdot H_2O$ 50 mg/l, $NaHCO_3$ 1 g/l, glucose 1 g/l) adjusted to pH 7.4 at 37° C and connected via a Grass FTO3C force transducer to a recorder. Contractions were observed when 10^{-7} M acetylcholine was applied to the muscle in the presence of 10^{-5} M eserine. This response was observed before, simultaneously with, and after treatment of the muscle with 10^{-5} M hycanthone.

Acetylcholinesterase (ACHE) preparations from *S. mansoni* were made according to the method of Bueding;[17] this method consists of homogenization followed by centrifugation. Enzyme preparations from *Necator americanus* and *Nippostrongylus brasiliensis* were a gift from Dr. B. Ogilvie of the National Institute for Medical Research, London. *Electrophorus electricus* and bovine erythrocyte enzymes were purchased from Sigma Chemical Co.

The enzyme was assayed by the method of Ellman et al.,[18] using acetylthiocholine as the substrate and measuring the production of cholinethiol by continuous reaction with 5-5′-dithiobis (2-nitrobenzoic acid) (DTNB). Solutions were made either in 0.15 M Na phosphate buffer or in Fischer's Medium; no difference could be detected in the results with these two methods. The reactions were monitored with a Gilford recording spectrophotometer for a period of 5 to 10 minutes following mixing of the reagents.

RESULTS

The effects of hycanthone (Hyc) on schistosome motor activity and on responses of the worms to serotonin (5HT) are shown in Figure 2. 5HT is a strong stimulator of somatic activity of the parasite (Fig. 2A). Effects can be noted by means of the activity monitor apparatus at 10^{-6} M; vigorous stimulation is generally achieved at 10^{-4} M. Experiments shown utilized 10^{-3} M 5HT to achieve uniformly intense stimulation of the neuromuscular system. Some variation in degree of responses was noted from preparation to preparation, which undoubtedly reflects changes in physiological condition or size of worm pairs recovered from the mouse host. Hycanthone rather weakly stimulates worms at 10^{-6} or 10^{-5} M concentration (Fig. 2B and 2C), while concentrations greater than 10^{-4} M result in no change or depression of activity (Fig. 2D). When 5HT follows preincubation with Hyc, the resultant motor activity is abnormally large (Figs. 2B and 2C), but the 5HT response is depressed by large

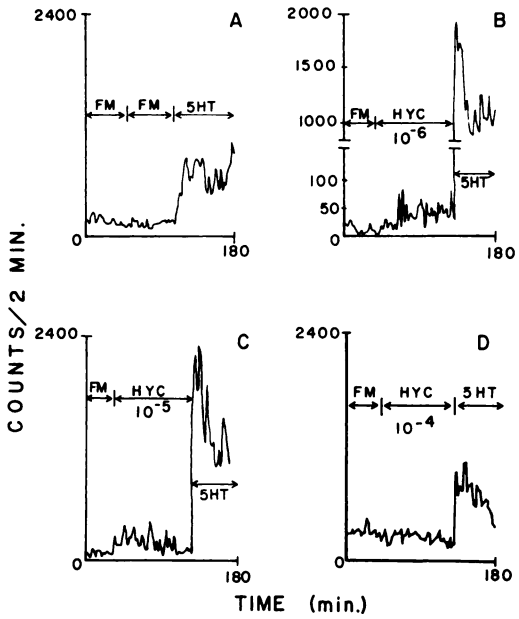

FIGURE 2. Effects of hycanthone on motor activity of *S. mansoni*. The number of movements counted per 2-minute interval are plotted as a function of time. Movements were recorded automatically, as described in the text. Drugs were applied for the periods indicated. All drug solutions were made in Fischer's Cell Culture Medium, our basic maintenance medium. FM, Fischer's Medium, supplemented as described in the text, with no drugs added; 5HT, 5-hydroxytryptamine (serotonin), 1×10^{-3} M; HYC, hycanthone, at the indicated concentration. Modest stimulation of worm activity is noted at hycanthone concentrations of 10^{-6} and 10^{-5} M. At 10^{-4} M hycanthone no stimulation of activity is seen.

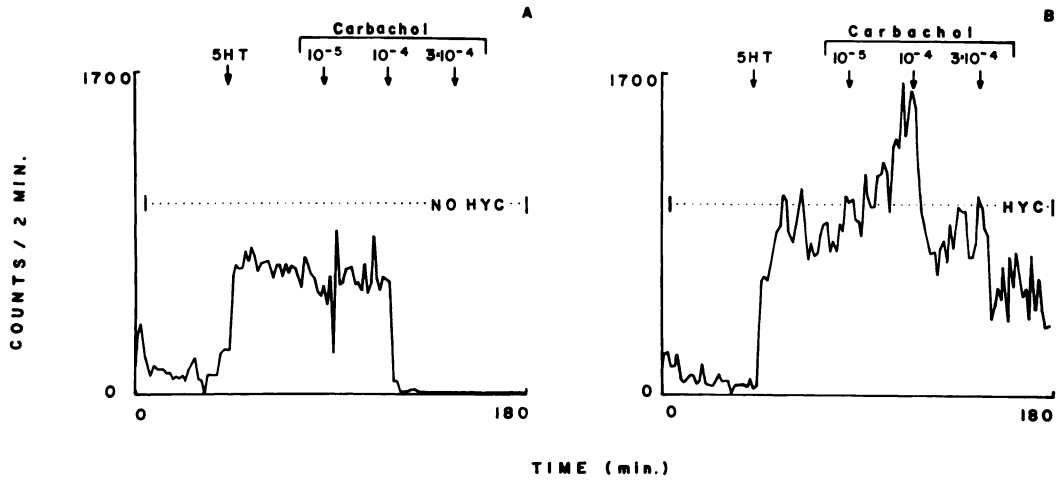

FIGURE 3. Effects of hycanthone on the carbachol dose response. Carbachol was added at increasing concentrations, as shown. **A**, no hycanthone present; **B**, hycanthone (10^{-5} M) was present throughout the experiment.

amounts of Hyc (Fig. 2D). When hycanthone is continuously present during administration of 5HT, motor activity is not sustained, but falls off gradually.

The effects of hycanthone on the response of this trematode to carbachol (CCH), a cholinomimetic, are shown in Figure 3. CCH at 10^{-4} M causes immediate cessation of motor activity of the worms, even when they are maximally stimulated by millimolar serotonin (Fig. 3A). However, when hycanthone (10^{-5} M) is present, the action of carbachol is delayed and inhibited (Fig. 3B and Table 1).

The responses induced by hycanthone are qualitatively similar to those produced by atropine (ATR). The normal response to CCH (shown in Fig. 4A) is partially blocked by atropine at 10^{-5} M (Fig. 4B; Table 1); however, the effect of atropine is not large. Higher concentrations of atropine cause considerable stimulation of schistosome activity (Fig. 4C).

The effects of hycanthone on the physostigmine (eserine) response are shown in Figure 5. Eserine depresses activity in worms (Fig. 5A), presumably by inhibiting hydrolysis of intrinsic acetylcholine by acetylcholinesterase (ACHE). Worms with a high basal activity level were used for this experiment. In the presence of hycanthone the

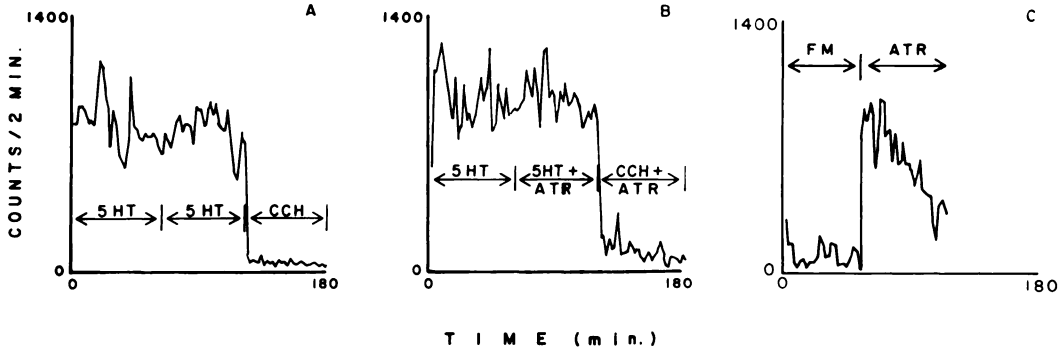

FIGURE 4. Effects of atropine on the paralytic effect of carbachol. 5HT, serotonin, 1×10^{-5} M in **A** and **B**; CCH, carbachol (carbamylcholine), 1×10^{-4} in **A** and **B**; ATR, atropine, 1×10^{-5} M in **B** and 1×10^{-4} M in **C**.

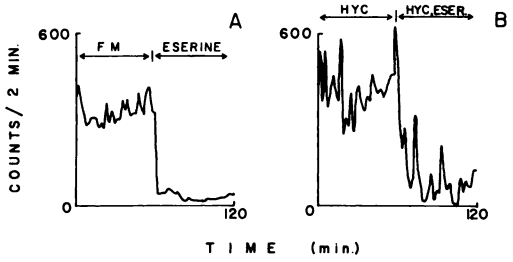

FIGURE 5. Effect of hycanthone on the paralytic effect of eserine. FM, Fischer's Medium, no drug added; HYC, hycanthone 1×10^{-5} M; ESER, eserine (physostigmine) 1×10^{-5} M. Both drugs are present in the last period of **B**.

effect of eserine is reduced (Fig. 5B), although complete abolition of its effects could not be achieved. The mean rate of movement after eserine treatment was 38.8 ± 2.6 without hycanthone (Fig. 5A) and 104.7 ± 15.7 with hycanthone (Fig. 5B). Those values, along with the slopes of plotted activity, are significantly different ($P < .05$).

The typical effect of lucanthone on *S. mansoni* in vitro is shown in Figure 6. It is seen that lucanthone at 10^{-4} M, 10^{-5} M, or 10^{-6} M does not stimulate worms as hycanthone does. Trials were made at 10^{-7} to 10^{-3} M lucanthone. Some preparations showed slight, transient increases in motility following lucanthone, but the magnitude is considerably less than that induced by hycanthone ($P < .001$). Also noted was the tendency for the 5HT response to diminish rapidly when Luc and 5HT were administered together. In this respect Luc and Hyc have similar properties.

A question of considerable importance concerning the action of hycanthone is the onset of its action on schistosomes in vivo. The first grossly visible toxic effect, a hepatic shift of the worms, is seen some days after the host animal is given a single injection of hycanthone. If the anticholinergic action of hycanthone is related to its antischistosomal action, one would expect to find that worms treated several days previously with hycanthone would be resistant to the actions of carbachol. This has been found to be the case. Infected mice were injected once with 80 mg/kg hycanthone, intraperitoneally. Five to 7 days later, as expected, most of the worms were found to have shifted to the liver or to the portal vein. These worms recovered from host mice and placed in the motility monitor revealed a diminished and delayed carbachol response similar to that seen with in vitro hycanthone administration. The 5HT-induced motor response of these worms resembled that of normal, untreated worms.

Attempts were made to detect a possible anticholinergic effect of hycanthone in mammalian systems. Hycanthone was unable to prevent the hypotensive effect of acetylcholine administered intravenously in rats (N = 3). In addition, hycanthone did not alter the contractile response of isolated guinea pig ileum (N = 5) to acetylcholine.

Using homogenates, hycanthone was found to inhibit acetylcholinesterase strongly in schistosome and hookworm preparations. Bovine and electric eel acetylcholinesterase were less susceptible to blockage by hycanthone, but were considerably more inhibited by eserine. Figure 7 illustrates the relative inhibition of these four species of enzyme by hycanthone and eserine. Figure 8 shows a Lineweaver-Burk plot of inhibition of *S. mansoni* enzyme by hycanthone at 10^{-5} M. This plot suggests a noncompetitive type of inhibition. From 43 determinations at concentrations of Hyc ranging from 10^{-6} to 5×10^{-5} a competitive K_i of 1.2×10^{-5} M and a non-

TABLE 1

Effect of hycanthone and atropine on response of S. mansoni *to carbachol**

Treatment	Mean rate	P† mean	Slope‡	P† slope
Control	66.8 ± 1.4§	–	−0.2 ± 0.1	–
Hycanthone 10^{-5} M	180.6 ± 34.2	<.001	−18.6 ± 2.2	<.005
Atropine 10^{-5}	125.9 ± 14.7	<.001	−6.99 ± 1.16	<.001

* All worms were first treated with serotonin 10^{-3} M, then with carbachol 3×10^{-4} M. Rates and slopes were determined during the carbachol period.

† Probability values determined by t-test for mean or slope compared to control value.

‡ "Slope" is the least-squares estimate of the change in motility rate per counting interval.

§ Standard error of the mean.

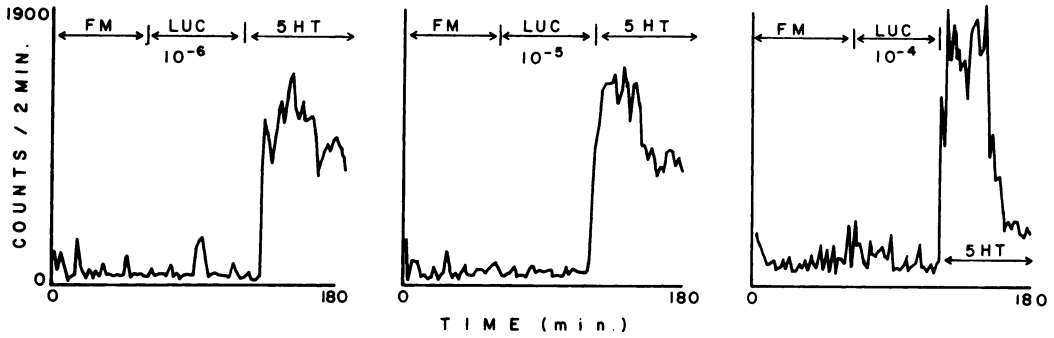

FIGURE 6. Effect of lucanthone on schistosome motility. Worms exposed to 10^{-6} or 10^{-5} lucanthone show no change in motility and exhibit a strong response to 5HT (10^{-3} M). There is some suggestion of stimulation of worms by lucanthone at 10^{-4} M; in addition, the motor response to serotonin declines after an initial period of stimulation.

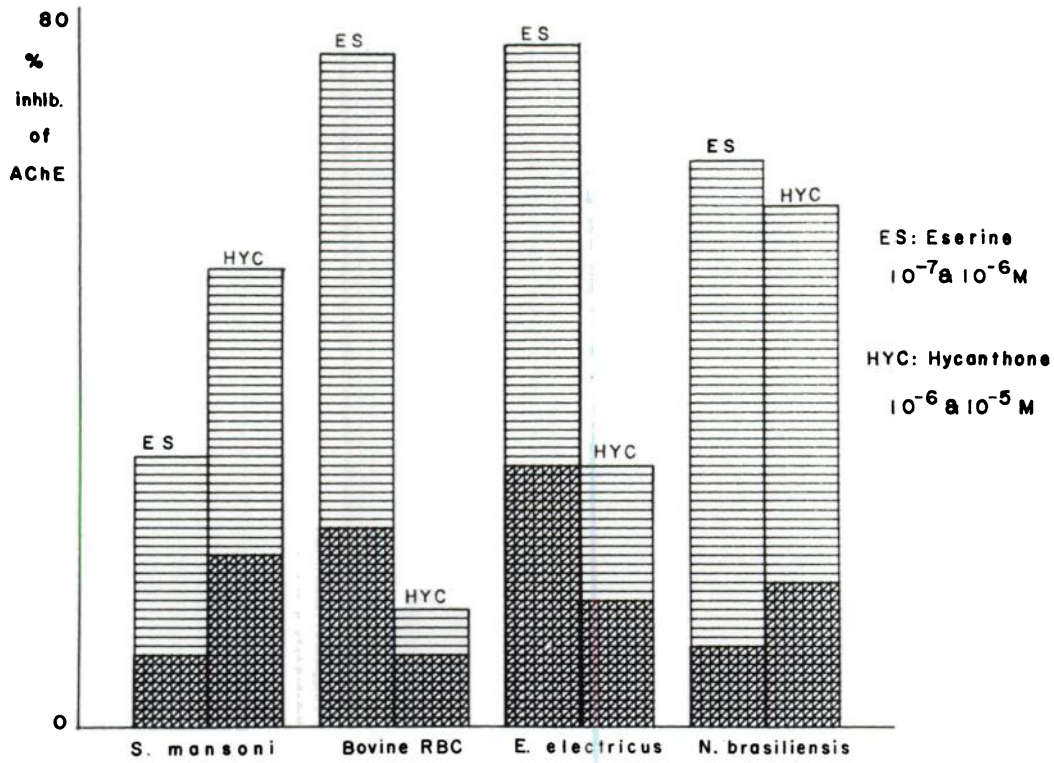

FIGURE 7. Comparative susceptibility of acetylcholinesterase (ACHE) from four different animal species to inhibition by eserine or hycanthone. *S. mansoni* ACHE is seen to be inhibited more strongly by hycanthone than by eserine at dosages shown. In contrast, *Electrophorus electricus* (electric eel) and bovine ACHE are more inhibited by eserine than by hycanthone. *Nippostrongylus brasiliensis* enzyme has an intermediate response to inhibition by eserine and hycanthone. The reactions of *Necator americanus* ACHE, which are not shown on this graph, were very similar to those of *N. brasiliensis*. Cross-hatching refers to the lower concentration of each drug used.

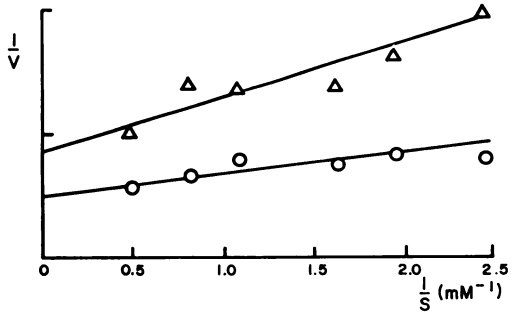

FIGURE 8. Double reciprocal plot of inhibition of acetylcholinesterase by hycanthone. Circles, no hycanthone present; triangles, 10^{-5} M hycanthone present. Rates were determined by spectrophotometric measurement of the hydrolysis of acetylthiocholine. Lines represent computer-determined estimates of the least square regression lines for the data points.

competitive K_i of 1.3×10^{-5} M were calculated. These results contrast with those of Chou et al., who reported no effect of Hyc on schistosome ACHE when this was measured by a histochemical method.[10]

DISCUSSION

There are several actions of hycanthone which can be interpreted in terms of two related features: 1) binding of the drug to acetylcholine receptor sites, i.e., acting to block acetylcholine; 2) binding of Hyc to acetylcholine esterase, i.e., acting to inhibit destruction of intrinsic acetylcholine. The action of hycanthone on the worm might therefore be related to whether the first or the second type of inhibition predominates at the dosage to which the parasite is exposed.

The direct effect of hycanthone, at low dosage, is to stimulate,[9] and this is as expected if the drug is a blocker of an inhibitory neurotransmitter. Atropine, a known acetylcholine blocker, also was found to stimulate activity of intact worms, although effective doses were higher than those commonly employed in mammalian tissue preparations.

The blockade of carbachol (CCh) by hycanthone was seldom absolute. It is possible that complete inactivation of CCh by Hyc could occur at higher Hyc concentrations, but such a function might be masked by the known paralytic effect of large amounts of hycanthone. It was noted that at low concentrations of CCh ($<10^{-4}$ M) its inhibition by Hyc was more pronounced. These observations are consistent with, but do not by themselves verify, a partial agonist model of the action of hycanthone on acetylcholine receptors.

It is sometimes assumed that schistosomes must have "muscarinic" acetylcholine receptors since acetylcholine and carbachol paralyze the worms and atropine stimulates them. Muscarine itself is ineffective, however, as are nicotine, d-tubocurarine, and decamethonium.[11] Our preliminary experiments with mammalian smooth muscle preparations show little sensitivity to hycanthone. These findings suggest that schistosome acetylcholine receptors might not be sterically identical to either "muscarinic" or "nicotinic" acetylcholine receptors. If this is the case, anticholinergic drugs might in the future be identified which have low toxicity in the mammalian host, while exhibiting profound antischistosomal effects.

Inhibition of ACHE by Hyc also showed a strong species specificity; mammalian types of the enzyme were only slightly inactivated by Hyc, although they were quite susceptible to eserine, while the helminth esterases were strongly inactivated by Hyc, but were less inhibited by eserine than were the mammalian enzymes. The fact that Hyc is an effective inhibitor of trematode ACHE may explain the previously published report that hycanthone has a biphasic effect on schistosomes, stimulating them at 10^{-6} M while depressing activity at or above 10^{-4} M.[9] At the lower concentration the drug might bind on or near the ACh receptor, preventing access of endogenous ACh for hyperpolarizing the parasite muscle. Stimulation by intrinsic 5HT would predominate and the worms would increase motility. At concentrations higher than 10^{-4} M, hycanthone might inactivate the worms' ACHE with the result that acetylcholine near the myoneural junction would rise to high levels, finally overcoming the blocking action of hycanthone and resulting in a depression of worm motility. In a clinical therapeutic dose, hycanthone has been estimated to be present in the serum at about 10^{-6} M,[19] at which concentration only the stimulatory, but not the depressant phase of drug activity would predominate.

It is possible, therefore, that the toxicity of hycanthone to the worms rests on its ability to disrupt the ACh receptor or the ACHE system, or both. If this is shown to be the case, then the

design of alternative hycanthone-like drugs should be made somewhat more rational by knowing the target towards which such a putative antischistosomal drug ought to be directed. We are aware of the report of Chou et al.[10] which claims that exposure of flukes in vivo to hycanthone results in worms which both contain more endogenous 5HT and also take up more serotonin than do controls. The data reported in the present paper present a view that Hyc may primarily perturb acetylcholine physiology rather than that of serotonin. In order to resolve these apparently conflicting interpretations, it is obvious that further confirmation of published experimental findings will be required, particularly since the implications for chemotherapy are considerably different if one looks for an anticholinergic as opposed to antiserotonin compounds.

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