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FLUORESCENT PROBES OF ACETYLCHOLINE BINDING SITES—INDICATORS OF DRUG ACTION IN *SCHISTOSOMA MANSONI*

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Abstract—Dimethyl DNS-chol (DDNS), a dansylated analog of acetylcholine, binds with specific sites in *Schistosoma mansoni*. This treatment provides fluorescent labeling of well-defined areas within the organism. It is believed that these regions of fluorescence reflect areas receiving cholinergic innervation; these regions might include both nervous and non-nervous (effector) tissue, and the DDNS binding sites might include both prejunctional amd postjunctional acetylcholine binding sites. Hycanthone (Hyc), an antischistosomal drug, blocks this labeling by DDNS. Atropine and other known antagonists of acetylcholine also prevent DDNS fluorescent labeling. Measurements of somatic motor activity demonstrate that Hyc and DDNS partially block the paralytic effects of the cholinomimetic drug carbamylcholine on *S. mansoni*. These data further support the hypothesis that Hyc is an anticholinergic agent, and show that DDNS may be useful as a histochemical label in studies involving structures receiving cholinergic innervation.

Acetylcholine (ACh) is generally recognized as a neurotransmitter in *Schistosoma mansoni*, controlling movements of the body musculature and the gut [1]. Various cholinomimetic and anticholinergic drugs have proved to affect the nervous system of schistosomes. However, some drugs, such as muscarine, nicotine and *d*-tubocurarine, which are highly active at mammalian cholinergic synapses, are conspicuously inactive in schistosomes [1], although ACh and carbachol (CCh) are immediately effective. This finding suggests that the ACh receptor in schistosomes is different from those found in nicotinic or muscarinic mammalian synapses.

Studies with acetylcholinesterase inhibitors have shown that drugs which alter cholinergic function can have useful chemotherapeutic effects [2–4]. However, to date, little attention has been devoted to the identification of anticholinergic drugs with specificity for schistosomal receptors. Our laboratory has suggested recently [5–7] that hycanthone (Hyc), a powerful antischistosomal agent, may have ACh-blocking actions in schistosomes. This theory was based on the demonstration that Hyc can block the paralytic action of carbachol and the stimulatory action of atropine. This suggestion was supported by a study [7] in which adult worms were treated with the fluorescent ACh analog, DNS-chol, which has specific affinity for ACh receptors [8, 9]. The labeling of the schistosomal anterior region by DNS-chol can be observed by fluorescence microscopy; this specific labeling was prevented by Hyc [7]. In the present study, we have further developed this approach, introducing a new fluorescent ACh analog superior to DNS-chol for fluorescence histochemistry.

An alternative theory of the mode of Hyc action has been proposed by Chou *et al.* [10], who described changes in serotonin (5-HT) dynamics caused by Hyc. We have undertaken the present studies in order to provide further, more stringent testing of our hypothesis that the primary site of Hyc action involves ACh function.

MATERIALS AND METHODS

Female CF[1] mice were infected by tail immersion with approximately 200 *S. mansoni* (Puerto Rican strain) cercariae. After 42–60 days, mice were injected i.p. with heparin, etherized and sacrificed by cervical fracture. Worm pairs were recovered by gentle hook dissection from the portal and mesenteric veins. The worms were washed and maintained at 37° in Fischer's cell culture medium (FM) (Grand Island Biological Co., Grand Island, N.Y.) to which 1 mg/ml of $NaHCO_3$, 10 mM Tricine [(*N*-Tris-hydroxymethyl) methyl glycine, Sigma Chemical Co., St. Louis, Mo.], 15 μg streptomycin and 15 units penicillin/ml have been added.

Hycanthone mesylate (Sterling-Winthrop, No. RO15-FF) was obtained through the kindness of the Sterling–Winthrop Research Institute. DNS-chol was purchased from Pierce Chemical Co., Rockford, Ill. Ethopropazine was a gift from the Warner Lambert Research Institute.

Dimethyl DNS-chol (DDNS) was synthesized by a method related to that previously reported [8]; it is an intermediate in the synthesis of DNS-chol. 5-Dimethylaminonaphthylsulfonyl chloride (1.6 g, 6.73 m-moles) was refluxed with unsymmetrical dimethylethylenediamine (1.06 g, 6.8 m-moles) in 20 ml acetone for 1 hr. The acetone was evaporated to dryness at reduced pressure, and the resulting residue was washed with 100 ml diethyl ether. The resulting hydrochloride salt was neutralized with 20 ml of 3% sodium carbonate solution. It was then repeatedly extracted with diethyl ether (3 × 50 ml) and water was removed by the addition of $MgSO_4$. The solid

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was removed by filtration and the filtrate was cooled in an ice bath and bubbled with excess HCl gas. The solution was concentrated to an oil which slowly solidified to yield analytically pure material, 300 mg (12%), m.p. 130–152°. The product was highly hygroscopic and tended to oil on exposure to air. A single component was seen by paper electrophoresis at pH 11. Thin-layer chromatography on Eastman Silica gel plates, developed with 100% ethanol, also revealed only a single component with an R_f of 0.50. The compound had fluorescence excitation and emission spectra similar to those reported for DNS-chol [8]: $u.v._{max}$ (H_2O) 246 nm (E = 15,600), 332 nm (E = 3,780); $u.v._{max}$ (pH 1) 288 nm (E = 7,620), 321.5 nm (E = 1,750); $u.v._{max}$ (pH 11) 238 nm (E = 15,660), 315 nm (E = 4,580). Analysis calcd. for $C_{16}H_{23}N_3SO_2 \cdot HCl \cdot 0.25\ H_2O$: C, 53.17; H, 6.55; N, 11.63. Found: C, 53.36; H, 6.82; N, 11.47.

Fluorescent labeling. The worms obtained from a single infected mouse were divided into equal groups and placed in FM containing the drug to be tested or no drug (control) for 30 min at 37°. After this preincubation period, DDNS was added, to a final concentration of 10^{-5} M DDNS. The incubation was continued for 1 hr. After this total incubation period of about 1.5 hr, the worms were quickly washed in saline and placed on a glass microscope slide in a drop of saline. The worms were observed and photographed through an Olympus XTR stereomicroscope, using u.v. illumination from a Zeiss mercury lamp witha type III (u.v.-transmitting) filter. In some experiments, a higher magnification, and a brighter image, were achieved by the use of an Olympus Vanox microscope equipped with a mercury light source and vertical illumination optics. Excitation was restricted to the u.v. region by a UGl filter, while a barrier interference filter permitted only fluorescence in the yellow–green region to be observed and photographed.

To provide quantitative measurements of the amount of fluorescent material absorbed by the worms, DDNS was extracted from DDNS-exposed worms into an organic solvent. For each measurement, 15 worm pairs were incubated with DDNS as described above. After this treatment they were rinsed briefly in saline and placed in 1 ml of 5% KOH. After 10 hr of gentle shaking at 37°, the worms were entirely dissolved. The solution was then extracted by shaking it vigorously with 5 ml ether. This procedure was repeated four times, the ether extracts were combined, and the volume of extract was reduced to 1.5 ml by evaporation. Fluorescence measurements were made with an Aminco–Bowman spectrophotofluorometer. By extracting identical tubes to which were added known amounts of DDNS in 5% KOH, it was estimated that this extraction and assay procedure resulted in the recovery of about 94 per cent of the DDNS absorbed by the worms.

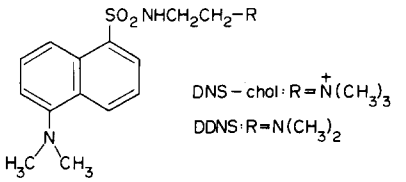

Fig. 1. Structure of DDNS and of DNS-chol.

Motility measurements. The motor response of *S. mansoni* to drugs was determined by the "microactivity cage" apparatus, described in detail by us previously [11, 12]. Worm pairs are incubated in glass-bottomed cells which are placed over an array of CdSe photocells. The movements of the worms in the cells obscure a light beam, and photocells register the light intensity fluctuations caused by these movements. The resultant electronic changes are translated into numerical "counts" which are proportional to the total amount of movement. Worms are incubated in FM. This apparatus allows the performance of four simultaneous experiments. After each 2 min of motility measurement, new medium (with or without added drug) is flushed through the chambers. Data are accumulated automatically and are plotted by a

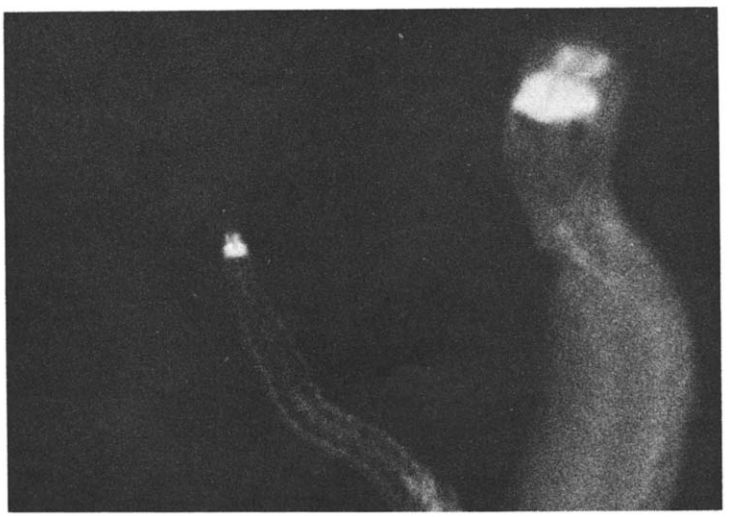

Fig. 2. Fluorescent labeling by DDNS. An *S. mansoni* pair was treated for 1 hr with DDNS in Fischer's cell culture medium, and photographed under u.v. light. Only the anterior portions of the worms are shown.

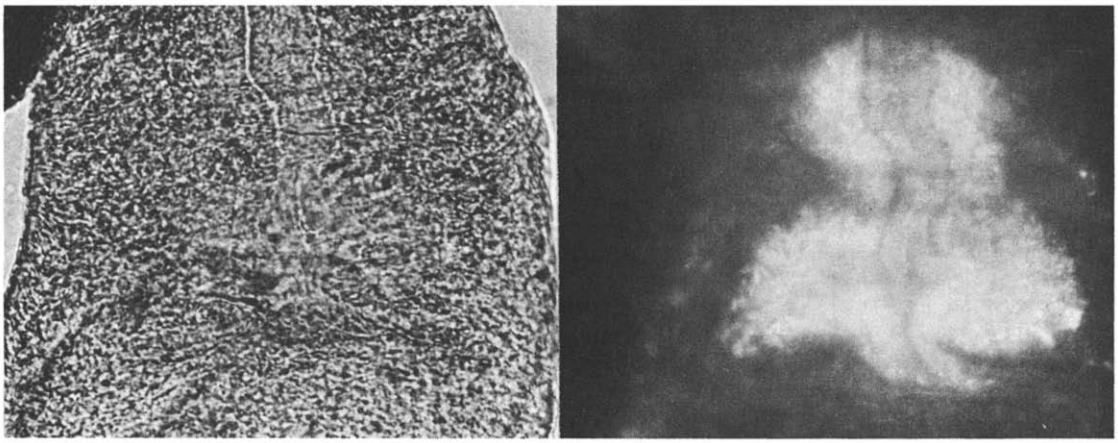

Fig. 3. Higher magnification view of a DDNS-labeled schistosome. Head of a male *S. mansoni* is shown; width of head is about 250 μm. The worm was treated with DDNS 10^{-5} M; no other stain was used. Left: photograph taken using visible bright-field transmitted illumination. Right: reflected-light fluorescence illumination, with u.v. excitation and yellow-green emission.

Wang 700B computer to give a graph of overall movement rates and patterns.

RESULTS

DDNS has fluorescent properties similar to those of DNS-chol [8]. In water, DDNS, when excited at 344 nm, has a fluorescence emission maximum of 548 nm. DDNS shows a 12-fold increase in fluorescent intensity in ether, and the emission maximum shifts to 488 nm when measured at the excitation maximum (348 nm). DNS-chol is insoluble in ether and, therefore, has limited capability as a hydrophobic probe. Both structures are shown in Fig. 1.

Schistosoma mansoni worm pairs, after 1 hr of incubation in 10^{-5} M DDNS, reveal a bright, well-defined region in the head when viewed under ultraviolet light (Fig. 2). This fluorescent labeling includes some regions previously known to contain nervous tissue [2, 13, 14], but does not correspond closely to the staining shown in these previous reports. This fact is not surprising, since the previous work [2, 13, 14] was based on staining for functions other than ACh binding (i.e. catecholamines [13, 14] or acetylcholinesterase [2]) which would not be expected to have identical localization with ACh binding sites, and since our worms are alive at the time of observation, while those of previous workers are fixed. The distribution of fluorescence in the head of a male worm is shown at higher magnification in Fig. 3; a conventional bright field photomicrograph of the same preparation is also shown for reference. The female head region is also clearly outlined by DDNS (Fig. 2), and resembles the male's in structure.

Diffuse fluorescence is seen in the body of the worm, especially after prolonged staining periods. Many preparations of male worms, such as that in Fig. 6, also show a brightly fluorescent structure that

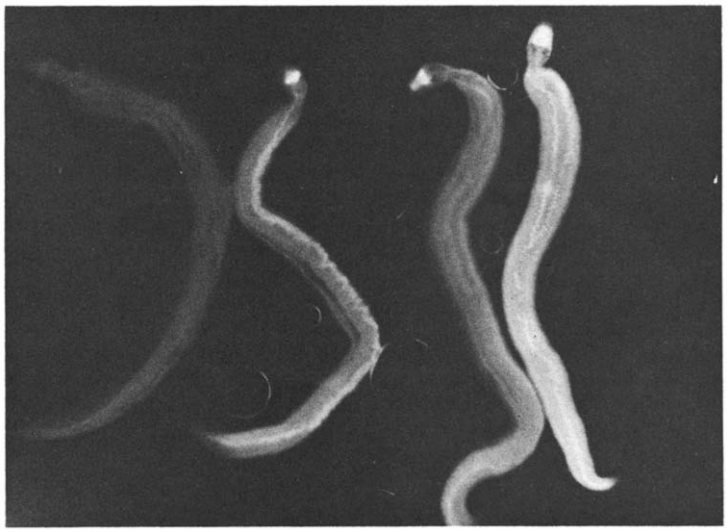

Fig. 4. Blockage by hycanthone of fluorescent labeling. All worms were treated with DDNS 10^{-5} M for 1 hr. For 0.5 hr prior to, as well as during, DDNS treatment, the worms were also exposed to (left to right): 10^{-5}, 10^{-6} and 10^{-7} M, and zero hycanthone.

corresponds anatomically to the gut. There is also strong DDNS labeling of the body of the female, but, unlike the effects mentioned above, this concentration of fluorescence appeals to represent non-specific labeling of the vitelline material, since this labeling is not prevented by any drugs we have tested.

DNS-chol also labels the head of the worms [7] but this staining is achieved only at concentrations about one hundred times greater than the amount of DDNS needed to stain the head equivalently. DDNS not only allows labeling of the head at much lower concentrations than DNS-chol, but the structure within the head is clearly seen only with DDNS. This improved labeling performance may account for the fact, noted earlier [7], that better results were achieved with a DNS-chol synthesized in our laboratory than with a commercial sample; it seems probable that the DNS-chol prepared by us [7] was contaminated with a small amount of DDNS.

To determine whether the fluorescent labeling was specific for ACh-related fluors, we treated schistosomes with dansylated compounds unrelated to ACh. Dansyl glycine and sodium dansylate were tested at concentrations of 1 mM. Both compounds produced a small degree of diffuse staining, but there was no localization of fluorescence in the head region or elsewhere in the schistosome. It was concluded that the choline-like portion of DNS-chol or DDNS was essential for the dansyl labeling.

Hyc effectively blocks the staining of the head of the worm by both fluorescent ACh analogs, i.e. DNS-chol and DDNS. Body fluorescence is also reduced. Hyc at 10^{-5} M inhibited labeling even by 10^{-3} M DDNS. Hyc is not fluorescent under these conditions and does not directly alter the fluorescent properties of dansyl compounds in the spectrophotofluorometer. Increased dosages of Hyc resulted in decreased fluorescence of the head when the worms were treated with equal amounts of DDNS (Fig. 4). Partial blockage of 10^{-5} M DDNS labeling is observable at 10^{-6} and 10^{-7} M Hyc; this is the approximate level of the drug in the bloodstream after a therapeutic dose.

An experiment was conducted in which schistosome-infected mice were injected i.m. with 80 mg/kg of Hyc mesylate. Worms were recovered 5 days later and were exposed *in vitro* to 10^{-5} M DDNS. These worms were compared directly with DDNS-stained worms from untreated mice. A distinct reduction in fluorescence intensity was seen in the worms from the Hyc-treated mice (Fig. 5).

Several known ACh antagonists were tested (Table 1) for their effect on DDNS-chol and DNS-chol fluorescent staining of schistosomes. Atropine (ATR) 10^{-3} M greatly reduced head brightness (Fig. 6) and overall body fluorescence. Atropine at 10^{-4} M had a smaller effect. A wide range of potencies was seen among the tested drugs.

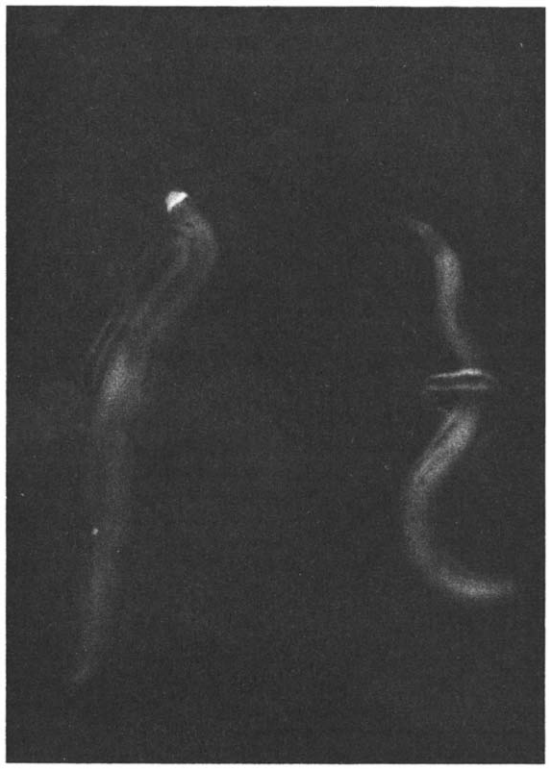

Fig. 5. Effect of Hyc treatment *in vivo* on DDNS labeling. Both worms were treated with DDNS 10^{-5} M. Left: worm taken from untreated mouse. Right: worm from mouse injected once with 80 mg/kg of Hyc, 5 days previously.

Significantly, the relative potencies of these drugs as blockers of DDNS fluorescence do not correspond to their relative anticholinergic activities in mammalian systems. For example, atropine is a stronger anticholinergic than ethopropazine in mammalian gut preparations [15], but the reverse order of potency is true with schistosomes. This observation suggests that significant host–parasite differences in ACh receptor specificity may exist.

The effect of atropine or hycanthone treatment on extractable DDNS levels is shown in Table 2. As one would expect from the above findings, both drugs caused a reduction in the total DDNS content of the worms, presumably by occupying some of the DDNS

Table 1. DDNS-chol staining blocked by acetylcholine-related drugs*

	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Physostigmine	+				
Scopolamine	+				
Carbachol	++	+			
Mecamylamine	++	+			
Atropine	++	++	+		
Ethopropazine	+++	+++	++	+	
Hycanthone	+++	+++	+++	++	+

* Key: +, weak, but definite blockage; ++, partial blockage; and +++, strong blockage.

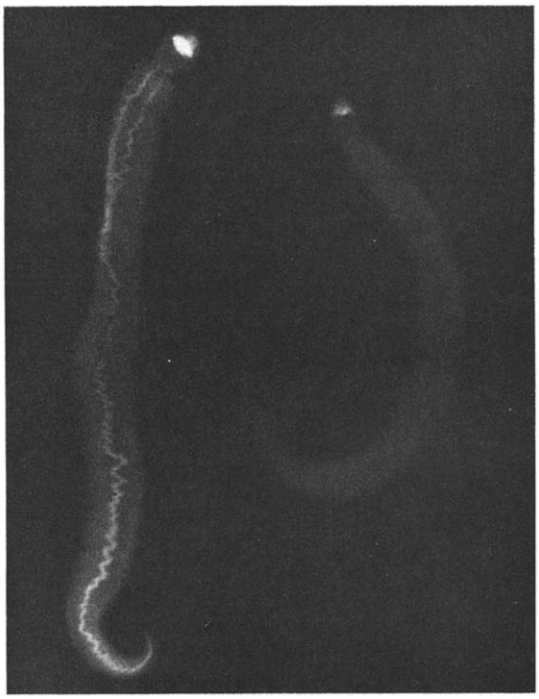

Fig. 6. Blockage by atropine of fluorescent labeling. Treatment protocol as in Fig. 4. Right: atropine 10^{-3} M. Left: control.

binding sites. However, the relative DDNS contents of atropine- or Hyc-treated worms shown in Table 2 do not correspond to the relative fluorescence of the worms, as shown in Figs. 4 and 6. Specifically, Hyc, which causes almost a complete abolition of fluorescence, causes only a small decrease in total schistosome DDNS content. On the other hand, atropine, a relatively weak fluorescence blocker, causes a larger decrease in DDNS content. This finding demonstrates that one apparently cannot obtain reliable estimates of worm fluorescence by extracting DDNS from worm homogenates. Clearly, the effect of anticholinergic drugs on fluorescence can be best seen in intact worms. The probable explanation of this finding lies in our observation that a non-polar environment greatly enhances the fluorescence of DDNS. It is likely that DDNS binds to hydrophobic receptor sites, resulting in the intense fluorescence seen in the photographs. Displacement by Hyc of DDNS from these receptors, without exclusion of DDNS from the body of the worm, may account for the fluorescence-blocking action of Hyc.

Motility measurements. Carbachol (CCh), a cholinomimetic agent, causes immediate paralysis of schistosomes at 10^{-4} M [1, 11]. This effect is observed even in the presence of 1 mM serotonin (5-HT), a powerful stimulant of somatic movement. Our laboratory has shown that Hyc partially blocks this paralysis of the worms by CCh [5], suggesting that these drugs act competitively at the same active site. DDNS and DNS-chol also partially block the paralytic effects of CCh (Fig. 7), also suggesting that these compounds bind to the same receptor site as Hyc and CCh. This observation was made on two worm pairs, each with a control experiment, on each of three independent occasions.

DISCUSSION

A new fluorescent ACh analog, DDNS, which may provide histochemical labeling of ACh binding sites in *S. mansoni*, has been identified. DDNS fluorescence is concentrated in the head of the worm. DDNS differs chemically from the previously known compound, DNS-chol, by having increased lipid solubility. This difference has a substantial effect on the usefulness of the compound, permitting greatly improved staining with DDNS at much lower concentrations than are required to obtain fluorescence with DNS-chol. It is likely that DDNS will prove useful as a histochemical tool in animals other than the schistosome, although we have not yet explored this possibility. Preliminary observations have shown, however, that DDNS administered to non-infected mice i.p. causes extensive fluorescence of the mouse brain tissue, whereas i.p. injections of DNS-chol result in negligible brain fluorescence. A possible explanation is that the highly charged DNS-chol penetrates the blood–brain barrier poorly in contrast to DDNS, which is more lipid soluble.

Fluorescent labeling is seen in the anterior portion of the schistosome's head. Other workers have reported [2, 13, 14, 16] that nervous structures are present in the head, but in view of the differences between their techniques and ours it is not possible to be certain of how much overlap exists between our labeled structures and theirs.

The distribution of fluorescence in our preparations does not correspond in detail to that shown by these previous workers, nor should it be expected to do so. In some cases [13, 14], these workers were reporting fluorescence histology of biogenic amines. Norepinephrine and serotonin would not have the same distribution as ACh receptors; in most animals the

Table 2. Effect of Drugs on extractable DDNS content

Drug	DDNS content (ng/worm pair)	$\frac{\text{DDNS (drug treated)}}{\text{DDNS (control)}}$†	P (t)‡
None (DDNS 10^{-5} M alone)	24.4 ± 4.9		
Hycanthone 10^{-5} M	21.3 ± 5.7	0.87 ± 0.13	0.055
Atropine 10^{-3} M	14.5 ± 5.2	0.56 ± 0.23	0.0064

* Mean and standard deviation of six values using worms of different sizes from different host mice.

† Ratios calculated from six experiments each using control and drug-treated worms of the same size from the same host mouse.

‡ Probability of t calculated from a t-test of drug treated vs control DDNS levels.

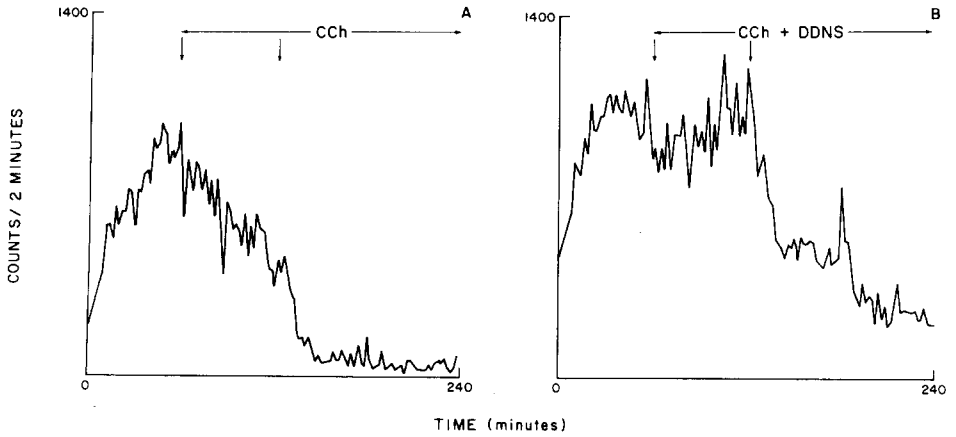

Fig. 7. Effects of DDNS on paralysis by carbachol. Chart shows movements recorded/2 min over a 4-hr period. Worms were first treated with 10^{-4} M serotonin, then with 3×10^{-5} M carbachol (CCh) (first arrow) and 10^{-4} M carbachol (second arrow). (A) control. (B) DDNS (10^{-4} M) present during CCh period.

cholinergic, adrenergic and serotoninergic systems have different localizations.

Other previous work [2, 16] is based on staining for acetylcholinesterase, an enzyme known to be present in axons as well as in nerve endings; therefore, the distribution of staining for this enzyme might be different from labeling of acetylcholine receptors and binding sites, although some overlap would be expected. Staining for acetylcholinesterase would, therefore, be expected to delineate nerve tracts as well as regions containing many nerve junctions. DDNS, on the other hand, would not be expected to label conducting fiber tracts, but would label any tissue receiving cholinergic innervation. One would expect the gut and the head region to be labeled, as appears to be the case.

Another factor of possible importance is that our preparations are alive and moving at the time they are photographed, while those of other workers [2, 13, 14, 16] are fixed. It is not known to what extent the tissue fixation and staining procedures used by these workers may affect the appearance of the nervous structures within the worm.

The fluorescent structures in the head may include not only neural but also effector tissues, such as muscles or secretory tissue, and therefore may not be identical with the head ganglion reported by others. It is known, for example [17], that the "esophageal gland" of the schistosome, which may be within the DDNS-labeled region, contains what appears to be a nerve network. More refined microscopic procedures are presently being employed to determine the identity of the fluorescent structures. This information, when it becomes available, may assist greatly in understanding the physiological mechanisms by which a blockage by Hyc results in harmful effects on the parasites.

It was established by other workers [8, 9] that DNS-chol has binding affinity for ACh receptors from vertebrate tissues. Consistent evidence was obtained in schistosomes [7] by demonstrating that the fluorescent staining is blocked by atropine and that dansylated compounds unrelated to ACh do not have staining properties similar to DNS-chol. This pharmacologic specificity is also demonstrated by DDNS, a close structural analog of DNS-chol. The idea that these fluorescent compounds bind at ACh synapses was further examined by recording their effects on schistosome motor activity. These compounds partially blocked the paralytic action of carbachol, a further indication that they bind to ACh receptors.

While this evidence suggests that DDNS binds at postsynaptic ACh receptors, it by no means rules out the possibility that DDNS also binds at other ACh binding sites. Cholinergic synapses presumably contain several types of proteins with ACh-specific binding sites, having various functions such as transport or storage of ACh; DNS-chol and DDNS may label some of these macromolecules as well as the ACh receptor proteins of the postsynaptic membrane. Future studies of the subcellular distribution of DDNS labeling, and of the binding of DDNS to purified proteins from cholinergic synapses, may help to identify the biochemical sites of labeling.

Recent reports from our laboratory [5, 6] have suggested, on the basis of motility experiments, that Hyc is an ACh blocker in schistosomes. The present report provides further support for this theory, resting on evidence entirely different from the effects on motor activity. The experiment shown in Fig. 4 shows that Hyc prevents DDNS from labeling the worms, presumably by blocking ACh binding sites.

Investigators in our laboratory, who have had the opportunity to observe many hundreds of labeled worms, could easily distinguish between the fluorescent characteristics of the four worms in Fig. 4. However, less experienced observers may have difficulty in making such distinctions, particularly from a photographic reproduction; and of course accurate quantitation is not possible by eye. Therefore, we have recently established a system for making reliable fluorometric measurements from the head region or body of a male schistosome. This system, which consists of a photomultiplier photometer fitted to our stereomicroscope, will be described fully in a publication planned for the near future. Initial results with

this new apparatus fully confirm our interpretation of Fig. 4 as a graded response to Hyc. Hyc concentrations of 10^{-7}, 10^{-6} and 10^{-5} M, used under the same experimental protocol as in Fig. 4, produce, respectively, 17, 42 and 80 per cent reduction (averages based on at least eight experiments at each concentration) in fluorescent intensity of the head of the worm. Additional preliminary studies have shown that added Hyc can displace DDNS from previously labeled worms, causing the fluorescence to disappear in less than 5 min.

Our theory of Hyc action contrasts with that proposed by other workers [10], who reported that Hyc stimulated serotonin uptake by schistosomes. The effects reported by these workers are not necessarily inconsistent with ours; it is not uncommon for a drug to affect more than one neurotransmitter system. However, we have reported recently [18] the inability to reproduce the findings of elevated 5-HT uptake; no effect of Hyc on 5-HT uptake or levels could be seen. At present, therefore, an action at the ACh receptors of schistosomes seems to constitute the most plausible hypothesis for the mechanism of action of hycanthone.

The present situation illustrates a classical problem in pharmacology—that of demonstrating that a drug effect *in vitro* is causally related to the therapeutic efficacy of that drug. In order to establish such a fact unequivocally, more information is required than is currently available. At present, therefore, we may only state that Hyc has anticholinergic activity in schistosomes, and that this activity may be involved in its therapeutic action. Our laboratory is presently attempting to design further tests of this hypothesis. While the therapeutic efficacy of Hyc seems certain, a variety of adverse effects have been reported in laboratory animals, although some of these have not been confirmed in studies of human populations treated with Hyc. This debate has been summarized recently [5]. In view of this situation, it would certainly be desirable to develop new drugs with efficacy similar to that of Hyc but with less toxicity. The rational design and identification of such drugs are possible only if it is understood at what site within the schistosome Hyc acts.

One means of challenging the above-stated hypothesis is to ask whether it can predict the actions of other drugs. The data in Table 1 show that ethopropazine, a phenothiazine strikingly resembling Hyc, has Hyc-like action in the fluorescence test. Preliminary results (G. R. Hillman and W. B. Gibler, manuscript in preparation) show that ethopropazine, administered to infected mice, causes both a hepatic shift of the worms and a reduction in egg count. Pursuit of this theory has, therefore, resulted in the identification of a drug with previously unrecognized antischistosomal activity.

The low potency of atropine as an anticholinergic agent in the DDNS system and in other schistosomal systems may be a particularly significant aspect of this work, since atropine is a very potent anticholinergic in mammals. Hillman and Senft [5] have reported that Hyc has no detectable antimuscarinic activity in mammalian preparations. These observations suggest the hypothesis that schistosomal ACh receptors have significantly different binding properties from their mammalian counterparts. While a broad variety of mammalian nicotinic and muscarinic agents are known which have no effect on schistosomes, it is possible that Hyc is the first agent to be identified having selective activity in schistosomes but not mammals. If this theory proves to be correct, it may provide the rationale for the development of chemotherapeutic agents, not only for schistosomiasis, but for infections by other parasitic helminths as well.

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