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Disulfiram—a compound that selectively induces abnormal egg production and lowers norepinephrine levels in *Schistosoma mansoni*

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Disulfiram (DS) has been used for many years in avoidance therapy for certain patients with chronic alcoholism. The use of this drug is based upon the observation that DS inhibits aldehyde dehydrogenase, which results in acetaldehyde accumulation upon ingestion of ethanol [1]. Increased blood acetaldehyde levels are apparently responsible for the unpleasant symptoms observed after the consumption of ethanol. In addition, DS via its reduced metabolite diethyldithiocarbamate (DDC) has been shown to be an effective inhibitor of dopamine-beta-hydroxylase (DBH) [2], an enzyme which is responsible for the conversion of dopamine (DA) to norepinephrine (NE) in mammalian brain. It is thought that the mechanism of this inhibition of DBH is due to DDC's chelation of the copper ion in this metalloenzyme [3].

Recent studies indicate that DA and NE may function as neurotransmitter substances in the parasitic trematode *Schistosoma mansoni* [4, 5]. Since it has been shown that DS can lower the concentration of NE in mammalian brain, via its inhibition of DBH, we wanted to determine if NE levels in *S. mansoni* could be lowered when mice infected with this parasite were treated with DS. In addition, we wanted to examine the effects of DS (a copper chelator) on egg production by the female schistosome since a copper-containing enzyme (e.g. phenol oxidase) is thought to be involved in the production of eggs by trematodes [6].

Female albino mice (Spartan Farms) were infected with 150 *S. mansoni* cercaria (Puerto Rican strain) by tail immersion. DS, or DDC, suspended in 1% methyl cellulose, was injected intraperitoneally into mice which had been exposed to cercaria 42–54 days previously. In the dietary study, infected mice (five/cage) were placed on a ground diet (Wayne Lab-blox) containing various concentrations of DS. Parasites were removed from infected mice as previously described by Bennett and Seed [7]. Female parasites were separated from the male by incubating paired worms in Eagle's medium (Grand Island Biological Co., BME Earle's powder, No. G-11) containing 0.05% sodium pentobarbital for 5 min at room temperature. After separation the method of Moore and Phillipson [8] was used to measure the concentration of catecholamines within male and female parasites. Egg shell formation in the female was determined by u.v. microscopy as previously described by Kelly and von Lichtenberg [9]. Oxygen consumption by ten paired schistosomes was measured by placing the worms in a nylon basket which in turn was placed in a small chamber, containing Eagle's medium kept at 37°, with an oxygen (Clark) electrode attached to the bottom. To measure lactic acid production by parasites we incubated ten paired worms in 1.0 ml Eagle's medium for 1 hr and then measured the lactic acid in the medium by the method of Barker and Summerson [10]. Acetaldehyde in serum from infected mice was determined according to the method of Summerson [11]. Phenol oxidase activity in female schistosomes was determined by the method of Mansour[12] with the following modifications: Fifty female schistosomes were placed in a homogenizing vessel containing 2 ml of 0.01 M phosphate buffer, pH 6.8, and then homogenized with a Teflon pestle. The homogenate was then centrifuged at 5000 *g* for 5 min. The supernatant was discarded while the pellet was resuspended in 2 ml of the buffer. All of the above procedures were performed at 4°. A sample of the resuspended pellet was then placed in a chamber (Rank Brothers, Cambridge, England), kept at 37°, with an oxygen (Clark) electrode attached to the bottom. Three and a half min later dopamine (2×10^{-3} M) was added through a small port (0.5 mm) at the top of the chamber. Oxygen consumption was measured during the first 5 min after addition of dopamine and expressed as μmoles/mg of protein/min. Protein was measured by the method of Lowry *et al.* [13].

P values were calculated using the unpaired Student's *t*-test [14].

From Table 1 it can be observed that DS given to infected mice by diet can reduce NE levels in both male and female parasites while DA levels were elevated in both sexes. In addition, we observed that females exposed to DS were not producing eggs but they were releasing egg precursor material into their ootype and uterus. Sub-

Table 1. Effects of disulfiram on the concentration of catecholamines in *S. mansoni*, and brain of infected mice, and on egg production by female *S. mansoni*

Conc. of disulfiram in diet (%)	Conc. of catecholamine (μg/g)						Per cent inhibition of egg production
	Female		Male		Mouse forebrain		
	Dopamine	Norepinephrine	Dopamine	Norepinephrine	Dopamine	Norepinephrine	
Control	0.16 ± 0.02	0.33 ± 0.03	0.09 ± 0.001	0.20 ± 0.01	0.36 ± 0.01	0.089 ± 0.003	0
1†	1.10 ± 0.27	0.00	0.34 ± 0.16	0.00			100
0.2†	0.48 ± 0.18	0.00	0.16 ± 0.02	0.00	0.42 ± 0.01	0.083 ± 0.008	100
0.05†							56
0.01†	0.36 ± 0.10	0.00	0.19 ± 0.04	0.00			0

* Mice fed disulfiram for 24 days.
† Mice fed disulfiram for 7 days.

sequent experiments revealed these effects on egg production when mice were kept on a diet consisting of 0.5 or 0.2% DS, but only partially at 0.05% (Table 1). In addition, we observed that all females were producing abnormal eggs 10–180 min after the intraperitoneal injection of 200, 100 or 50 mg/kg of DS or DDC to infected mice, but not after a 10 mg/kg dose. The effects of DS on egg production were completely reversible since normal eggs were being produced by the female parasites within 24 hr after a 200 mg/kg injection or after the removal of the infected mice from the DS diet.

Since DS disrupts female egg production, we examined the effects of this drug on other schistosome physiological and biochemical parameters. Adult paired worms, isolated from mice fed DS (0.2 per cent in diet) for 7 days, consumed the same amount of oxygen (DS = 1.74 ± 0.12; control = 2.02 ± 0.19 μmoles/mg of protein/min) and weighed (DS = 0.62 ± 0.05; control = 0.57 ± 0.07 mg wet weight/ten worm pairs) the same as control worms not exposed to DS. Lactic acid production was significantly ($P < 0.01$) higher in worms (435 ± 55 μg/mg of protein/hr) from mice treated with DS as compared to their controls (155 ± 20 μg/mg of protein/hr). The distribution of paired schistosomes between the mesenteric and portal veins of the mouse was the same in DS-treated and control mice.

Since DS is a potent inhibitor of mammalian aldehyde dehydrogenase, its effect on female egg production could be due to an elevation, to toxic levels, of aldehydes in the serum of the host. We fed infected mice DS (0.2 per cent in diet) for 7 days and then measured the levels of acetaldehyde in the serum of these mice. We did not detect any acetaldehyde in the serum of these mice or their respective controls.

Because DS blocks egg production, we wanted to determine if DS could alter the pathology of this disease in mice by treating infected mice with a 1 per cent diet of this drug from day 27 to day 51 after exposure to 150 cercaria. On the day 51 we sacrificed the mice and observed that the livers and to a greater extent the spleens of infected mice treated with DS weighed 21 and 58 per cent less, respectively, than livers and spleens from infected mice not treated with DS. In addition, the textures of the livers removed from DS-treated mice were smooth and red whereas the surfaces of livers from nontreated mice were rough and gray, indicating the presence of granulomatous material (Fig. 1).

To determine if there was a relationship between the effect of DS on female schistosome egg production and its effect upon the NE levels of the parasite, we measured the concentration of NE in worms removed from mice that were kept on a diet containing a level of DS (0.01 per cent) that would not affect egg production. Worms removed from mice kept on this diet for 7 days contained no detectable amount of NE (Table 1); thus, since egg production was normal, it appears that NE is not involved in this process.

Because the enzyme phenol oxidase is thought to be involved in the production of eggs by trematodes [6], we examined the effects of DS on female schistosome phenol oxidase. Thirty min after the administration of DS (200 mg/kg, i.p.) to infected mice we removed and isolated the female parasites and determined if phenol oxidase activity was present. We observed that phenol oxidase activity in disulfiram-treated female worms (3.1 ± 0.2 μmoles oxygen/min/mg of protein) was significantly lower ($P < 0.01$) than in control worms (7.8 ± 0.3 μmoles oxygen/min/mg of protein) isolated from mice not exposed to DS.

Trematode phenol oxidases appear to be copper-containing enzymes [12, 15] since they can be inhibited by copper-chelating agents. For example, Mansour [12] demonstrated that the phenol oxidase of the trematode of *F. hepatica* could be inhibited by the copper chelator DDC. Since DS is metabolized by mice to the potent copper chelator DDC [3, 16], we examined the effects of DDC and various copper chelators on female schistosome phenol oxidase activity and on egg production by *S. mansoni* (Table 2). We observed that DDC was the only copper chelator, among the three we examined, that affected both egg production and inhibited the parasite's phenol oxidase. A previous report [15] describing the effects of similar copper chelators on another copper-containing phenol oxidase has also observed results similar to ours. Since many of the phenol oxidases are membrane bound [3, 15, 16], it has been suggested that the lipophilic properties of DDC (as compared to the hydrophilic properties of cysteine and penicillamine) allow it to penetrate into the membrane and thus concentrate at those sites where the enzyme is located.

In addition to examining the effects of DS on adult

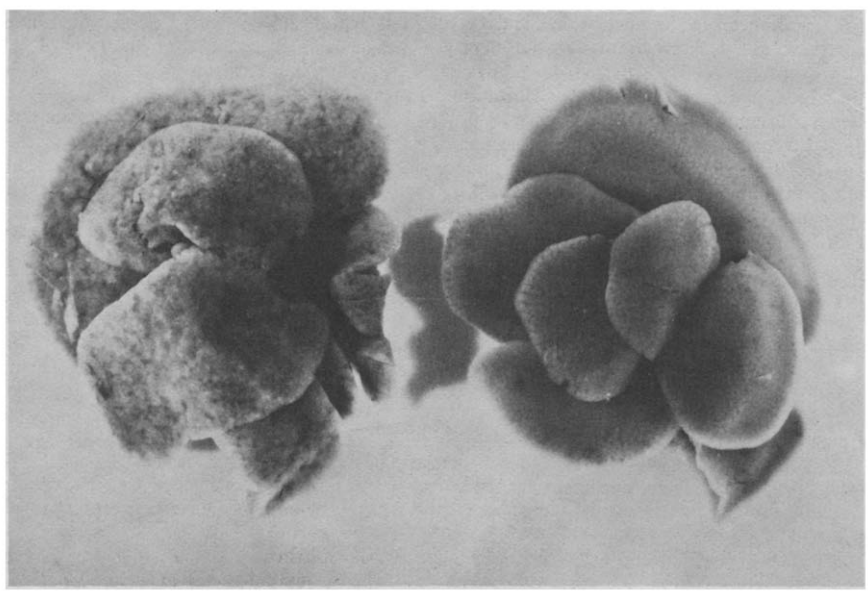

Fig. 1. Liver from an infected mouse not given disulfiram is on the left, while liver from infected mouse given disulfiram is on the right.

Table 2. Effect of compounds which bind copper on female schistosome phenol oxidase activity and egg production

Compound (10^{-4} M)	Phenol oxidase activity as per cent of control*	Per cent of females producing abnormal eggs†
Control	100	0
Diethyldithiocarbamate	29 ± 6	100
Cysteine	79 ± 7	0
Penicillamine	115 ± 16	0

* Females were incubated for 4 hr in Eagle's medium and then homogenized as described in the text. Above drugs were added to homogenate and incubated for 3.5 min before addition of 2×10^{-3} M dopamine.

† Egg production was measured 30 min after i.p. injection of 100 mg/kg of each drug.

worms, we examined the effect of DS on the development of the parasite within mice. Three days before exposure to cercaria, mice were fed a 0.2 per cent diet of DS and maintained on this diet until the worms should have reached the adult stage. Thus, about 42 days after exposure to cercaria the non-drug-treated mice were sacrificed and the number of adult paired schistosomes in their portal and mesenteric veins and liver were compared to the number in mice treated with DS. The mean number of adult worms in DS-treated mice (4.5 ± 0.8; $N = 6$) was significantly ($P < 0.01$) lower than the mean number obtained from mice not exposed to DS (12.5 ± 1.3; $N = 6$). Thus it appears that DS has an effect upon the development of *S. mansoni* within mice.

Our results indicate that DS can lower norepinephrine in *S. mansoni*. In addition, this drug induces the production of abnormal eggs by the female and can reduce the pathological consequences of this disease when given chronically to recently infected mice. The DS-induced disruption of female egg production was not related to the drug's ability to lower the concentration of norepinephrine within the female, since the drug's effect upon worm norepinephrine levels could be observed at doses that would not affect egg production.

Excluding the effects mentioned above, DS did not appear to be overtly toxic or lethal to the adult parasite, i.e. weight and oxygen consumption by worms exposed to DS was the same as worms not exposed to DS. The fact that worms exposed to DS produced more lactic acid than their controls is difficult to interpret. It is known that schistosomes derive most of their energy (i.e. ATP) from the glycolytic pathway [17] and that many anti-schistosomal compounds will depress schistosome lactic acid production [17]. Thus, if this rise in lactic acid production is an indicator of the toxic effects of DS, this effect on lactic acid production is the opposite from what would have been predicted.

The effects of DS on egg production by the female schistosome are not novel, since Machado *et al.* [18] reported that an allyl-thiourea, thiosinamine, could selectively suppress egg production by female *S. mansoni*. Using a histochemical technique for localization of phenol oxidase, they reported that thiosinamine inhibited this enzyme and that this inhibition led to the production of abnormal eggs. In addition, recent work on female *Schistosoma japonicum* has demonstrated that thiourea will selectively inhibit egg production [19]. The authors did not investigate the mechanism by which thiourea interferes with egg production. We observed that phenol oxidase activity was decreased in female worms isolated from mice given DS, and that DDC was a potent inhibitor of this enzyme. DDC is also a potent inhibitor of the phenol oxidase of *F. hepatica* [12], a parasite that is similar to *S. mansoni* (i.e. they are both trematodes). Thus, our work combined with previous reports on female schistosome phenol oxidase suggests that this enzyme can be inhibited by a series of structurally related compounds (DDC, DS, thiourea and thiosinamine) and that this inhibition is correlated with disruption of female egg production.

We previously mentioned that mammalian DBH can be inhibited by DDC, and that inhibition of this enzyme elevates dopamine and decreases norepinephrine, respectively, in the brain of rats given either DDC or DS. Since the parasite's levels of dopamine and norepinephrine also increased and decreased, respectively, after exposure to DS, it appears that the parasite's DBH can be inhibited by this drug. In fact, this enzyme appears to be much more sensitive to the drug than mouse brain DBH because the concentration of norepinephrine in the brain of infected mice given 0.02% DS did not decrease below control values whereas the norepinephrine levels in worms from these drug-treated mice were significantly below the control levels (Table 1). It is important to note that DS ability to markedly lower the concentration of NE in schistosomes without causing any observable change in the parasite (e.g. motor activity) is evidence that this putative neurotransmitter substance [20] does not play a critical role in the physiological processes of this parasite.

The effect of DS on the developing parasite is difficult to assess because we do not know if the effects of this drug are due to a retardation of the parasite's growth or to a direct lethal effect upon the immature parasites. We are presently attempting to define more clearly the effects of this drug on developing parasites.

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Accumulation and metabolism of [^{14}C]histamine by rat lung *in vivo*

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The ability of the mammalian lung to provide a metabolic as well as a ventilatory function has been established over the past decade [1–4]. Included among those substances which can be accumulated and/or metabolized by this organ are the following: norepinephrine [5–7], epinephrine [5], isoproterenol [8], 5-hydroxytryptamine [6, 9, 10], bradykinin [11] and prostaglandins of the E and F series [12]. While rat lung inactivates norepinephrine [5, 7], epinephrine [5] and 5-hydroxytryptamine [9, 10], it has been suggested that this tissue does not remove histamine from the circulation [13, 14]. Since chopped lung from this species has been shown to metabolize this amine [15], the ability *in vivo* of rat lung to accumulate and/or degrade exogenous histamine has been re-examined using a sensitive radiometric technique.

Adult male Sprague–Dawley rats (Charles River) weighing 150–250 g were injected i.v. with 1 μCi/200 g of [ring 2-^{14}C]histamine (sp. act. 57–59 mCi/m-mole, Amersham/Searle Corp., Arlington Heights, IL). At varying time intervals after injection, the animals were decapitated; the lungs were removed or the blood was collected in a heparinized centrifuge tube and the plasma prepared. After resection, the lungs were perfused through the pulmonary artery with ice-cold 0.9% NaCl, minced with scissors and homogenized in 10 vols of 0.4 N $HClO_4$. After standing for 15 min in the cold (4°), the homogenate was centrifuged at 1000 *g* for 15 min. A 100-μl aliquot of the resultant supernatant was counted in a liquid scintillation spectrometer (Packard Instrument Co.) for determination of total ^{14}C. The remainder of the supernatant was extracted according to the technique of Snyder *et al.* [16] for determination of [^{14}C]histamine. Plasma was mixed with an equal volume of 0.8 N $HClO_4$, allowed to stand for 15 min, centrifuged at 1000 *g* for 15 min and then treated in a manner identical

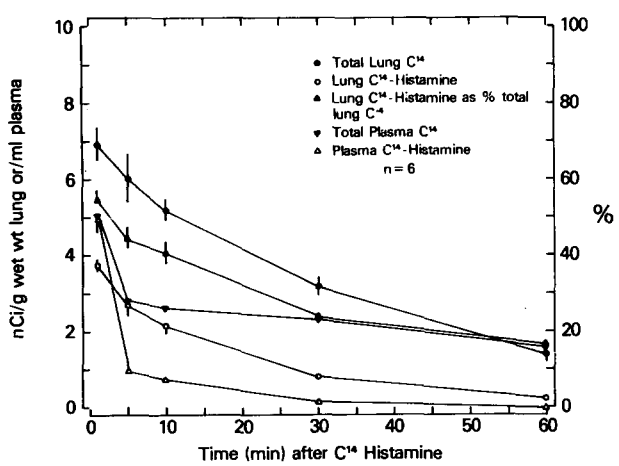

Fig. 1. Time course of lung and plasma levels of total ^{14}C and [^{14}C]histamine after the i.v. administration of 1 μCi/200 g of [^{14}C]histamine to rats; n = number of animals. Values are mean ± S.E.M. Where the S.E.M. is omitted, it was smaller than the symbol for the mean.