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Inhibition by metrifonate and dichlorvos of cholinesterases in schistosomes

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Summary

1. No species differences between *Schistosoma haematobium* and *Schistosoma mansoni* were detected when the I50 of metrifonate for the acetylcholinesterases (AChE) and the cholinesterases (ChE) of these two trematodes were determined in isolated enzyme preparations or following exposure of the intact worms to this drug *in vitro*.

2. *S. haematobium* appeared to be more affected by AChE inhibition because, after administration of metrifonate to hamsters, a hepatic shift of the parasites was observed with a dose of metrifonate (150 mg (0·6 mmol) per kg) which produced no shift of *S. mansoni*, although AChE inhibition was comparable in both species.

3. Administration of a possible metabolite of metrifonate, dichlorvos, to hamsters resulted in a greater inhibition of AChE and ChE activities of *S. haematobium* than those of *S. mansoni*. Furthermore, when schistosomes were incubated with dichlorvos, inhibition of AChE activity of female *S. haematobium* was significantly greater ($P<0{\cdot}005$) than that of both sexes of *S. mansoni* and of male *S. haematobium*.

4. The discrepancy between the lack of a significant chemotherapeutic effect of metrifonate in hamsters infected with *S. haematobium* and the clinical results obtained with this organophosphorus compound in human schistosomiasis *haematobium* is discussed, and the need to conduct similar studies in primates is pointed out.

Introduction

The organophosphorus derivative metrifonate (*O,O*-dimethyl-2,2,2, trichloro-1-hydroxyethylphosphonate ; Dipterex ; Trichlorfon) is effective in the treatment of human urinary schistosomiasis caused by *Schistosoma haematobium*, but lacks activity in animals and human subjects infected with *Schistosoma mansoni* (Beheyt, LeBrun, Cerf, Dierickx & Degroote, 1961 ; Cerf, LeBrun & Dierickx, 1962 ; Abdalla, Saif, Taha, Ashmawy, Tawfik, Abdel-Fattah, Sabet & Abdel-Meguid, 1965 ; Hanna, Basmy, Selim, Shoeb & Awny, 1966 ; Forsyth & Rashid, 1967 ; Katz, Pellegrino & Pereira, 1968 ; Davis & Bailey, 1969). Since many organophosphorus compounds are potent inhibitors of various cholinesterases and since there is evidence that acetylcholine may be a humoral neurotransmitter in

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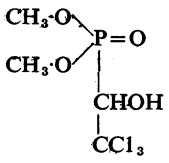
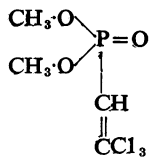
schistosomes (Bueding, 1952; Barker, Bueding & Timms, 1966), it is possible that inhibitors of acetylcholinesterase may interfere with the functional integrity of the parasite.

Accordingly, the effects of metrifonate and of a presumed metabolite thereof, dichlorvos,



Metrifonate



Dichlorvos

on schistosome cholinesterases were studied. It was found that differences in the chemotherapeutic activities of metrifonate in human subjects infected with *S. haematobium*, on the one hand, and with *S. mansoni* on the other, could not be accounted for by differences in the inhibitory potency of metrifonate on the enzymes catalyzing the hydrolysis of acetylcholine in the two species of trematodes.

Methods

Female hamsters weighing 75 to 120 g were infected by exposing the shaved abdomen of each animal (previously anaesthetized by the intraperitoneal injection of 75 mg of sodium pentobarbitol/kg) to 125 cercariae of *S. mansoni*. Hamsters infected with *S. haematobium* were kindly supplied by Dr. Elmer Berry, University of Michigan, through the U.S. Japan-Cooperative Medical Science Program.

The hepatic shift of the worms, a sensitive criterion for antischistosomal activity (Bang & Hairston, 1946; Kikuth & Goennert, 1948; Standen, 1953; Buttle & Khayyal, 1962), is represented by the percentage of the total number of worms found in the liver. In untreated control hamsters the number of worms in the liver was negligible.

For determination of AChE and ChE activities, adult worms were removed from the mesenteric and portal veins or from the liver sinuses and placed in 75% horse serum. After three transfers in a buffered solution containing 50% Earle's medium (Earle, 1943) and 0·02 M glycylglycine buffer, pH 7·8, the worms were homogenized, in a beaker containing ice, with 0·05 M phosphate buffer, pH 7·3 (40 worms per ml), and centrifuged at 18,000×*g* for 90 min at 2 to 4° C. In this manner, an acetylcholinesterase (AChE, acetylcholine acetyl-hydrolase, E.C. 3.1.1.7) can be separated from one or several other cholinesterases (ChE, acetylcholine acyl-hydrolase, E.C. 3.1.1.8) of schistosomes (Bueding, 1952). The supernatant, containing ChE, was removed; the sediment, in which AChE is localized, was suspended in a volume of phosphate buffer equal to that of the supernatant.

Brains from female mice (Swiss-Webster, weighing 18 to 24 g) were homogenized in 0·05 M phosphate buffer, pH 7·3. After centrifugation (18,000×*g*, for 30 min, 2 to 4° C), the sediment was suspended in the same buffer. The suspended sediment did not catalyze the hydrolysis of butyrylcholine and thus contained no ChE activity.

The activities of AChE and ChE were determined by a modification of the method of Ellman, Courtney, Andres & Featherstone (1961), with an optimal

concentration of acetylthiocholine. In inhibition studies 0·04 ml of the enzyme preparations were preincubated with or without varying concentrations of the organophosphorus compound in a final volume of 0·1 ml at 25° C for 10 minutes. Thereafter acetylcholine, 5,5-dithiodinitro-bis-(2-nitrobenzoate) (DTNB) and phosphate buffer were added and the volume was brought to 1·0 ml with water. The final molar concentrations of the constituents of the reaction mixture for the determinations of AChE and ChE activities were as follows: acetylthiocholine: 0·05 M for the schistosome, and 0·005 M for the brain preparations; DTNB: 0·1 mM; phosphate buffer 0·05 M (pH 7·3). Enzymic activity was measured by the increase in optical density at 412 nm, recorded with a Zeiss PMQ-spectrophotometer every minute for 5 min at 25° C. Appropriate corrections were made by subtracting the increase in optical density of the reaction mixture without enzyme preparation and for tissue blanks. Over a period of 5 min enzyme activities both in the absence and the presence of inhibitors were linear with time.

The histochemical distribution of AChE in schistosomes was determined by a procedure described previously (Bueding, Schiller & Bourgeois, 1967).

Protein concentrations were determined according to Lowry, Rosebrough, Farr & Randall (1951).

Results

The AChE activities per mg protein of both sexes of *S. mansoni* and of male *S. haematobium* were approximately half, while AChE activity of female *S. haematobium* equalled that of mouse brain. The latter amounted to 103 ± 7 nmol acetylthiocholine hydrolyzed per min and per mg protein (25° C).

The AChE activities of schistosomes were 4 times more sensitive to the inhibitory effects of metrifonate than the AChE activity of mouse brain and the inhibitory potency of metrifonate was the same for the AChE activities of both sexes of *S. mansoni* and of *S. haematobium* (Table 1). The latter applied also to the effects of metrifonate on ChE activities of these two species but a slightly higher concentration was required for the inhibition of ChE than that of AChE (Table 1).

TABLE 1. *Inhibitory effects of metrifonate on cholinesterase activities in schistosomes and mouse brain*

Preparation tested	Species or tissue	Sex	I50* AChE	I50* ChE
Fractionated tissue homogenate†	*S. mansoni*	♂	$7{\cdot}5\times10^{-5}$	1×10^{-4}
		♀	$7{\cdot}5\times10^{-5}$	1×10^{-4}
	S. haematobium	♂	$7{\cdot}5\times10^{-5}$	1×10^{-4}
		♀	$7{\cdot}5\times10^{-5}$	1×10^{-4}
	Mouse brain		3×10^{-4}	
Intact worms‡	*S. mansoni*	♂	$3{\cdot}5\times10^{-6}$	3×10^{-5}
		♀	4×10^{-6}	3×10^{-5}
	S. haematobium	♂	4×10^{-6}	3×10^{-5}
		♀	4×10^{-6}	3×10^{-5}

* Molar concentration of metrifonate producing 50% inhibition of enzyme activity. Each figure represents the mean of 8 experiments. † Incubation with metrifonate at 25° C for 10 min before addition of substrate. ‡ The schistosomes were incubated in 75% horse serum at 37° C for 90 min without and with varying concentrations of metrifonate. Subsequently the parasites were homogenized and their AChE and ChE activities were determined.
For details, see **Methods.**

When AChE and ChE activities were determined following incubation at 37° C of intact schistosomes of both species in 75% horse serum containing metrifonate, the AChE and ChE activities of both species were inhibited to the same extent by exposure to $3{\cdot}5\text{–}4\times10^{-6}$M and 3×10^{-5}M metrifonate, respectively (Table 1). Complete paralysis of the acetabulum and of the oral sucker of *S. haematobium* was observed at a metrifonate concentration of 2×10^{-5}M; that of the body musculature at 1×10^{-4}M. These concentrations were the same as those producing this effect in *S. mansoni*. At both these levels of metrifonate in the medium, inhibition of AChE activities in both species was nearly complete (90–100%).

In a limited number of experiments metrifonate, at three dose levels, was administered orally to hamsters infected with either *S. mansoni* or with *S. haematobium*. At each dose level, an initial, dose-dependent, inhibition of AChE and ChE activities was observed (Table 2). These levels of inhibition gradually declined over a period of a week and the original activity returned in a month. In every case inhibition of AChE activity was greater than that of ChE activity. There was no significant difference in the degree of inhibition of AChE of *S. mansoni* and of *S. haematobium* when the effect of the same dose was determined at the same time interval. However, *S. haematobium* appeared to be more affected by a given dose of metrifonate because this species shifted to the liver more readily than *S. mansoni* (Table 2). When, after intervals ranging from 2 to 4 weeks, administration of metrifonate was repeated a second and then a third time, these effects and their time course remained essentially the same. In every case, even at the highest dose level (200 mg (0·8 mmol)/kg), there was, following inhibition, a gradual return of AChE activity and when this started the hepatic shift disappeared. In no instance were dead worms found in the liver, nor was there a significant reduction in the number of live worms. Histochemical determination of AChE activity was in agreement with enzyme assays. For example, a significant decrease of AChE activity in the central ganglia, the nerve trunks and the two sucker organs of the worms was observable 17 h after the administration of 150 mg/kg of metrifonate, but the normal histochemical appearance returned nine days thereafter. It should be noted that the two higher doses used, 150 and 200 mg/kg, approached the maximum tolerated dose because the host animals exhibited many signs of cholinergic stimulation such as increased salivary and bron-

TABLE 2. *Effect of the oral administration of metrifonate to hamsters infested with schistosomes*

Dose (oral)	Time after drug administration	*S. mansoni* % inhibition of AChE	*S. mansoni* % inhibition of ChE	*S. mansoni* Hepatic shift %	*S. haematobium* % inhibition of AChE	*S. haematobium* % inhibition of ChE	*S. haematobium* Hepatic shift %
100 mg	24 h	58 (±2)	43 (±2)	0	61 (±2)	20 (±2)	0
(0·4 mmol)	96 h	52 (±2)	12 (±1)	0	54 (±3)	30 (±2)	0
/kg	22 days	0	0	0	0	0	0
150 mg	24 h	81 (±3)	42 (±1)	0	84 (±2)	37 (±1)	50
(0·6 mmol)	48 h	78 (±2)	45 (±2)	0	84 (±1)	45 (±3)	50
/kg	90 h	57 (±2)	56 (±3)	0			
	24 h	88 (±1)	57 (±2)	50	92 (±2)	45 (±1)	100
200 mg	48 h	83 (±2)	56 (±3)	0	73 (±2)	35 (±2)	50
(0·8 mmol)	9 days				32 (±1)	12 (±1)	0
/kg	20 days	31 (±1)	22 (±1)	0			
	31 days	16 (±1)	7 (±1)	0			

The figures in each column are means of three experiments, with S.E.M. in parentheses.

chial secretions, miosis, diarrhoea, fasciculation, generalized perspiration, muscular weakness and respiratory difficulties. These effects subsided after several hours.

Dichlorvos was more potent than metrifonate as a cholinesterase inhibitor (Table 3). The AChE activity of mouse brain was approximately 5 times less susceptible to inhibition by this compound than AChE of schistosomes. The AChE activities of the female worms, especially in the case of *S. haematobium*, were slightly more sensitive ($P<0.02$) to inhibition than those of the males (Table 3). In both species AChE was more readily inhibited than was ChE. While dichlorvos was approximately equally potent as an inhibitor of the ChE activities of male and female *S. mansoni* and of male *S. haematobium*, the activity of female *S. haematobium* ChE was more than twice as susceptible to inhibition as that of the males ($P<0.005$).

Incubation of schistosomes with dichlorvos revealed that the AChE activities of female *S. haematobium* were almost three times more susceptible to inhibition by this compound than the corresponding activities of either male or female *S. mansoni* (Table 3). The AChE of male *S. haematobium* was also significantly different in this respect from that of *S. mansoni*; but the difference was less pronounced ($P<0.05$).

The highest single non-lethal oral dose of dichlorvos for hamsters was 20 mg (0·04 mmol)/kg. Administration of this dose did not result in a hepatic shift

TABLE 3. *Inhibitory effects of dichlorvos on cholinesterase activities in schistosomes and mouse brain*

Preparation tested	Species or tissue	Sex	I50* AChE	I50* ChE
Fractionated tissue homogenates†	*S. mansoni*	♂	1.75×10^{-7}	2.5×10^{-7}
		♀	1.5×10^{-7}	2.5×10^{-7}
	S. haematobium	♂	1.75×10^{-7}	4×10^{-7}
		♀	1.25×10^{-7}	1.5×10^{-7}
	Mouse brain		7.0×10^{-7}	
Intact worms‡	*S. mansoni*	♂	2×10^{-6}	3×10^{-6}
		♀	2×10^{-6}	3×10^{-6}
	S. haematobium	♂	1.5×10^{-6}	2×10^{-6}
		♀	7.5×10^{-7}	2×10^{-6}

* Molar concentration of dichlorvos producing 50% inhibition of enzyme activity. Each figure represents the mean of 8 experiments. † Incubation with dichlorvos at 25° C for 10 min before addition of substrate. ‡ The schistosomes were incubated in 75% horse serum at 37° C for 90 min without and with varying concentrations of dichlorvos. Subsequently, the parasites were homogenized and their AChE and ChE activities were determined.
For details, see **Methods**.

TABLE 4. *Effect of the oral administration of 20 mg (0·04 mmol)/kg dichlorvos to hamsters infected with schistosomes*

Time after drug administration	*S. mansoni* % inhibition of AChE ♂	AChE ♀	ChE ♂	ChE ♀	*S. haematobium* % inhibition of AChE ♂	AChE ♀	ChE ♂	ChE ♀
2 h	44	50	30	46	62	65	42	52
96 h	29	32	19	20	44	46	37	47
7 days	—	—	—	—	12	12	27	14

(3 hamsters were used in each group)

of the worms, but, in contrast to metrifonate, the activities of the AChE and of ChE of both sexes of *S. haematobium* were inhibited to a greater extent than those of *S. mansoni* (Table 4).

Discussion

In schistosomes only a small proportion of their total proteins is contributed by the nervous tissue where AChE is predominantly, if not exclusively, located (Bueding *et al.*, 1967). Since the activity of schistosome AChE per mg of protein approaches that of mammalian nervous tissue (mouse brain), the activity of this enzyme in the nervous structures of these trematodes must certainly be of a high order.

No species differences between *S. haematobium* and *S. mansoni* were detectable with respect to the susceptibilities of their AChE and ChE activities to inhibition by metrifonate. Therefore, the clinical effectiveness of metrifonate in human subjects infected with *S. haematobium*, contrasted with its lack of activity against *S. mansoni* in man, cannot be explained by a higher susceptibility of the cholinesterases of *S. haematobium* to this drug. Furthermore, the difference in the clinical effectiveness of metrifonate against these two schistosome species cannot be accounted for by a greater permeability of *S. haematobium* for this drug because the inhibitory potencies of metrifonate on the cholinesterase activities of both species were the same when the intact worms were exposed to metrifonate *in vitro*. However, it should be noted that *S. haematobium* appears to be more susceptible to the consequences of AChE inhibition because it responded with a hepatic shift to a dose of metrifonate (150 mg/kg) which produced no shift of *S. mansoni*.

Exposure of schistosomes to dichlorvos *in vitro* and *in vivo* revealed some differences between *S. haematobium* and *S. mansoni*. Administration of the same dose of dichlorvos to hamsters resulted in a greater inhibition of AChE and ChE activities of *S. haematobium* than of those of *S. mansoni*. In addition, when the worms were incubated with dichlorvos, inhibition of AChE activity of female *S. haematobium* was significantly greater than that of AChE activities of both sexes of *S. mansoni* and of male *S. haematobium*. While it is by no means established that dichlorvos is a major metabolite of metrifonate in animals or man, conceivably AChE of *S. haematobium* may be more susceptible also to other analogues of metrifonate, one or some of which may be metabolites of the drug in a given host.

The possibility cannot be excluded that in man the chemotherapeutic activity of metrifonate against *S. haematobium* is brought about by mechanisms other than AChE or ChE inhibition. However, a correlation between inhibition of AChE activity and an effect on the worms was observed in hamsters. When inhibition of AChE activity had reached levels between 80 and 90%, the parasites shifted to the liver. Conversely, a subsequent return of enzyme activity was associated with a return of the worms to the mesenteric veins. Since there is evidence for a physiological role of acetylcholine in schistosomes (Bueding, 1952; Barker *et al.*, 1966) and since administration of another antischistosomal compound, i.e., *p*-rosaniline, results in an inhibition of AChE activity of *S. mansoni* (Bueding *et al.*, 1967), it is possible that AChE inhibition produces functional damage to the worm.

It is noteworthy that inhibition of schistosome AChE activity by metrifonate is not very selective because AChE of mouse brain is only four times less sensitive to inhibition by this drug than are the isofunctional enzymes of schistosomes. This compares unfavourably with other chemotherapeutic agents which are inhibitors of parasite enzymes. For example, 70 to 80 times higher concentrations of trivalent organic antimonials are required to inhibit the activity of mammalian phosphofructokinases to the same extent as that of the isofunctional enzyme of *S. mansoni* (Mansour & Bueding, 1954; Bueding & Fisher, 1966). Furthermore, dihydrofolate reductase of *Plasmodium berghei* is 2,000 times more sensitive to inhibition by pyrimethamine than the enzyme catalyzing the same reaction in the tissues of the host (Ferone, Burchall & Hitchings, 1969).

Even at highest tolerated doses administration of metrifonate to hamsters infected with *S. haematobium* resulted only in a temporary hepatic shift, with no elimination of worms. In fact, it is most unlikely that, on the basis of its effect in hamsters, this compound would have been considered for trials in human subjects. Several factors may account for the discrepancy between the low antischistosomal activity of the drug in hamsters and its observed chemotherapeutic effectiveness and low toxicity in man. In man, metrifonate may be metabolized to a compound which has a greater selectivity for the acetylcholinesterase of the parasite. Furthermore, in the hamster, *S. haematobium* is located in the mesenteric veins, while in man the adult parasite establishes itself in the pelvic veins. In the latter the concentration of either metrifonate or of an active metabolite may be higher. Also, it is possible that metrifonate or an active metabolite is concentrated in red cells; since schistosomes ingest these cells, the effect of an AChE inhibitor may be prolonged because of the longer half-life of erythrocytes in larger animals.

Evidence for the chemotherapeutic activity of metrifonate against *S. haematobium* in man is based on the disappearance of eggs from the urine. This could have been caused either by the destruction of the worms or by a shift of the functionally damaged parasites to an 'ectopic' site, such as the lung (Abdalla *et al.*, 1965; Forsyth & Rashid, 1967; Davis & Bailey, 1969). In the latter eventuality, it has not been ascertained with certainty whether or not subsequently the schistosomes are eliminated.

The problems discussed above may be resolved, at least in part, by studies of the effects of metrifonate in primates infected with *S. haematobium*, because in these host animals the trematode establishes itself in the pelvic veins. Furthermore, investigations on the mode of the chemotherapeutic action of metrifonate in human urinary schistosomiasis would be of particular significance because this compound has been found to be not only effective, but also well tolerated in this infection (Davis & Bailey, 1969). In addition, it has no mutagenic, teratogenic or carcinogenic effects in animals (D. Lorke: personal communication). Therefore, metrifonate appears to be not only a suitable alternative to, but also has a considerably lower risk-to-benefit ratio than a widely used antischistosomal compound, hycanthone, whose mutagenic properties have been demonstrated in bacterial and mammalian cell systems *in vitro* (Hartman, Levine, Hartman & Berger, 1971; Clive, Flamm & Machesko, 1972), as well as in a host-mediated mammalian cell assay (G. A. Fischer: personal communication) and which has been found to be teratogenic in mice (Moore, 1972). These factors should receive especial attention

when the treatment of urinary schistosomiasis is considered for children and other individuals with a long life expectancy.

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