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THE BIOCHEMICAL MECHANISM OF ACTION OF THE ANTINEMATODAL DRUG TETRAMISOLE

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Abstract—The effect of tetramisole was studied on succinate dehydrogenase activity of the nematodes *Ascaris suum*, *Ascaridia galli*, *Toxocara cati* and *Dictyocaulus viviparus*, the cestodes *Taenia taeniaeformis*, *Taenia pisiformis* and *Dipylidium caninum*, and also on rat liver and pigeon breast muscle. Administered to nematode preparations at low concentrations, it reduced the succinate, NAD and ATP levels and increased the fumarate, $NADH_2$ and inorganic phosphate levels. In all experiments the laevo-isomer was a more potent inhibitor of the fumarate-succinate system than the dextro-isomer.

Although tetramisole inhibited $NADH_2$ oxidation in coenzyme-free pulps of nematodes, there was no effect upon $NADH_2$ oxidation in cestodes under the same conditions. Similarly, although tetramisole inhibited succinate oxidation in coenzyme-free pulps of nematodes, there was no effect upon succinate oxidation in pigeon breast muscle at the drug-concentrations tested. Inhibition of succinate oxidation was observed in rat liver, but at drug-concentrations fifty times higher than those necessary to inhibit fumarate reduction to the same extent in the nematodes studied.

The experiments suggest that at low concentrations tetramisole inhibited stereospecifically "fumarate reductase" activity in nematodes, although succinate dehydrogenase activity was unaffected in the other organisms tested.

THE SUCCINATE dehydrogenase of tissues dependent upon aerobic metabolism catalyses particularly the oxidation of succinate,[1] whereas that of parasites living in predominantly anaerobic habitats (e.g. the small intestine) seems to catalyse particularly the reverse reaction, the reduction of fumarate, as described for *Ascaris* muscle.[2] As this reduction appears to serve as an important source of ATP for *Ascaris* muscular contraction,[3] inhibition of the reaction should result in paralysis.

Tetramisole,* has broad spectrum activity against almost all gastrointestinal and pulmonary nematodes, but is almost inactive against cestodes and trematodes.[4, 5] It paralyses susceptible nematode species *in vitro* and *in vivo*[4] and there is good evidence that this results from inhibition of the succinate dehydrogenase[6] and that the laevorotatory S-isomer, which is largely responsible for the anthelmintic activity of racemic tetramisole,[7] is a more potent inhibitor of *Ascaris* muscle succinic dehydrogenase than the dextro-isomer.[6]

If tetramisole does act as a specific succinate dehydrogenase inhibitor, then two conditions must be fulfilled:

(1). In susceptible organisms, it must decrease the concentrations of succinate, ATP and NAD, and increase the concentrations of fumarate, inorganic phosphate and $NADH_2$.

* Generic name for the hydrochloride of racemic 2,3,5,6-tetrahydro-6-phenyl-imidazo [2,1-b] thiazole.

(2). In non-susceptible organisms, it must not inhibit the succinate dehydrogenase enzyme system.

The present paper describes experiments investigating the extent to which these criteria are met by tetramisole and by its optical isomers.

MATERIALS

Reagents. NAD (DPN 15300), crystalline fumarase (FUM 15155) and the crystalline disodium salts of fumaric acid (FU 15684) and succinic acid (SUC 15147) were obtained from Boehringer & Soehne, Mannheim, Germany; $NADH_2$ (β, Grade III) from Sigma Chemical Co., St. Louis, U.S.A.; and bovine serum albumin (fraction V, Grade B) from Calbiochem., California, U.S.A.

Biological material. Nematode species comprised *Ascaris suum* from pigs, *Dictyocaulus viviparus* from calves, *Toxocara cati* from cats and *Ascaridia galli* from chickens; cestode species comprised *Taenia taeniaeformis* from cats and *Taenia pisiformis* and *Dipylidium caninum* from dogs. All worms were thoroughly rinsed in Epps physiological saline[8] and kept in an incubation medium[9] at 37° until required. In all cases they were used within 4 hr of collection. Pigeon breast muscle and rat liver were also used after removal of fat and connective tissue.

METHODS

Succinate, Inorganic phosphate and ATP determinations. Ascaridia galli were incubated in a buffered glucose-salt medium[9] for 15 hr at 37° in an atmosphere of 5% CO_2 and 95% N_2. Each Erlenmeyer flask contained 5 worms in 19 ml of medium, to which 1 ml of tetramisole solution (made up in 0·1 M Tris-buffer of pH 7·4) or 1 ml of buffer solution alone was added.

For the determination of succinate, the parasites were blotted, weighed and homogenised in 5 ml of 5% perchloric acid with an Ultra–Turrax Disintegrator. After centrifugation for 30 min at 10,000 *g*, an aliquote of the supernatant was treated as described by Bueding *et al.*[9] and the succinate content determined according to Umbreit *et al.*,[10] using a conventional Warburg apparatus. To take account also of the succinate excreted, the medium was similarly analysed, except that treatment with perchloric acid was omitted.

For the determination of inorganic phosphate content, the worms were incubated as above and homogenised in the incubation medium, and 5 ml of the homogenate was treated with 1 ml of 25% trichloroacetic acid. After centrifugation at 25,000 *g* for 10 min, the inorganic phosphate content was determined by an automated sodium molybdate technique.[11]

For the determination of ATP content, the worms were incubated and homogenised in the incubation medium as previously, and 5 ml of 5% perchloric acid was added to the homogenate. After centrifugation at 12,000 *g* for 20 min, the ATP content was found using the Boehringer "Biochimica-Test Combination" (No. 15979, Boehringer & Soehne, Mannheim, Germany).

Fumarate and NAD determinations. Ascaridia suum coenzyme-free muscle pulps were made by the method of Bueding and Charms.[12] Other pulps were obtained as follows: whole worms, pigeon breast and rat liver were cut into small pieces, homogenised by a modified Ultra–Turrax at low speed in ice-water, and a coenzyme-free pulp prepared as described by Bonner.[13]

Acetone powders were made by the method of Singer *et al.*[14] A 2% suspension of the powder in 0·08 M Tris-buffer of pH 8·5 was first blended at 2° for 45 min and then vigorously stirred for 30 min. The slightly opalescent solution obtained on centrifugation at 3000 *g* for 20 min contained the succinate dehydrogenase in soluble form.

Anaerobic reduction of fumarate and oxidation of $NADH_2$ in the coenzyme-free material was carried out for 60 min at 37°, under nitrogen and in the presence of 0·5 ml of fumaric acid (6 μmole), 0·5 ml of $NADH_2$ (10 μmole), 0·5 ml of $MnCl_2$ (0·5 μ mole), 0·5 ml of tetramisole solution or buffer (0·1 M Tris, pH 7·4) and 0·5 ml of buffer (0·08 M Tris, pH 8·5). The coenzyme-free material comprised either 0·5 ml of pulp or 0·5 ml of acetone powder extract in 0·08 M Tris (pH 8·5).

For the determination of fumarate content, the reaction mixtures were deproteinised with 1 ml of 5% perchloric acid and centrifuged at 10,000 *g* for 20 min. The pH of the supernatant was adjusted to 7·0 by the addition of 5 N KOH. After standing for 60 min at 4°, the $KClO_3$ was removed by centrifugation at 10,000 *g* for 10 min. The supernatant was treated with Norit as described by Kmetec and Bueding[2] and after further centrifugation at 10,000 *g* for 10 min, the fumarate content was determined spectrophotometrically at a wavelength of 240 mμ, at room temperature.[2] To find the amount of fumaric acid converted, the measured value was subtracted from the initial value.

For the determination of NAD content, the reaction mixtures were deproteinised with 5% trichloroacetic acid and centrifuged at 10,000 *g* for 10 min, and the trichloroacetic acid was extracted with ether. The NAD content was found by the methyl ethyl ketone fluorimetric method (excitation: 360 mμ, fluorescence: 460 mμ),[15] using a Spectrofluorimetro (model DC/3000-CGA).

Succinic dehydrogenase determination. One millilitre of coenzyme-free pulp was incubated for 60 min at 37° under nitrogen, with 1 ml of tetramisole solution or buffer (0·1 M Tris, pH 7·4). After 1 hr, the activity of the succinic dehydrogenase was determined in an aliquot (usually 0·1 ml) of the reaction mixture by the ferricyanide method of Keilin and King.[16] The assay mixture (total volume: 3·5 ml) comprised 0·1 M-phosphate buffer (pH 7·8), 6m M-potassium ferricyanide, 3 mg of bovine serum albumin and 0·04 M-succinate.

In all cases, protein was determined by the Biuret method.[17]

RESULTS

The effects of tetramisole and its optical isomers upon succinate, inorganic phosphate, ATP, fumarate and NAD levels, and upon succinic dehydrogenase activity are presented in Tables 1–6 respectively, for various nematode and cestode species and for rat liver and pigeon breast muscle.

TABLE 1. DECREASE IN THE SUCCINATE PRODUCTION OF *ASCARIDIA GALLI* DUE TO TETRAMISOLE

Amount of *dl*-tetramisole added (mμ moles/ml of incubation mixture)	Succinate level (μ moles/g of worm)			
	Worms	Medium	Total	Difference from controls
0	4·58	2·67	7·25	
2·6	3·18	1·73	4·91	−2·34
5·2	2·87	1·30	4·17	−3·08
10·4	0·58	2·08	2·66	−4·59

TABLE 2. INCREASE IN THE INORGANIC PHOSPHATE CONTENT OF *ASCARIDIA GALLI* DUE TO TETRAMISOLE

Amount of tetramisole added (mμ moles/ml of incubation mixture)	Isomer(s)	Inorganic phosphate level (μ moles/g of worm)	
		Total	Difference from controls
0	*dl-*	11·06	
2·6	*dl-*	14·54	+3·48
2·6	*l-*	16·01	+4·95
5·2	*dl-*	15·80	+4·74
5·2	*l-*	17·21	+6·15
10·4	*dl-*	18·54	+7·48

TABLE 3. DECREASE IN THE ATP CONTENT OF *ASCARIDIA GALLI* DUE TO TETRAMISOLE

Amount of tetramisole added (mμ moles/ml of incubation mixture)	Isomer(s)	ATP level (μ moles/g of worm)	
		Total	Difference from controls
0		11·19	
2·6	*dl-*	9·14	−2·05
2·6	*l-*	8·68	−2·51
5·2	*dl-*	7·23	−3·96
5·2	*l-*	6·80	−4·39
10·4	*dl-*	6·55	−4·64

The results show that, under the experimental conditions, tetramisole decreased the concentrations of succinate, ATP and NAD, and increased those of fumarate, inorganic phosphate and $NADH_2$ in the nematode species studied, but there was no evident effect upon NAD production in the presence of fumarate in cestodes (Table 5) or upon succinic dehydrogenase activity in pigeon heart muscle (Table 6) at the concentrations used. Although there was apparently some inhibition of the enzyme system in rat liver pulp (Table 6), this occurred at drug levels 50 times higher than those necessary to inhibit fumarate reduction in nematodes to the same extent.

In all cases, the laevo-isomer of tetramisole was more potent than the dextro-isomer.

DISCUSSION

The results obtained agree well with the working hypothesis that tetramisole acts, under the present conditions, as a specific inhibitor of succinic dehydrogenase activity in nematodes. Moreover, the superior potency of the laevo- over the dextro-isomer was confirmed, indicating a stereospecific action of the drug.

Comparison of tetramisole inhibition of fumarate reduction (Table 4) and of succinate oxidation (Table 6) shows that the drug was more potent as an inhibitor of fumarate conversion and accordingly may be better considered as a "fumarate reductase" inhibitor than as a succinic dehydrogenase inhibitor in the present experiments.

Although cestodes (including *Taenia taeniaeformis*,[18] here studied) form succinate, presumably by the same pathway as that found for *Ascaris*[20, 21], tetramisole was

TABLE 4. DECREASE IN FUMARATE REDUCTION IN THREE NEMATODE SPECIES DUE TO TETRAMISOLE*

Species	Preparation	Amount of protein in incubation mixture (mg)	Amount of tetramisole added (μ moles/ml of incubation mixture)		Fumarate converted† Absolute (μ moles/ml of incubation mixture)	Fumarate converted† % (control = 100)
Ascaris suum	Coenzyme-free muscle pulp	13·19	0		0·755	100
			0·138	*l*-	0·562	74·4
			0·276	*l*-	0·234	30·9
Ascaris suum	Acetone powder	4·05	0		0·231	100
			1·1	*d*-	0·199	86·1
			1·1	*l*-	0·019	8·2
Toxocara cati	Coenzyme-free whole worm pulp	8·52	0		0·224	100
			0·138	*l*-	0·099	44·2
			0·276	*l*-	0·085	38·0
Dictyocaulus viviparus	Coenzyme-free whole worm pulp	5·5	0		0·375	100
			1·1	*d*-	0·375	100
			1·1	*l*-	0·243	64·8

* Values are means of three determinations.
† The initial concentration of fumarate was 2 μmole/ml of incubation mixture.

TABLE 5. EFFECT OF TETRAMISOLE UPON NAD PRODUCTION IN NEMATODES AND CESTODES

	Species	Preparation	Amount of tetramisole added (μmoles/ml of incubation mixture)	NAD Production*			
				After *d*-tetramisole		After *l*-tetramisole	
				Absolute (μmoles/mg of protein)	% (control = 100)	Absolute (μmoles/mg of protein)	% (control = 100)
NEMATODES	*Ascaris suum*	Coenzyme-free muscle pulp	0·0	0·219	100	0·219	100
			0·145	0·206	94	0·175	79·9
			0·285	0·190	86·7	0·149	68·0
			0·533	0·164	74·8	0·091	41·6
			1·621			0·084	38·3
	Ascaris suum	Acetone powder	0·0	0·451	100	0·451	100
			0·083	0·379	84	0·282	62·5
			0·166	0·206	45·6	0·072	15·9
	Ascaridia galli	Acetone powder	0·0	0·312	100	0·312	100
			0·066	0·295	94·5	0·255	81·7
			0·133	0·252	80·7	0·217	69·5
	Toxocara cati	Coenzyme-free whole worm pulp	0·0	0·134	100	0·134	100
			0·083	0·132	98·5	0·115	85·8
			0·166	0·113	84·3	0·102	76·1
CESTODES	*Taenia taeniaeformis*	Coenzyme-free whole worm pulp	0·0			0·188	100
			0·668			0·198	105·3
			1·339			0·190	101·0
	Taenia pisiformis	Coenzyme-free whole worm pulp	0·0			0·164	100
			0·208			0·169	103
			0·831			0·164	100
	Dipylidium caninum	Coenzyme-free whole worm pulp	0·0			0·230	100
			0·099			0·239	103·9

* Average of duplicate samples.

TABLE 6. EFFECT OF TETRAMISOLE UPON THE SUCCINIC DEHYDROGENASE ACTIVITY OF VARIOUS TISSUES

Species	Preparation	Amount of *l*-tetramisole added (μ moles/ml of incubation mixture)	Succinic dehydrogenase activity*	
			Absolute†	% (control = 100)
Ascaris suum	Coenzyme-free muscle pump	0	150	100
		0·146	142	94·6
		0·292	116	77·3
		0·561	110	73·3
Dictyocaulus viviparus	Coenzyme-free whole worm pulp	0	60·3	100
		4·0	38·6	64
Rat	Coenzyme-free liver pulp	0	64·0	100
		6·24	60·5	94·5
		12·48	51·3	80·1
Pigeon	Coenzyme-free breast muscle pulp	0	44·8	100
		6·24	44·8	100
		12·48	44·6	99·5

* Average of 2 experiments.

† The activities are expressed as mμmoles of succinate converted/min/mg of protein at 20°.

inactive against all cestode species tested *in vivo*[4, 5] and also failed to affect the $NADH_2$ oxidation in the presence of fumarate of these helminths *in vitro* (Table 5). It is possible that the molecular properties of the enzyme in nematodes and cestodes differ, despite the similarity of catalytic function, as in the case of schistosomal and mammalian phosphofructokinases, in which the differences between two analogues are reflected in their differential susceptibility to antimonials.[22, 23] Such a possibility naturally does not preclude the existence of other pathways for the recuperation of NAD and the production of energy.

Currently only a few studies are reported concerning the biochemistry of lungworms. Frick[24] showed that application of methylene blue, a known electron-acceptor from reduced flavoprotein enzymes, increased oxygen uptake by adult *D. viviparus*, and Polyakova[25] found that sexually-mature *D. filaria* from sheep bronchi had relatively high catalase and peroxidase activities. Such observations suggest that *Dictyocaulus* species are adapted to life under aerobic conditions. However, carbohydrate catabolism may still be anaerobic: adult *Schistosoma mansoni*,[26, 27] which lives in venous blood, and *Nippostrongylus brasiliensis*,[28] which lives in intimate contact with the jejunal mucosa, both have well-oxygenated environments but, nonetheless, their catabolism is essentially anaerobic.

In the current experiments, coenzyme-free extracts of *D. viviparus* catalysed fumarate reduction under anaerobic conditions in the presence of $NADH_2$ (Table 4) and succinate oxidation under aerobic conditions (Table 6). As in the Ascarididae, tetramisole inhibited both reactions, but the fumarate reduction more readily. It therefore appears that the fumarate-succinate system in the lungworm *Dictyocaulus* is comparable to that in the other nematodes studied.

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