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Biochemical Effects of Niridazole on *Schistosoma mansoni*

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SUMMARY

Administration to the host of the nitrothiazole derivative niridazole, an antischistosomal drug, results in a reduction in the glycogen levels of male schistosomes. This effect precedes observable functional damage to the worm, e.g., a hepatic shift, and is accounted for by a reduced rate of the conversion of active schistosome glycogen phosphorylase to its inactive form. This niridazole-induced effect is not completely selective, because the rate of glycogen phosphorylase inactivation in the host's skeletal muscle is also decreased following administration of niridazole, although to a much lesser degree than in the parasite.

INTRODUCTION

The chemotherapeutic effect of niridazole in experimental animals and man infected with schistosomes has been well documented (1–7), but little is known about the mode of the antischistosomal action of this drug. In this paper the early onset of a biochemical effect following the administration of niridazole is reported. It consists of inhibition of glycogen phosphorylase inactivation in *Schistosoma mansoni* and accounts for the glycogen depletion of the parasite observed under these conditions.

MATERIALS AND METHODS

Adult female Swiss albino mice were infected with 100 cercariae of *S. mansoni* (Puerto Rican strain) by tail immersion. The animals were used 7–8 weeks thereafter. Worms removed from the mesenteric veins or from the portal vein were placed in 75% horse serum (diluted with distilled water) for subsequent studies.

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Niridazole (kindly supplied by Dr. C. R. Lambert of Ciba, Basle) was administered by stomach tube in an aqueous suspension containing 0.9% NaCl and 0.1% Tween 80. Worms from mice which had been infected on the same day by the same number of cercariae, but which had received no drug, were used as controls. Variations in the glycogen levels of male worms from each control group did not exceed ±7.5%, but were considerably greater when one group was compared with others. These levels ranged from 16 to 23 μg of glycogen per worm.

The female reproductive system of the worms was examined by means of an *intra vitam* staining procedure reported previously (8).

Commercially available rabbit muscle glycogen (Nutritional Biochemicals) was purified by stirring for 10 min 50 ml of a 4% aqueous solution of this material with 24 g of Dowex 1-Cl^-, filtering the mixture through a sintered, coarse glass filter, and recovering the glycogen from the filtrate by precipitation with 1 volume of ethanol and a few crystals of lithium bromide.

Phosphoglucomutase and glucose 6-phosphate dehydrogenase were obtained from Boehringer.

For the determination of glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) activity of schistosomes, the worms were homogenized in a medium containing 20% glycerol in 0.05 M glycylglycine buffer (pH 7.4), five worm pairs being used per 0.1 ml of homogenizing medium. Enzymatic activity was assayed by a modification of a previously reported procedure (9) based on the spectrophotometric measurement of the rate of glucose 1-phosphate formation from glycogen in the presence of P_i. The homogenate was diluted 5-fold with a solution containing 0.02 M NaF and 0.05 M glycylglycine (pH 7.4) and centrifuged at 10,000 × *g* for 10 min; 0.05 ml of the supernatant fraction was added to 0.75 ml of a reaction mixture which contained 4 mg of glycogen, 120 μmoles of potassium phosphate (pH 7.2), 32 μmoles of imidazole (pH 7.2), 0.8 μmole of $MgCl_2$, 0.3 μmole of NADP, 0.005 μmole of glucose 1,6-diphosphate, 8 μmoles of NaF, 0.7 unit of phosphoglucomutase, and 0.28 unit of glucose 6-phosphate dehydrogenase. The rate of glucose 1-phosphate formation was shown by the increase in absorbance at 340 mμ due to the reduction of NADP by the combined catalytic activities of phosphoglucomutase and glucose 6-phosphate dehydrogenase. This increase in optical density was recorded between 3 and 13 min after addition of the homogenate. During this period enzymatic activity was proportional with time and enzyme concentration. Enzyme activity is expressed as nanomoles of glucose 1-phosphate produced per minute per milligram of protein.

For the determination of glycogen phosphorylase activities of mouse tissues, the animals were killed by the intraperitoneal administration of sodium pentobarbital, 200 mg/kg of body weight. The tissue samples to be used were frozen *in situ* by means of a precooled clamp and placed in liquid nitrogen. In the case of heart muscle, the organ was cut before clamping in order to reduce contamination with blood. Weighed tissue samples were homogenized in 10 volumes (heart and brain), 20 volumes (liver), or 40 volumes (skeletal muscle) of 20% glycerol in 0.05 M glycylglycine buffer (pH 7.4). Phosphorylase activity was determined in the same manner as described for schistosome homogenates, except that in the case of brain 0.1 ml instead of 0.05 ml of the diluted homogenate was used.

Glycogen was determined by a specific enzymatic method (10, 11); protein, according to Lowry *et al.* (12); and glucose utilization by the schistosomes and the levels of phosphate esters in the worms, by previously described procedures (8, 13).

RESULTS

Niridazole is a relatively slowly acting drug; a hepatic shift, which is considered one of the first manifestations of antischistosomal action, begins only 72 hr after the oral administration of chemotherapeutically effective doses of niridazole to mice infected with *S. mansoni* has been initiated. After the daily administration of 200 mg/kg to mice for 5 consecutive days, no schistosomes are detectable in the host 17 days after the initial dose (1).

A partial shift of the worms toward the liver is detectable after the daily oral administration of 200 mg/kg to mice for 3 days when autopsy is performed 4 days after the first dose. An attempt has been made to determine earlier biochemical changes in the worms. If they are to be related to the mode of antischistosomal action of niridazole, they should precede the functional damage reflected in the loss of attachment of schistosomes to the internal wall of the mesenteric veins.

Prior to or after the start of the hepatic shift brought about by the administration of niridazole, there was no evidence for inhibition of the activity of phosphofructokinase in the worms, because there was no change in the ratios of the levels of hexose monophosphate to hexose diphosphate and triose phosphate levels in the worms (Table 1). Such a change is an early effect of the administration of trivalent organic antimonials and is brought about by the inhibition of the phosphofructokinase activity of the worms (13, 14). In addition, the rate of glycolysis of schistosomes was

TABLE 1

Ratios of hexose monophosphates to fructose diphosphate + triose phosphate in S. mansoni following hepatic shift produced by potassium antimony tartrate and niridazole

Drug	Glucose 6-phosphate + fructose 6-phosphate (A)	Fructose diphosphate + triose phosphate (B)	A:B
	μmoles/g		
None	221	78	2.8
Potassium antimony tartrate[a]	485	45	10.8
None	234	86	2.7
Niridazole[b]	222	82	2.7

[a] Two hours after the intraperitoneal administration of 25 mg/kg.

[b] A dose of 200 mg/kg was administered orally on each of 3 successive days. The worms were analyzed 24 hr after the last dose.

not affected by the administration of niridazole. The lack of these effects after the administration of niridazole indicates that the mode of antischistosomal action of this drug differs from that of antimonials. Furthermore, niridazole administration did not alter the activity of acetylcholinesterase of *S. mansoni* and thus differed in this respect from the antischistosomal action of *p*-rosaniline (8). Therefore, niridazole must exert its chemotherapeutic activity against *S. mansoni* through an effect distinct from those of antimonials and of *p*-rosaniline; elucidation of its mode of action would reveal, within the parasite, another mechanism essential for its functional integrity and susceptible to selective inhibition by a chemical agent.

After the administration of niridazole, the shift of the worms to the liver was preceded by a decrease in the glycogen levels of male worms. There was a dose-effect relationship, the size of the dose ranging from 11 to 200 mg/kg administered on 3 successive days (Fig. 1). Four days after the first dose there was a partial, but significant, hepatic shift with the two highest doses (200 and 100 mg/kg) of niridazole, but this was not observed with the lower doses. Furthermore, 24 hr after a single dose of 200 mg/kg of niridazole there was already a significant reduction in the glycogen levels (Fig. 2), but no hepatic shift was observed.

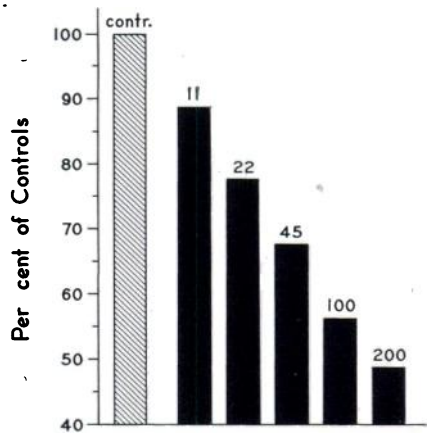

FIG. 1. *Glycogen levels of male schistosomes (as percentage of controls) following three oral doses of niridazole administered to the mouse host on 3 successive days*

The worms were removed for analysis 24 hr after the last dose. The figures on top of each bar indicate the single dose in milligrams per kilogram. Each bar represents the average of 12 determinations.

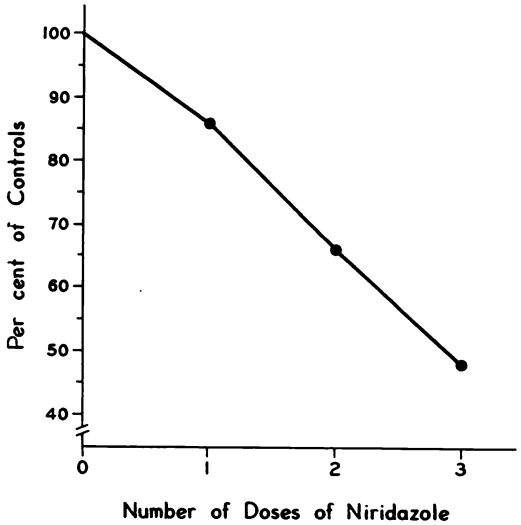

FIG. 2. *Glycogen levels of male schistosomes (as percentage of controls) 24 hr after one, two, or three oral doses of 200 mg/kg of niridazole administered at daily intervals to mice infected with S. mansoni*

The figures on the abscissa indicate the number of doses. Each point on the graph represents the average of nine determinations.

Therefore, glycogen depletion was an earlier and more sensitive indicator of niridazole action than the hepatic shift.

The observed niridazole-induced glycogen loss could have been caused by a reduction of glycogen synthesis, i.e., by inhibition of uridine diphosphate glucose-glycogen synthetase activity following the administration of the drug. However, after niridazole administration had already produced a marked glycogen loss, the activities of the two forms of the glycogen synthetase of the worm did not change.[1] Since there was no evidence of interference with glycogen synthesis, the possibility of activation of glycogenolysis was considered. *S. mansoni* has high glycogen phosphorylase activity. In freshly prepared homogenates of adult schistosomes, phosphorylase activity was almost as high in the absence as in the presence of AMP. However, unless fluoride was present, incubation of these homogenates at 30° resulted in a rapid decrease in phosphorylase activity, especially when assayed in the absence of AMP. After addition of ATP, Mg^{++}, and fluoride, glycogen phosphorylase activity was restored within a few minutes; in fact, under these conditions phosphorylase activity was greater than that of the initial homogenate (Table 2). These observations suggest the presence in the worm of both an active and an inactive phosphorylase and a rapid interconversion of the two forms. The rapid activation of phosphorylase by ATP and Mg^{++} indicates the action of a phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38), while it appears that the fluoride-sensitive inactivation of phosphorylase is catalyzed by a phosphorylase phosphatase (phosphorylase phosphohydrolase, EC 3.1.3.17).

After the administration of niridazole to mice infected with *S. mansoni*, the rate of inactivation of schistosome phosphorylase, assayed in the absence of AMP, was reduced. As in the case of the reduction of the glycogen stores produced by this drug, there was a dose-effect relationship; i.e., the higher the dose, the greater was the reduction of phosphorylase inactivation (Fig. 3).

[1] S. H. Rogers and E. Bueding, unpublished observations.

This biochemical effect of niridazole on *S. mansoni* was reversible. If, after one to three daily oral doses of 200 mg/kg of niridazole, the administration of the drug was discontinued, inhibition of phosphorylase inactivation became more pronounced for 48–

TABLE 2

Glycogen phosphorylase activity of S. mansoni

Schistosome homogenates were incubated at 30° for 45 min. Incubation was continued after a solution was added yielding a final concentration of 2 mM ATP, 4 mM $MgCl_2$, and 20 mM NaF.

Incubation period (30°) before addition of ATP, Mg^{++}, and NaF	Time after addition of ATP, Mg^{++}, and NaF	Phosphorylase activity	
		Without AMP	3 mM AMP
min	*min*	*mμmoles glucose 1-phosphate/min/mg protein*	
0		8.5	9.7
45		0.8	4.0
	2	5.7	8.0
	5	7.9	9.9
	30	10.2	11.4

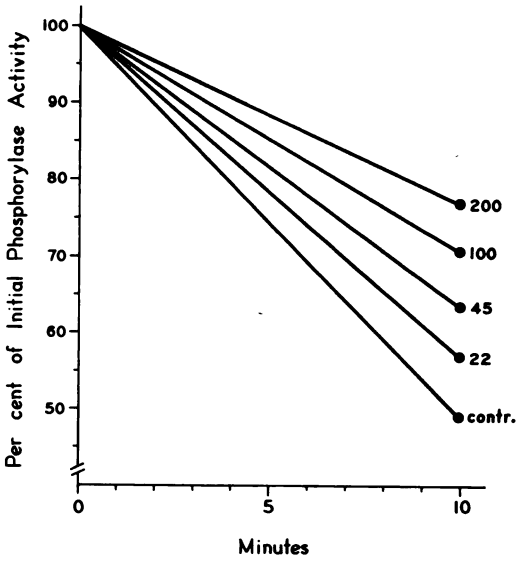

FIG. 3. *Rate of inactivation of glycogen phosphorylase following incubation of homogenates of schistosomes for 10 min (30°) before and after oral administration of three doses of niridazole to the host on 3 successive days*

The worms were removed 24 hr after the last dose. The figures at the right of each line indicate the single dose in milligrams per kilogram. Each line represents the average of eight experiments.

TABLE 3

Effect of administration of niridazole on glycogen phosphorylase inactivation, glycogen phosphorylase activity, and glycogen levels of S. mansoni

Niridazole (200 mg/kg) was administered orally once, twice, or three times (at 24-hr intervals) to mice infected with *S. mansoni*. Glycogen phosphorylase activity of schistosome homogenates was determined before and after incubation (30°) for 10 and 30 min. The percentage decrease in phosphorylase activity observed after 10 min (column a) was compared with that of the homogenate of control worms. Similarly, the percentage of residual phosphorylase activity after incubation (30°) for 30 min (column b), the initial phosphorylase activity (column c), and the glycogen levels of the male worms (column d) were compared with those of the controls. Each value represents the average of at least three experiments.

No. of doses of niridazole	Days after last dose	(a) Inhibition of phosphorylase inactivation (10 min)	(b) Increase in residual phosphorylase activity after 30 min	(c) Increase in initial phosphorylase activity	d) Decrease in glycogen levels
		%	%	%	%
1	1	19	22	12	19
	2	31	67	32	36
	3	35	130	44	47
	4	48	225	48	43
	5	27	50	48	27
	10	20	38	15	8
2	1	28	150	17	33
	2	47	186	43	38
	3	45	180	40	42
	4	49	214	48	47
	5	34	154	50	38
	10	31	100	24	33
	16	7	13	0	0
3	1	50	255	37	58
	2	66	470	67	67
	3	73	450	64	71
	4	65	320	58	63
	7	46	272	70	49
	8	44	186	68	40
	11	28	108	41	31
	14	17	44	19	19
	16	13	36	18	15
	19	5	11	0	7

72 hr and thereafter diminished progressively until inhibition was no longer detectable after another 10–14 days (Table 3). This niridazole-induced reduction of phosphorylase inactivation was also reflected in the percentage of residual phosphorylase activity following incubation of schistosome homogenates for 30 min at 30°. Under these conditions the residual phosphorylase activity of control homogenates amounted to only 15–25% of that present originally, prior to incubation. After niridazole administration, this percentage was considerably greater (Table 3). Reduction of phosphorylase inactivation was observable as early as 12 hr after the oral administration of a single dose of niridazole to the host (Fig. 4).

Reduction of phosphorylase inactivation by the administration of niridazole should increase the activity of active phosphorylase in the worm, as was observed (Table 3). When, following incubation of schistosome homogenates with ATP and Mg^{++}, inactive phosphorylase was converted to the active form of the enzyme, however, the activity

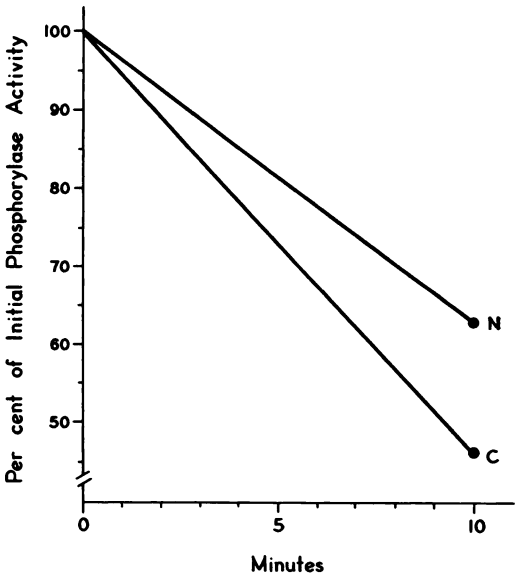

FIG. 4. *Glycogen phosphorylase activity before and after incubation (10 min, 30°) of the worm homogenate*

C, controls; *N*, 12 hr after the administration of a single oral dose (100 mg/kg) to the host. Each line represents the average of six experiments.

was the same in homogenates of worms removed from niridazole-treated animals and from controls. Therefore, niridazole administration did not affect *total* phosphorylase activity, but merely increased the proportion of active phosphorylase.

Following the administration of niridazole, usually both the increase in phosphorylase activity and the reduction in the glycogen levels of the worms were preceded by the inhibition of phosphorylase inactivation. Otherwise the time course of these three biochemical changes and their return to the control values occurred at similar time intervals after cessation of drug administration (Table 3).

A decrease of phosphorylase inactivation was demonstrable not only after the administration of niridazole to the host, but also after incubation of the worms with the drug *in vitro*. However, the concentrations required to produce this effect *in vitro* were higher than those prevailing in the portal venous blood after administration of niridazole to the host (15). Therefore, it appears that a metabolite of niridazole is responsible for the antischistosomal action of this drug. Fully 24 hr after the administration of a single dose of niridazole to the host, Faigle and Keberle (15) could detect only unidentified metabolites, but not the drug itself, in the tissues of the host and of the parasite. Yet, it was found that the inhibition of phosphorylase inactivation progressively increased for at least 48 hr under these conditions (Table 3). The antischistosomal activity of one or of several metabolites of niridazole produced by the host or by the parasite (or both) could explain such a time course.

Administration of niridazole resulted in characteristic changes in the female reproductive system, detectable by an *intra vitam* staining technique (8). The time course of these changes was noteworthy. The onset of demonstrable damage to the female reproductive system coincided with the inhibition of phosphorylase inactivation

TABLE 4

Glycogen phosphorylase inactivation in mouse tissues before and after daily oral administration of niridazole (200 mg/kg) for 4 and 7 days

Rates of inactivation are expressed in terms of the average percentage decrease of initial phosphorylase activity following incubation of the tissue homogenates at 30°. Figures in parentheses in the second column indicate the number of experiments.

Tissue	No. of doses of niridazole	Average decrease in phosphorylase activity after			
		10 min	15 min	20 min	30 min
		%	%	%	%
Liver	0 (6)			48 ± 2	65 ± 1
	4 (5)			49 ± 3	66 ± 2
	p			>0.5	>0.5
	7 (5)			49 ± 2	67 ± 2
	p			>0.5	>0.5
Skeletal muscle	0 (5)	40 ± 2	54 ± 3		
	4 (5)	34 ± 1	47 ± 2		
	p	<0.025	<0.01		
	7 (5)	30 ± 1	43 ± 1		
	p	<0.001	<0.005		
Brain	0 (6)			40 ± 1	48 ± 2
	4 (6)			43 ± 3	49 ± 2
	p			>0.5	>0.5
	7 (6)			41 ± 1	49 ± 2
	p			>0.5	>0.5
Heart muscle	0 (5)		42 ± 2	52 ± 2	61 ± 2
	7 (5)		41 ± 2	52 ± 2	60 ± 2
	p		>0.4	>0.5	>0.5

and became more pronounced as this inhibition became greater, and the return of the phosphorylase inactivation to control values preceded the disappearance of abnormalities in the female reproductive system.

Since administration of niridazole decreased phosphorylase inactivation in the parasite, an attempt was made to ascertain whether this drug would bring about similar changes in the tissues of the host. It was found that after the daily oral administration of 200 mg/kg of niridazole to mice for 7 days, the rate of phosphorylase inactivation remained unchanged in mouse liver, brain, and heart homogenates. However, in skeletal muscle there was some reduction in this rate after four doses, and this was more pronounced after 7 days (Table 4). This inhibitory effect was much smaller than that produced by the same dosage schedule of niridazole on phosphorylase inactivation in the worm. However, this degree of inhibition seems sufficient to account for the glycogen depletion observed in the skeletal muscle of rhesus monkeys following the administration of niridazole (16).

DISCUSSION

A reduction in the glycogen stores of male schistosomes observed shortly after the administration of niridazole to the host could be accounted for by the decreased phosphorylase inactivation produced by this drug. This reduction results in an increase in active phosphorylase, which in turn enhances the rate of glycogenolysis. It remains to be determined whether this glycogen loss induces functional damage to the worm and can thus account for the mode of the antischistosomal action of niridazole. Since the rate of glycolysis of intact schistosomes was not affected by niridazole, glucose provided by the plasma of the host surrounding a worm should be an adequate source of energy, unless in certain cell compartments of the worm glycogen, in contrast to glucose, is an obligatory substrate. On the other hand, the possibility should be considered that glycogen, besides being a source of metabolic energy, may have another function in the parasite. Since this polysaccharide interacts with proteins and possibly other tissue constituents, the latter may be protected from degradation by enzymes present in the cytoplasm. If this were the case, degradation of glycogen would result in the release and subsequent destruction of a constituent essential for the functional integrity of the parasite.

It appears that the phosphorylase-inactivating systems of three host tissues, liver, brain, and heart, differ from that of *S. mansoni*, because the former were not inhibited under conditions under which the enzyme of the parasite was markedly inactivated following the administration of niridazole. However, the enzyme catalyzing phosphorylase inactivation in muscle was affected under the same conditions, although to a much smaller degree than the enzyme of the parasite. Thus, this action of niridazole was not completely selective. Its inhibitory effect on phosphorylase inactivation in skeletal muscle can account for the pronounced glycogen depletion in the muscle of rhesus monkeys to which niridazole had been administered (16). This effect of niridazole on the host is contrasted by the action of another antischistosomal nitroheterocyclic compound, *trans*-5-amino-3-[2-(5-nitro-2-furyl)-vinyl]-1,2,4-oxadiazole (SQ 18,506) (17, 18). Its administration resulted in a reduction of phosphorylase inactivation in *S. mansoni*, but had no effect on the activity of this system in skeletal muscle.[2] Therefore, structural modifications resulted in greater selectivity of the drug effect on an enzyme of the parasite.

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