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The Role of Cyclic AMP-Mediated Regulation of Glycogen Metabolism in Levamisole-Perfused *Ascaris suum* Muscle

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SUMMARY

The effects of levamisole on muscle contraction and glycogen metabolism have been examined in isolated muscle-cuticle sections of the roundworm *Ascaris suum*. Muscle contraction occurred when various levels of levamisole were perfused through the preparation. At a levamisole concentration of 0.42 mM, the period of contraction lasted only about 6 min and was followed by a period of relaxation. During this relaxation period, there was an activation of glycogen synthase (EC 2.4.1.11), as evidenced by a decrease in the K_a values of glucose 6-phosphate for glycogen synthase to 0.26 mM from control values of 0.50 mM. The glycogen phosphorylase (EC 2.4.1.1) activity ratio decreased from 0.85 to 0.65, which indicated an inactivation of this enzyme. Concomitant with this activation of glycogen synthase and inactivation of phosphorylase there was an increased synthesis of glycogen. In addition, the presence of levamisole prevented both the serotonin-induced cyclic AMP accumulation and the activation of the cyclic AMP-dependent protein kinase (EC 2.7.1.37). However, levamisole did not significantly affect the changes in glycogen synthase and phosphorylase brought about by perfusion with the neurostimulator acetylcholine. Collectively, the data indicated that levamisole caused a transient muscle contraction followed by muscle relaxation, and the muscle relaxation effect appeared to be the result of a levamisole-inhibited cyclic AMP-mediated pathway of glycogen utilization.

INTRODUCTION

Levamisole is the L-isomer of tetramisole [2,3,5,6-tetrahydro-6-phenylimidazo(2,1-*b*)thiazole hydrochloride] and is a more active anthelmintic drug than the racemic mixture (1). Levamisole has been found to be highly effective against a broad spectrum of nematodes. Levamisole paralyzes live *Ascaris suum* in 3 min and causes a sustained contraction of isolated somatic muscle cells of this worm when given continuously (2). However, this muscle contraction appears to be transient. Coles *et al.* (3) demonstrated that *A. suum* bathed in levamisole recovered from paralysis and regained muscular movement after approximately 12 hr. When the drug was administered continuously to adult filariids, muscular contraction was followed in 10–20 min by relaxation (4). From these results, it has been postulated that the initial effect of levamisole is on the neuromuscular system of the nematode, and the relaxation which was observed may be due to an inhibition of intermediary metabolism. Verhoeven *et al.* (1) have investigated the enzyme fumarate reductase in *Ascaris* muscle and have shown that high concentrations of levamisole inhibit fumarate reductase. Komuniecki and Saz (5) have observed an activation of glycogen synthase during the relaxation period in adult filariids incubated in levamisole.

In a series of reports from this laboratory (6–10) glycogen metabolism in *A. suum* muscle has been investigated. An *in vitro* muscle perfusion system has been developed to examine the role(s) of cyclic AMP and calcium in the regulation of glycogen metabolism (7–10). The cyclic AMP-dependent regulation appears to be mediated via serotonin (8), whereas the cyclic AMP-independent pathway may be controlled concomitantly with muscle contraction by the neurotransmitter, acetylcholine (10). Since levamisole caused muscle contraction in parasites, and also appeared to stimulate glycogen synthesis, (5), it was of interest to test the effect of this drug on glycogen metabolism in the perfusion system of *A. suum* muscle. The present results demonstrate that levamisole causes muscle contraction followed by spontaneous relaxation. During this period of relaxation, gly-

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cogen synthase is activated, phosphorylase is inactivated, and glycogen synthesis occurs. In addition, levamisole inhibits the serotonin-mediated increase in cyclic AMP levels and cyclic AMP-dependent protein kinase.

METHODS

Animals

A. suum specimens were collected at a slaughterhouse and transported to the laboratory in a salt solution (11). Adult female parasites that were 25–30 cm long were used in these experiments.

Maintenance

The worms were maintained in a holding system (6) which contained *Ascaris* saline (11) modified to contain 111 mM NaCl, 10 mM $NaHCO_3$, 24 mM KCl, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 14 mM NH_4Cl, and 0.5 mM KH_2PO_4 (pH 7.0). The solution was maintained at 37° and saturated with 95% N_2/5% CO_2. Fed worms were maintained for at least 24 hr in *Ascaris* saline containing 27 mM glucose, while starved worms were maintained similarly except without glucose.

Perfusion System

The perfusion system was essentially that described by Donahue *et al.* (7). Briefly stated, the worms were taken from the holding vessel and placed in a dissecting pan containing modified *Ascaris* saline. The worms were then cut at points posterior to the genital pore and anterior to the intestinal attachment to the muscle wall. The intestinal and reproductive tracts were removed, leaving a hollow muscle-cuticle preparation approximately 10 cm long. This section of muscle-cuticle was cut in half, generating two 5-cm hollow tubes, each weighing about 0.4 g (muscle weight 0.3 g). One tube served as the control and the other as the experimental tissue. The muscle-cuticle ends were cannulated and ligated to polyethylene tips, which were mounted on glass tubes inserted through rubber inlet and outlet stoppers in the perfusion apparatus. The system was continually gassed with 95% N_2/5% CO_2 and was maintained in a water bath at 37°. The muscle-cuticle preparation was perfused at a rate of 1.25–1.50 ml/min with incubation saline containing 100 mM NaCl, 20 mM KCl, 6 mM $CaCl_2$, 5 mM $MgSO_4$, 5 mM NH_4Cl, 5 mM $NaHCO_3$, and 5 mM imidazole HCl (pH 6.7 at 37°). When glucose was included, it was at a level of 10 mM. When [^{14}C]glucose was perfused, labeled glucose was added to the nonlabeled glucose to a level of 0.01 μCi/μmole. The incubation medium was also maintained at 37° and saturated with 95% N_2/5% CO_2.

Muscle Contraction

Each muscle segment preparation was connected by a surgical thread to a muscle transducer (Myograph B-4178), and contractions were recorded on a Narco Bio-Systems physiograph, Model Four-A.

An equilibration period of perfusion with saline was allowed for at least 15 min. When the experimental muscle sections were perfused with saline plus drug, the adjacent control sections were perfused with *Ascaris* saline alone.

Preparation of Extract

At appropriate time intervals after the introduction of the drug, the muscle sections were frozen, using a Wollenberger freeze-clamp device precooled in liquid nitrogen. The frozen muscle was stored at −80° until biochemical assays were performed, or was powdered immediately with a precooled mortar and pestle. The frozen, powdered tissue from each muscle preparation was weighed in a cold room and transferred to a Potter-Elvehjem homogenization tube. The powder was homogenized at 0° in 20 volumes of 50 mM triethanolamine HCl (pH 7.0), 5 mM EDTA, 20 mM NaF, and 40 mM 2-mercaptoethanol. The homogenate was centrifuged for 10 min at 10,000 × *g* and the supernatant fraction was filtered through cheesecloth and stored in the cold. Enzymatic assays were carried out on the supernatant fraction within 1 hr of homogenization. The glycogen content was determined on the crude homogenate.

Assays

Glycogen synthase. The enzyme activity was assayed by measuring the incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into glycogen, using a modification of the method of Thomas *et al.* (12). The supernatant fraction was diluted (1:10) with homogenization buffer so that the rate of incorporation of glucose into glycogen was linear with respect to time and concentration of protein. This solution was added to an assay mixture containing a final concentration of 50 mM triethanolamine HCl (pH 7.0), 1% *Ascaris* glycogen, and 6.7 mM UDP-[^{14}C]glucose (0.45 Ci/mole) in a total volume of 0.1 ml. One unit of enzyme activity corresponded to that amount of enzyme activity which catalyzed the incorporation of 1 μmole of [^{14}C]glucose into glycogen per minute at 30°.

Determination of the apparent activation constant of glucose 6-phosphate was made by the methods of Kaslow *et al.* (13) and Dietz *et al.* (14), which correlated inactivation of glycogen synthase with an increasing K_a for glucose 6-phosphate. These values were obtained by plotting the ratio $v - v_0/V_{max} - v$ versus glucose 6-phosphate concentrations (0.1–10 mM). The concentration giving a ratio of 1 was taken at the K_a. The following values were used: v_0 = counts per minute at zero glucose 6-phosphate; V_{max} = counts per minute at 10 mM glucose 6-phosphate; v = counts per minute at a given glucose 6-phosphate concentration.

Phosphorylase. The activity of glycogen phosphorylase was determined according to the method of Gilboe *et al.* (15). The diluted enzyme (1:40) was added to an assay mixture containing a final concentration of 15 mM [^{14}C]glucose 1-phosphate (0.45 μCi/mole), 1% *Ascaris* glycogen, 1.0 mM 5′-AMP, 25 mM imidazole HCl (pH 6.1), 1 mM EDTA, and 10 mM 2-mercaptoethanol. One unit of activity is defined as that amount of enzyme which catalyzed the conversion of 1 μmole of [^{14}C]glucose incorporated into glycogen per minute at 30°. The phosphorylase activity ratio was expressed as the ratio of the activity in the absence of AMP to that in its presence, i.e., phosphorylase *a* activity/total phosphorylase activity. Dialysis of the extract or passage of the extract

through a Sephadex G-25 column did not significantly change the ratio.

Glycogen and [^{14}C]glycogen determination. Glycogen was precipitated on filter papers (12), hydrolyzed using amyloglucosidase at 30° and determined as glucose with the hexokinase/glucose-6-phosphate dehydrogenase method of Sölling and Esmann (16). Radioactivity in the glycogen was determined by extraction of the glycogen in 0.4 N $HClO_4$, precipitation by ethanol (66%), redissolution of the glycogen in water, and reprecipitation. The purified glycogen was dissolved in water, and an aliquot was spotted on filter paper, dried, and counted in a liquid scintillation counter.

Protein kinase assay. Protein kinase assays were performed by an adaptation of the methods of Reiman *et al.* (17). The reaction mixture contained 2 μmoles of 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8), 1 μmole of $MgCl_2$, and approximately 10 nmoles of [γ-^{32}P]ATP (50–100 dpm/pmole) in a total assay volume of 100 μl. The synthetic peptide PK-1 (Leu-Arg-Arg-Ala-Ser-Leu-Gly) (5 nmoles) was added as substrate. The concentration of cyclic AMP, when present, was 1 μM. The amount of heat-stable inhibitor protein, when present, was 100 μg. Appropriate aliquots containing enzyme were added as noted in individual experiments. All incubations were carried out at 30° for 10 min. The amount of ^{32}P-labeled product was determined by the method of Glass *et al.* (18).

Cyclic AMP assay. For cyclic AMP analyses, muscle was homogenized in 10 volumes of 10% trichloroacetic acid (w/v) and the protein precipitate was removed by centrifugation at 10,000 × *g* for 10 min. The clear supernatant fluid was acidified with 100 μl of 1 N HCl and extracted five times with 2 volumes of diethylether. Aliquots from the extracted fraction were diluted in 5 mM sodium acetate buffer (pH 4.75), acetylated with acetic anhydride, and assayed by the radioimmunoassay for cyclic AMP (10). Antibody was kindly provided by Dr. Gary Brooker, Georgetown University (Washington, D. C.). The assay procedures could detect 5 fmoles of cyclic AMP. In appropriate control experiments, the authenticity of the measured nucleotide was verified by Dowex 50-X8 chromatography or phosphodiesterase treatment of the supernatant fractions.

The [γ-^{32}P]ATP was prepared by the method of Walseth and Johnson (20). Glycogen for phosphorylase and glycogen synthase assays were prepared from *Ascaris* muscle (6). The synthetic peptide PK-1 was purchased from Peninsula Laboratories, Inc. (San Carlos, Calif.). The heat-stable protein inhibitor was prepared from bovine skeletal muscle through DE-52 chromatography (20). UDP-glucose ([U-^{14}C]glucose; 310 mCi/mmole) and D-[U-^{14}C]glucose (240 mCi/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). All protein assays were conducted by the method of Bradford (21), using bovine serum albumin as a standard.

RESULTS

In the perfused muscle preparation two distinct phases in the response to levamisole administration were observed, a contractile phase and a relaxation phase. Contraction of *A. suum* muscle-cuticle segments was dose-dependent when low concentrations of levamisole were perfused (Fig. 1). At levamisole levels below 4 μM no response was recorded. Maximal muscle contraction was observed with 20 μM levamisole. In experiments where the muscle segment was flushed with *Ascaris* saline after contraction in the presence of 20 μM levamisole, the muscle tension returned to baseline values. Subsequent addition of levamisole again generated a dose-dependent contractile response. No differences were observed between dose-response curves from muscle segments which were perfused with drug then washed with saline and perfused again with a higher concentration of drug, and those dose-response curves where a fresh muscle-cuticle section was used for each concentration point.

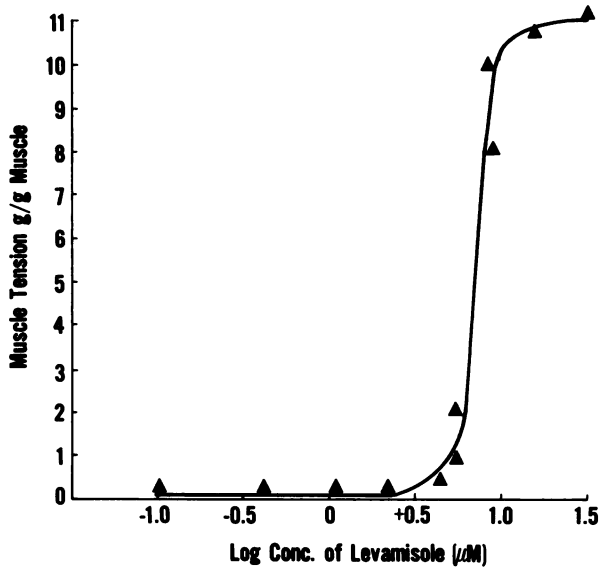

FIG. 1. *Dose-response curve for perfusion of levamisole through muscle-cuticle sections of Ascaris suum*

Isolated muscle-cuticle sections from *A. suum* perfused with various concentrations of levamisole and allowed to contract. The muscle-cuticle section was then washed with *Ascaris* saline until the muscle had returned to the baseline contractile value (5–10 min). After the muscle section had returned to a baseline value, the next higher concentration of drug was perfused. Each *point* represents the average of at least three muscle sections; the standard deviation was less than 10%.

The contractile phase was followed by a levamisole-dependent relaxed paralysis. When muscle-cuticle segments were perfused with 0.42 mM levamisole (Fig. 2), the muscle was contracted for 5.7 ± 0.4 min (n = 3). After this time the muscle began to relax, and after 4.8 ± 0.5 min the muscle was completely relaxed. The relaxed phase persisted for the duration of the experiment, i.e., 30 min. The time at which onset of the relaxed phase was observed was dependent on levamisole concentration. In contrast to the rapid onset of relaxation with 0.42 mM levamisole, no relaxation was observed for up to 15 min after the administration of levamisole at concentrations shown in Fig. 1. In other experiments, relaxation at the low levamisole concentrations was observed after 15–30 min of perfusion.[3]

The glycogen synthase and phosphorylase activity in muscle segments perfused with 0.42 mM levamisole was measured (Table 1). During the first 5 min, when the

[3] D. Yeltman and B. Harris, unpublished observations.

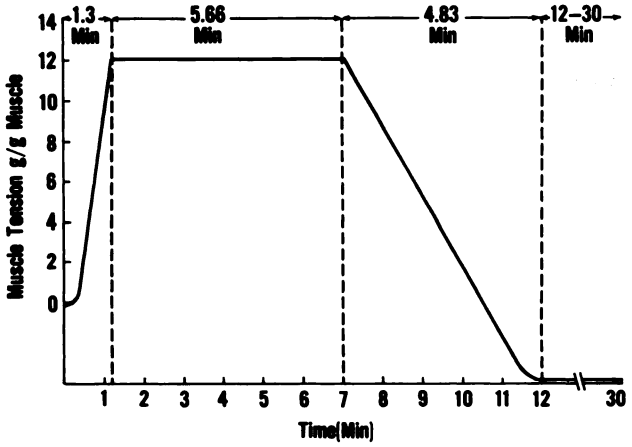

FIG. 2. *Time-dependent effects of perfusion of levamisole through muscle-cuticle sections of Ascaris suum*

Isolated muscle-cuticle sections from *A. suum* were perfused with 0.42 mM levamisole for 30 min and muscle contraction was recorded on a physiograph (see Methods). Represented is a typical muscle response over the experimental time course.

muscle was contracting, there was no change in the K_a of glucose 6-phosphate for glycogen synthase between the experimental muscle perfused with 10 mM glucose plus 0.42 mM levamisole and the control sections perfused with 10 mM glucose alone. After 15 min those muscles perfused with 10 mM glucose plus 0.42 mM levamisole exhibited relaxation and showed a decrease in the glycogen synthase K_a value from 0.50 mM to 0.36 mM, which indicated an activation of glycogen synthase (6). The K_a values decreased further during the 30- and 60-min perfusion times to levels of 0.28 and 0.26 mM, but no significant changes occurred in the control values. Levamisole alone did not activate glycogen synthase when added to enzyme assays.

During the initial 5-min period of perfusion, the phosphorylase activity ratio was 0.85 in the levamisole-perfused muscle and 0.80 in the control tissue (Table 1).

TABLE 1

Effect of perfusion of levamisole on glycogen synthase and phosphorylase activity in isolated muscle sections of Ascaris suum

Worms were starved for 24 hr prior to experimentation. Isolated muscle sections from starved worms were perfused with 10 mM levamisole for the specified times. The tissue was freeze-clamped and analyzed for the K_a of glucose 6-phosphate for glycogen synthase and the phosphorylase activity ratio as described under Methods.

Time	Glycogen synthase[a] K_a		Phosphorylase activity ratio[b]	
	Control	Experimental	Control	Experimental
	mM			
0	0.50 ± 0.04	0.50 ± 0.04	0.80	0.85
5	0.50 ± 0.04	0.50 ± 0.04	0.85	0.80
15[c]	0.46 ± 0.04	0.36 ± 0.03	1.00	0.65
30	0.48 ± 0.04	0.28 ± 0.03	0.81	0.70
60	0.44 ± 0.04	0.26 ± 0.03	0.83	0.65

[a] Results are averages ± standard deviation of three experiments.

[b] Results are averages of three experiments.

[c] The control and experimental numbers are significantly different at the $p < 0.05$ level for both glycogen synthase K_a and phosphorylase activity ratio at the 15-, 30-, and 60-min time periods.

When the levamisole perfusion was continued there was relaxation in the experimental muscle and a significant decrease in the phosphorylase activity ratio (to 0.65) from control. Levamisole did not affect the phosphorylase activity ratios when added to the enzyme assays.

The effect of levamisole on the absolute amount of glycogen synthesized and the incorporation of [^{14}C]glucose into glycogen was determined. Results are shown in Table 2. In the presence of levamisole, a 1.9-fold increase in the absolute amount of glycogen synthesized was observed. Glucose alone promoted glycogen synthesis at a rate of 20 μmoles/g of muscle-cuticle/hr, whereas the rate in the presence of levamisole was 39 μmoles/g/hr. Comparable results were obtained with [^{14}C]glucose. A 1.7-fold increase in [^{14}C]glycogen was observed when levamisole was present in the perfusion media.

Muscle sections from a single ascarid were next perfused with 0.42 mM levamisole, 0.05 mM serotonin, or 0.42 mM levamisole plus 0.05 mM serotonin. Table 3 shows that after 20 min of perfusion with serotonin the K_a for glucose 6-phosphate of glycogen synthase had increased to 0.83 mM from control values of 0.50 mM. The phosphorylase activity ratio in the serotonin-perfused muscle was 0.90, as compared with control values of 0.81. Concomitant with this there was an increase in cyclic AMP levels and an apparent activation of cyclic AMP-dependent protein kinase. However, perfusion of muscle sections with levamisole, or with levamisole and serotonin, resulted in an inhibition of the serotonin effects (Table 3). There was no inactivation of glycogen synthase or increase in the phosphorylase activity ratio, nor was there an increase in the cyclic AMP levels or an increase in the activation of cyclic AMP-dependent protein kinase. In addition, levamisole had no effect on the protein kinase assay when added *in vitro*.

Muscle sections were perfused with 0.42 mM levamisole, 0.05 mM acetylcholine, or 0.42 mM levamisole plus 0.05 mM acetylcholine. Perfusion with acetylcholine resulted in an increase in the K_a for glucose 6-phosphate from 0.5 mM to 0.75 mM (Table 3). The phosphorylase activity remained high, and there was no change in the cyclic AMP level or protein kinase activity. Inclusion of levamisole with acetylcholine resulted in no significant changes in any of the parameters measured, although there was a slight decrease in the glucose 6-phosphate

TABLE 2

Effect of levamisole on the incorporation of [^{14}C]glucose into glycogen on perfused muscle sections of Ascaris suum

Worms were starved 24 hr prior to experimentation. Isolated muscle sections were perfused for 60 min with saline, 10 mM [^{14}C]glucose (0.01 μCi/μmole), or 10 mM [^{14}C]glucose + 0.42 mM levamisole. The tissues were freeze-clamped and analyzed for radioactivity and glycogen as described under Methods.

Perfusion medium	Glycogen content[a]	[^{14}C]Glycogen[a]
	mg/g	*dpm/g*
Saline	38.1 ± 1.0	—
[^{14}C]Glucose	41.5 ± 1.1	2530 ± 126
[^{14}C]Glucose + levamisole	45.1 ± 0.9	4320 ± 207

[a] All values are means ± standard deviation ($n = 4$). All of the glycogen values are significantly different from each other at the $p < 0.05$ level.

TABLE 3

Effects of levamisole on glycogen synthase, K_a for glucose 6-phosphate, phosphorylase and protein kinase activity ratios, and cyclic AMP levels in muscle segments of Ascaris suum perfused with serotonin and acetylcholine

Worms were starved for 24 hr prior to experimentation. All drugs were dissolved in glucose-containing (10 mM) perfusion medium. Isolated muscle sections from starved worms were perfused with 0.42 mM levamisole, 50 μM serotonin, or 0.42 mM levamisole and 50 μM serotonin. All three sections were from the same worm. Three other isolated muscle sections, again from the same worm, were perfused with 0.42 mM levamisole, 50 μM acetylcholine, and 0.42 mM levamisole and 50 μM acetylcholine. All values are averages of three experiments; the standard deviation was less than 10%. The tissue was freeze-clamped after 20 min of perfusion and analyzed as described under Methods.

Substance	Glycogen synthase K_a	Phosphorylase activity ratio	Cyclic AMP	Cyclic AMP-dependent protein kinase activity ratio
	mM		*nmoles/g*	
Levamisole	0.31	0.63	0.42	39
Serotonin	0.83	0.90	1.84	85
Levamisole + serotonin	0.41	0.70	0.51	38
Levamisole	0.26	0.65	0.43	39
Acetylcholine	0.75	0.84	0.38	42
Levamisole + acetylcholine	0.57	0.95	0.38	38
Control[a]	0.50	0.81	0.37	38

[a] Glucose-containing perfusion medium.

K_a value for glycogen synthase. However, perfusion with levamisole alone again resulted in decreased K_a values as well as lowered phosphorylase activity ratios.

DISCUSSION

The effects of levamisole on muscle contraction in the perfused preparation paralleled the observed effects with intact parasites (1–5, 22). In all cases a contractile phase which preceded a flaccid paralysis was observed. At higher doses of levamisole the flaccid paralysis occurred in less than 10 min, but at lower levamisole doses this phase required 15–30 min to appear.[3]

The flaccid paralysis phase in levamisole-treated *Ascaris* muscle was correlated with an activation of glycogen synthase and an inactivation of phosphorylase. Komuniecki and Saz (5) have reported a similar influence on glycogen synthase in the levamisole-treated filarial worm, *Litomosoides carinii*. Consequently, in both the ascarid and filarial (5) systems, an increased incorporation of glucose into glycogen was noted. In the present system, levamisole inhibited the elevation of cyclic AMP brought about by serotonin, thereby preventing an increase in the cyclic AMP-dependent protein kinase activity ratio. These results provide evidence to link the flaccid paralysis effect of levamisole with a cyclic AMP-mediated pathway. The failure to observe an influence of levamisole on the acetylcholine response of glycogen metabolism further suggests that levamisole inhibits cyclic AMP-mediated, but not calcium-mediated, glycogenolysis. The inhibition of cyclic AMP accumulation in response to serotonin and the inhibition of the glycogenolytic cascade and concomitant increase in glycogen synthesis observed in the levamisole-treated muscle are consistent with a model in which levamisole inhibits the adenylate cyclase of the muscle membrane. However, the data do not distinguish which of the proteins of the adenylate cyclase complex is inhibited, nor does it exclude other molecular events which may be inhibited or activated by levamisole.

Thus, the over-all effects of levamisole on the ascarid muscle can be divided into two stages. First, the contractile stage appears to involve direct stimulation of the muscle. Lewis and co-workers (23) have shown that levamisole is a cholinergic agonist in the free-living nematode, *Caenorhabditis elegans*, and this appears to be true also for *A. suum*.[4] Therapeutic doses of levamisole (7.6 mg/kg) would result in a concentration of this compound of about 30 μM in the intestine (3). From Fig. 1 it can be seen that this level is more than sufficient to cause contraction of the muscle and, thus, loss of muscular coordination. Second, the flaccid paralysis stage is characterized by an inhibition of glycogen mobilization and an increase in glycogen synthesis. The results presented herein indicate that modest changes in total muscle glycogen content can be detected within 1 hr of levamisole perfusion, and a 30% increase in newly synthesized glycogen is detected when [^{14}C]glucose incorporation into the polymer is measured. The data support a rapid turnover of glycogen which is disrupted by levamisole. Since glycogen stores have been shown to provide the only known source of energy-producing substrates in this anaerobic organism (for citations, see refs. 6 and 10), disruption of the regulatory mechanisms which mobilize glycogen in response to a failing energy charge may play a central role in the anthelmintic process.

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