Biochemical Pharmacology, 1961, Vol. 5, pp. 311–322. Pergamon Press Ltd., Printed in Great Britain.

BIOCHEMICAL EFFECTS OF DITHIAZANINE ON THE CANINE WHIPWORM, *TRICHURIS VULPIS**

E. BUEDING,† E. KMETEC,‡ C. SWARTZWELDER, S. ABADIE and H. J. SAZ†

Department of Pharmacology and Department of Tropical Medicine, and Medical Parasitology, School of Medicine, Louisiana, State University, New Orleans, La.

(*Received* 25 *July* 1960)

Abstract—Lactic, acetic, propionic and *n*-valeric acids, carbon dioxide, and small quantities of formic and *n*-butyric acids, are products of the aerobic and anaerobic metabolism of adult *Trichuris vulpis*, the canine whipworm. Approximately 50 per cent of the glucose taken up by this parasite is accounted for by these products. The parasite is not dependent on aerobic metabolism. In an atmosphere containing CO_2 (from 2 to 5 per cent) in nitrogen, *T. vulpis* survives *in vitro* for considerably longer periods and metabolizes carbohydrate at a higher rate than in nitrogen alone, in air, or in a mixture of CO_2 (5 per cent) and oxygen (20 or 95 per cent).

Dithiazanine, a cyanine dye effective in the treatment of canine and human whipworm infections, in concentrations which do not affect the motility of *T. vulpis*, produces an irreversible inhibition of the uptake of glucose by the parasite and a marked reduction in the concentrations of free glucose and ATP, and in the size of the carbohydrate stores in the worm. It is concluded that dithiazanine interferes with the transport of glucose in *T. vulpis*. As a result, utilization of endogenous carbohydrate is increased. Depletion of the carbohydrate stores and inability to utilize exogenous glucose brings about a decrease in the generation of energy-rich phosphate bonds. These biochemical changes can account for the chemotherapeutic action of dithiazanine in trichuriasis.

RECENT reports have demonstrated the chemotherapeutic effectiveness of a cyanine dye, dithiazanine, in the treatment of human subjects infected with the whipworm, *Trichuris trichiura*,[1] as well as with other intestinal nematodes (*Strongyloides stercoralis*, *Enterobius vermicularis* and *Ascaris lumbricoides*).[2, 3] In the course of investigations designed to determine the biological properties of cyanine dyes[4] the anthelmintic activity of these compounds was uncovered during World War II.[5–7] In extremely low concentrations (5×10^{-8} M) cyanines inhibit the oxygen uptake of a number of helminths.[8–11] This effect accounts for the chemotherapeutic activity of cyanines against the filarial worm *Litomosoides carinii*. The structural property conferring these metabolic and chemotherapeutic actions of cyanines is the amidinium ion resonance system which is a common characteristic of this group of compounds.[12] Subsequent investigations have revealed that cyanine dyes have chemotherapeutic

* This investigation was conducted under the auspices of the Commission on Parasitic Diseases of the Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General of the Army and by research grants (E–668 and E–2347) from the National Institutes of Health, U.S. Public Health Service.

† Present address: Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland.

‡ Present address: Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland, Ohio.

activity against certain intestinal nematodes of dogs (*Ascaris*, *Trichuris* and *Ancylostoma*),[13] of rats and of mice (pinworms, strongyloids).[14] These observations led to the development of a cyanine dye, pyrvinium chloride, which proved highly effective in the treatment of human pinworm infections.[15, 16] Since the oxygen tension of the habitat of these helminths is extremely low and since most of the above-mentioned parasites depend for survival on anaerobic, rather than on aerobic, metabolism[17, 18] it appears that the chemotherapeutic actions of cyanine dyes on these organisms are brought about by a mechanism other than inhibition of oxidative reactions. The high activity of dithiazanine against similar types of intestinal nematodes provides additional support for this conclusion. In the present study an attempt has been made to determine the effects of dithiazanine on the anaerobic metabolism of *Trichuris vulpis*, the canine whipworm highly susceptible to the chemotherapeutic actions of this cyanine dye.

The observations reported below indicate that under both anaerobic and aerobic conditions sublethal concentrations of dithiazanine irreversibly inhibit the transport of glucose into *Trichuris vulpis*. Lack of availability precluded similar studies using the human whipworm, *Trichuris trichiura*.

METHODS AND MATERIALS

After removal of the whipworms from the caecal mucosa of dogs, the parasites were placed in a medium which had the following composition: 0·12 M NaCl, 0·005 M KCl, 0·001 M $CaCl_2$, 0·001 M $MgCl_2$, 0·02 M Tris*buffer (pH 7·7), 0·002 M sodium phosphate buffer (pH 7·7), 0·05 M glucose; penicillin, streptomycin and kanamycin, each in a concentration of 10 μg per ml; and mycostatin, 1 μg per ml. Subsequently, this medium will be referred to as the basic medium. The nematodes were freed of contaminating intestinal contents and tissue debris by three transfers into petri dishes containing the basic medium, and were then blotted (using Whatman filter paper no. 50), weighed, and placed in solutions in which their metabolic activities were to be determined. One milliliter of medium was used per worm. When CO_2 was used in the atmosphere, sodium bicarbonate was added to the basic medium, in order to readjust the pH to 7·7. The final concentration of $NaHCO_3$ was $4{\cdot}5 \times 10^{-2}$ M with 5 per cent CO_2, $1{\cdot}3 \times 10^{-2}$ M with 2 per cent CO_2 and 6×10^{-4} M with 0·3 per cent CO_2. With 0·1 per cent CO_2 no bicarbonate was required, since the decrease in the pH produced by this concentration of gas was not significant. Gassing and incubation were carried out in the conventional Warburg apparatus at 37 °C. Glucose was determined by the method of Somogyi[19], lactic acid according to Barker and Summerson[20], glycogen according to Hassid and Abraham[21] and total carbohydrate according to a method used in a previous study.[8] Volatile acids were isolated from the media by distillation *in vacuo* and subsequent concentration, as described previously;[22] they were then separated and tentatively identified by gas partition chromatography, according to James and Martin[23].

Survival of *T. vulpis in vitro* was determined at 37 °C by incubating each worm in a screw-capped culture tube containing 5 ml of medium. Each experimental condition employed five worms and transfer to fresh media was made every three or four days.

* In this paper the following abbreviations are used: ATP, adenosine triphosphate; Tris, tris-hydroxymethylamino methane; TPN, triphosphopyridine nucleotide.

When gas mixtures other than air were used, the medium in each tube was gassed for 1 min before, and for another minute after the parasite had been introduced. This was followed immediately by sealing the screw-capped culture tube. The worms were observed daily for motility, which was used as the criterion for survival.

Concentrations of glucose and of ATP in the worms were determined enzymatically, as proposed by Slein *et al.*[24] by measuring the reduction of TPN as a result of the combined actions of hexokinase and of glucose-6-phosphate dehydrogenase. For these analyses the parasites were homogenized with cold perchloric acid (final concentration, 4 per cent). The mixtures were centrifuged at 1000 *g* for 15 min and measured volumes of the supernatant fluids were adjusted with KOH (2 N) to a pH of 7·0. The insoluble potassium perchlorate was removed by centrifugation at 1000 × *g* for 15 min. All operations were carried out at temperatures between 0 and 4 °C. Glucose was determined in aliquots of the neutralized supernatant fractions in a reaction mixture containing 0·01 M $MgCl_2$, 0·05 M potassium glycylglycine buffer (pH 7·4), 0·0003 M TPN and 0·003 M ATP (pH 7·4) in a final volume of 1 ml. After glucose-6-phosphate dehydrogenase was added, the change in optical density was determined at 340 mμ in a Beckman DU spectrophotometer. On completion of the reaction, hexokinase was added and the reduction of TPN was measured again in the same manner in order to determine free glucose. The same procedure was used for the determination of ATP, except that glucose (final concentration: 0·0015 M) was added to the reaction mixture in place of ATP. Under these conditions, reduction of 1·0 μmole of TPN is equivalent to 1·0 μmole of glucose if an excess of ATP is provided, and to 1 μmole of ATP in the presence of an excess of added glucose. Addition of glucose-6-phosphate dehydrogenase prior to that of hexokinase insures the dehydrogenation of any glucose-6-phosphate which may be present initially in the reaction mixture. Crystalline hexokinase was prepared according to an unpublished method of Darrow and Colowick[25]. Glucose-6-phosphate dehydrogenase was obtained from Boehringer and Soehne. Of a twentyfold dilution of the hexokinase, 0·02 ml was used, as well as 0·02 ml of a fiftyfold dilution of the glucose-6-phosphate dehydrogenase suspension (in 0·05 M glycylglycine; pH 7·4); these amounts were adequate to carry the reactions to completion within from 4 to 5 min at room temperature.

Hexokinase activity in *T. vulpis* was determined after homogenizing the worms in 0·05 M glycylglycine buffer (pH 7·4) at 0 °C. The homogenate was prepared by the addition of 0·2 ml of buffer per 10 mg of tissue. The same reaction mixture as the one described in the preceding paragraph was used, except that NaF (final concentration: 0·05 M) and *both* glucose and ATP (final concentrations: 0·003 M and 0·0015 M, respectively) were included, and addition of the crystalline hexokinase preparation was omitted. Immediately after homogenization, 0·02 ml of the suspension or of a dilution thereof was added to start the reaction and the increase in the optical density due to the reduction of TPN was followed at a wavelength of 340 mμ for 4 min every 30 sec. Hexokinase activity was proportional to the amount of homogenate in the reaction mixture and was linear with time.

The potassium salt of ATP was obtained from Pabst Laboratories and TPN from Sigma Chemical Corporation. Dithiazanine (3:3′-diethylthiadicarbocyanineethyl sulfate) was supplied by Dr. Koert Gerzon of Eli Lilly Laboratories and 1-ethyl-3:6-dimethyl-2-phenyl-4-pyrimido-2-cyanine chloride (CC no. 863) by Dr. L. S. G. Brooker of Eastman Kodak Research Laboratories.

RESULTS

Carbohydrate utilization

In an atmosphere of nitrogen, uptake of exogenous glucose by *T. vulpis* varied between 0·35 and 0·55 μmoles per 24 hr and per mg. (wet weight). This rate of uptake is approximately one-tenth to one-twelfth of that of *Schistosoma mansoni*[9] and one-fifth of that of *Litomosoides carinii*.[8] In the absence of added glucose, the glycogen content of *T. vulpis* decreased at a rate which was about one-half the rate of glucose uptake. These values were obtained by the determination of the rate at which glycogen disappeared over a period of 4 hr. If the parasites were incubated without glucose for longer periods they gradually lost their motility, and survival was reduced to less than 1 day. In the absence of glucose the average rate of anaerobic glycogen utilization per mg wet wt. was 0·039 μmoles (in terms of glucose units) in 4 hr, as compared to from 0·058 to 0·089 μmoles of exogenous glucose uptake during the same period. There was no difference between the rates of anaerobic and aerobic glycogen utilization. The average intial glycogen content of the worms (wet weight) varied between 2·11 and 2·62 per cent (average: 2·35). After incubation for 4 hr (37 °C) in the absence of glucose the glycogen concentration fell to between 1·53 and 1·75 per cent (average: 1·62) under anerobic, and to between 1·50 and 1·82 (average: 1·69) under aerobic conditions.

TABLE 1. CARBON BALANCE OF ANAEROBIC GLUCOSE METABOLISM OF *Trichuris vulpis*

Atmosphere	100% N_2		5% CO_2, 95% N_2		5% CO_2, 95% N_2 0·1 μg dithiazanine/ml	
	μ moles*	% of glucose-carbon taken up	μ moles*	% of glucose-carbon taken up	μ moles*	% of glucose-carbon taken up
Glucose uptake	0·41	100	0·64	100	0·36	100
Products:						
Formic acid	0·025	1·0	0·032	0·8	0·012	0·6
Acetic acid	0 103	8·3	0·175	9·1	0·084	7·9
Propionic acid	0·112	13·8	0·181	14·2	0·091	12·7
Butyric acid	0·011	1·8	0·013	1·4	0·002	0·4
n-Valeric acid	0·045	9·2	0·076	9·9	0·034	8·0
C_6 acid (s)	0·015	3·7	0·024	3·7	0·007	2·0
Lactic acid	0·054	6·7	0·074	5·8	0·082	11·7
CO_2	0·25	10·2	0·25†	6·5	0·25†	11·0
Total		54·7		51·4		54·3

* Per mg (wet weight) and 24 hr.
† Estimated on the basis of CO_2 production determined in 100 per cent nitrogen.

Products of carbohydrate metabolism

Lactic acid production accounted for no more than from 5 to 10 per cent of the carbohydrate utilized under aerobic or anaerobic conditions. Other metabolic products formed by this parasite were acetic, propionic and *n*-valeric acids (Table 1). Smaller quantities of formic, *n*-butyric and of an as yet unidentified hexanoic acid also were produced. Approximately 10 per cent of the glucose metabolized under anaerobic conditions was converted to CO_2, indicating the occurrence of active

decarboxylation mechanisms. The above products accounted for approximately 50 per cent of the carbohydrate utilized by this parasite.

Effect of carbon dioxide on carbohydrate metabolism

When *T. vulpis* was incubated in the basic medium for several days in an atmosphere of air or of nitrogen, the rate of glucose uptake was not maintained at a constant level,

TABLE 2. EFFECT OF GASEOUS ATMOSPHERE ON GLUCOSE UPTAKE OF *Trichuris vulpis*

Gas	μ moles of glucose taken up in 24 hr, (per mg wet weight)		
	1st day	2nd day	3rd day
100% N_2	0·47 0·38	0·41 0·42	0·33 0·25
Air	0·42 0·55	0·36 0·56	0·29 0·41
100% O_2	0·48 0·32	0·17 0·26	0·04 0·16
5% CO_2 95% N_2	0·75 0·87	0·80 0·92	0·78 0·88
5% CO_2 95% O_2	0·46 0·68	0·22 0·31	0·16 0·24
5% CO_2 20% O_2 75% N_2	0·68 0·76	0·38 0·22	0·26 0·14

TABLE 3. EFFECT OF THE CO_2 CONCENTRATION ON THE ANAEROBIC UPTAKE OF GLUCOSE BY *Trichuris vulpis*

Concentration of CO_2 (in N_2)	μ moles of glucose taken up in 24 hr (per mg wet weight)		
	1st day	2nd day	3rd day
—	0·38 0·40	0·33 0·13	0·31 0·09
0·1	0·48 0·36	0·19 0·33	0·07 0·30
0·3	0·43 0·36	0·31 0·29	0·35 0·25
2	0·76 0·88	0·85 0·84	0·82 0·87
5	0·71 0·92 0·86	0·76 0·82 0·86	0·71 0·86 0·80

but decreased more or less markedly (Table 2). Under these conditions another irregularity was noted: the reduction in glucose uptake was not uniform even among worms originating from the same host. On the other hand, if these *in vitro* experiments were carried out in an atmosphere more closely approaching that prevailing in the

natural habitat of the parasite,[26–30] i.e. in a gas mixture containing 5 per cent CO_2 and 95 per cent N_2, the rate of glucose uptake not only was higher than in oxygen, in air, or in various mixtures containing CO_2 and O_2, but also remained at a constant level for a period of at least 3 days (Table 2). The minimal concentration of CO_2 in nitrogen required to produce this effect was found to lie between 0·3 and 2 per cent (Table 3). The increase in the rate of glucose uptake produced by CO_2 under anaerobic conditions was demonstrable already during shorter periods of incubation lasting only 4 hr. Elimination of metabolically produced CO_2 from the atmosphere by the use of alkali resulted in a further reduction in glucose utilization (Table 4). While CO_2 increased the production of lactic and of volatile acids from glucose, the ratios of these acids formed per mole of glucose taken up were not affected materially by the presence of CO_2 in the atmosphere (Table 1).

TABLE 4. EFFECT OF CO_2 ON ANAEROBIC GLUCOSE UPTAKE OF *Trichuris vulpis* DURING AN INCUBATION PERIOD OF 4 HR

Gas	μ moles of glucose taken up in 4 hr (per mg)
N_2*	0·070
	0·091
N_2	0·110
	0·117
5% CO_2, 95% N_2	0·162
	0·146

* Center cup of incubation vessel contained 0·1 ml of 40 per cent KOH in order to absorb the CO_2 produced in the closed system.

TABLE 5. SURVIVAL OF *Trichuris vulpis in vitro*

Medium	Survival in days			
	In air		in 5% CO_2, 95% N_2	
	Average	Limits	Average	Limits
Complete basic medium	6·0	4–7	16·2	13–17
Without potassium	4·2	3–6	6·8	2–10
Without calcium	3·2	2–4	8·6	7–12
Without magnesium	3·1	2–5	11·2	2–11
Without phosphate			12·8	7–17

Survival of T. vulpis in vitro

In an atmosphere of air the worms survived for a period of from 4 to 7 days in the basic medium.

The concentrations of the constituents of the basic medium were arrived at by varying the concentration of each cation until the optimal survival period was obtained. Omission of K^+, Ca^{2+} or Mg^{2+} resulted in a significant reduction of survival (Table 5).

In the absence of glucose, survival was reduced to less than 1 day. Glucose could be replaced by mannose, fructose, maltose or glycerol (Table 6), indicating that one or

several hexokinases, a glycerol kinase, a maltase and a phosphomannose isomerase were present in the worms. On the other hand, galactose, sucrose, trehalose and ribose did not support the survival of the worms (Table 6).

When air was replaced by a gas mixture containing 5 per cent CO_2 and 95 per cent nitrogen, survival of the worms was increased significantly (Table 7). As in the experiments concerned with glucose uptake, this effect of CO_2 required the absence of oxygen. When survival was extended to a period of 2 weeks by the use of the CO_2–N_2 gas mixture it became evident that glucose was more effective in supporting the survival of *T. vulpis in vitro* than were mannose, fructose, maltose or glycerol (Table 6). Under these conditions, the requirement of the worms for K^+, Ca^{2+} and Mg^{2+} was demonstrable also (Table 5).

TABLE 6. EFFECT OF VARIOUS SUGARS ON THE SURVIVAL OF *Trichuris vulpis in vitro*

Sugar	Concentration (mg/ml)	Survival in days			
		In air		in 5% CO_2, 95% N_2	
		Average	Limits	Average	Limits
—	—	0	0	0	0
Glucose	2	6·0	5–7	14·8	14–16
Fructose	2	5·6	5–6	7·2	5–8
Mannose	2	5·4	5–6	10·3	9–11
Glycerol	2	4·2	3–6	5·3	5–6
Galactose	2	0·4	0–1	—	—
Maltose	4	4·4	2–6	4·0	2–5
Sucrose	4	0	0	—	—
Trehalose	4	0	0	—	—
Ribose	2	0·2	0–1	—	—

TABLE 7. EFFECT OF THE GASEOUS ATMOSPHERE ON THE SURVIVAL OF *T. vulpis in vitro* 37 °C

Gas	Survival (days)	
	Average	Limits
Air	6·0	4–7
5% CO_2 in air	5·2	2–9
N_2	6·0	4–9
5% CO_2 in N_2	11·2	9–16

Respiratory enzymes

During incubation of *T. vulpis* in the basic medium with air as the atmosphere, the parasites took up 0·34 μmoles of oxygen per mg (wet weight) in 24 hr (37 °C). Evidence for the presence of the cytochrome system in *T. vulpis* was supplied by the observations summarized in Table 8. Homogenates of the worms catalyzed the reduction of cytochrome *c* and this rate was increased significantly by the addition of succinate. Furthermore, reduced cytochrome *c* was oxidized by homogenates of the worms and this reaction was sensitive to cyanide and heat. These observations suggest that the worms contain a succinoxidase which operates with the participation of the cytochrome system. The activities of both cytochrome *c* and of cytochrome oxidase in homogenates are sufficiently high to account for the rate of oxygen uptake of intact worms.

Addition of hydrogen peroxide to homogenates of *T. vulpis* resulted in the rapid release of oxygen (Table 8) indicating that these worms have high catalase activity.

Concentrated aqueous extracts of *T. vulpis* have a distinct red coloration. Using a cytochrome *c*-deficient rat liver succinoxidase preparation[31] it was found that this pigment was not identical with cytochrome *c*. The pigment had a maximum extinction

TABLE 8. SUCCINIC DEHYDROGENASE, CYTOCHROME OXIDASE AND CATALASE ACTIVITIES OF *Trichuris* HOMOGENATES

Exp. no.	Reaction	Measured component	Rate mμmoles/hr per mg
1	Endogenous ⟶ 2 cytochrome *c*	Cytochrome *c*	5·8
2	succinate ⟶ 2 cytochrome *c*	Cytochrome *c*	12·7
3	2 Cytochrome *c* ⟶ O_2	Cytochrome *c*	12·4
4	2 Cytochrome *c* ⟶ O_2 + KCN	Cytochrome *c*	4·7
5	2 H_2O_2 ⟶ 2 H_2O + O_2	O_2	229·0

Reaction mixtures in experiment nos. 1 to 4 contained 50 μmoles of phosphate buffer (pH 7·7), 0·04 μmoles of reduced or 0·05 μmoles of oxidized cytochrome *c*, 0·02 ml *Trichuris* homogenate and, where indicated, 1 μmole of KCN or 50 μmoles of sodium succinate, or both. Final volume: 1·0 ml. The homogenate was prepared in sucrose (20 per cent) containing 0·01 M phosphate buffer (pH 7·7) and 68 mg of tissue per ml. Measurements were taken in the Beckman DU spectrophotometer at 550 mμ at room temperature (25 °C). Experiment number 5 was carried out in the presence of 40 μmoles of phosphate buffer (pH 7·3), 3·0 μmoles of H_2O_2 and 0·2 ml of *Trichuris* homogenate. Controls were run in the presence and absence of homogenate and of H_2O_2. The *Trichuris* homogenate was prepared in sucrose (20 per cent) containing 0·05 M phosphate buffer (pH 7·3) and contained 121 mg of tissue per ml. Gas production was determined in the Warburg apparatus (37 °C) with 0·1 ml of 10 N KOH in the center well.

at a wavelength of 414 mμ, which was shifted to 426 mμ on reduction with a few crystals of sodium hydrosulfite. This behavior is more characteristic for a hemoglobin rather than for a cytochrome. Possibly this pigment originates from the red cells of the host because it has been reported that trichurids secure their food from mucosal tissue and blood.[32–35]

Effect of dithiazanine on metabolism of T. vulpis

Concentrations of dithiazanine lower than those which affected the motilityof the worms significantly inhibited their rate of anaerobic glucose uptake (Table 9). Therefore it appears that dithiazanine has a direct effect upon the carbohydrate metabolism of *T. vulpis* and that these biochemical changes are not secondary to a reduction in the motility of the worm. When the organisms were exposed for 24 hr to from 0·1 to 0·2 μg of the drug per ml and were then transferred into dithiazanine-free medium, the inhibition of glucose uptake persisted, and became even more pronounced in many experiments (Table 9). This metabolic effect of dithiazanine was found to be irreversible. The inhibitory effects of dithiazanine on glucose uptake were equally pronounced in the presence and in the absence of CO_2. Reduction of glucose uptake by dithiazanine was associated with a corresponding decrease in the amount of volatile acids produced, but there was no specific inhibition of the production of any particular volatile acid (Table 1).

Exposure of the worms to concentrations of dithiazanine which produced a reduction in glucose uptake resulted in a marked decrease in the concentrations of free glucose, of ATP and of total carbohydrate in the worms (Table 10).

Dithiazanine had no effect on the hexokinase activity of homogenates of *T. vulpis*. The highest drug concentration used was 5 μg/ml which is fifty times higher than the one which produced decreases in glucose uptake and in the glucose concentration in the worm.

Uptake of glucose by *T. vulpis* and the concentration of free glucose in the nematode were decreased also by another cyanine dye, 1-ethyl-3:6-dimethyl-2-phenyl-4-pyrimido-2-cyanine chloride. The inhibitory effects of this dye were much weaker and considerably higher concentrations were required. Furthermore, in contrast to dithiazanine, the actions of this cyanine dye were reversible.

TABLE 9. EFFECT OF DITHIAZANINE ON GLUCOSE UPTAKE BY *Trichuris vulpis* (Atmosphere: 5% CO_2, 95% N_2, 37 °C)

Dithiazanine concentration during first day* (μg/ml)	Glucose uptake (μ moles†)		
	1st day	2nd day	3rd day
—	0·66	0·63	0·61
—	0·77	0·65	0·69
—	0·68	0·65	0·68
0·1	0·60	0·42	0·46
0·1	0 48	0 35	0 33
0 1	0·55	0·35	0·28
0·15	0·42	0·38	0·30
0·15	0·56	0·26	0·22
0·2	0·48	0·36	0·27
0·2	0·40	0·22	0·16
0·2	0·44	0·29	0·14

* These worms were incubated for 24 hr in a medium containing dithiazanine and then transferred to the drug-free basic medium which was used throughout for the control worms.

† Per mg worm wet wt.

DISCUSSION

Since *T. vulpis* survives *in vitro* for considerably longer periods of time in an atmosphere of 5 per cent CO_2 in nitrogen than in one containing the same concentration of CO_2 in air or oxygen, it is evident that the energy required by *T. vulpis* is provided by anaerobic, rather than by aerobic, metabolism. The possibility that hydrogen peroxide produced during respiration may have a deleterious effect can be ruled out because catalase activity of the worms is far in excess of the rate of oxygen uptake. Therefore, even if all dehydrogenation reactions were mediated by a terminal electron acceptor catalyzing the production of hydrogen peroxide, the latter would be decomposed as soon as it was formed. It is doubtful whether hydrogen peroxide is a respiratory product of *T. vulpis* formed by a terminal flavoprotein, because cytochrome *c* and cytochrome oxidase activities are sufficiently high to mediate the respiration of the worm. The presence of the cytochrome system and of a cytochrome *c* linked succinoxidase

system distinguishes *T. vulpis* from two other nematodes, *Ascaris lumbricoides* and *Litomosoides carinii*, in which the cytochrome system could not be detected,[36] and from *Schistosoma mansoni* in which cytochrome *c* and cytochrome oxidase activities account for less than 10 per cent of the oxygen taken up by this trematode.[36] Since the habitat and metabolism of the adult whipworm are essentially anaerobic, the cytochrome system in this organism may play no physiological role, but may have been carried over from a previous, aerobic, stage in the life cycle of the nematode.

TABLE 10. EFFECT OF DITHIAZANINE ON THE CONCENTRATIONS OF GLUCOSE, ATP AND TOTAL CARBOHYDRATE IN *Trichuris vulpis*
(Atmosphere: 5% CO_2, 95% N_2, 37 °C)

Exp. no.	Incubation period (hr)	Dithiazanine (μg/ml)	Glucose (μmoles*)	ATP (μmoles*)	Total carbohydrate (μmoles*)
1	24	—	5·52	1·55	125
	24	0·1	1·46	0·89	64
2	24	—	8·70	1·53	146
	24	0·1	1·73	0·76	78
3	48	—	7·30	1·50	167
	48	0·1	1·58	0·48	36
4	48	—	6·67	1·66	152
	48	0·1	1·88	0·58	43
5	72	—	8·35	1·76	185
	72	0·1	0·92	0·36	41

For the first 24 hr the worms were incubated in a dithiazanine containing medium. At the end of this period they were transferred into a dithiazanine-free medium and incubated for another 24 (experiments 3 and 4) or 48 (experiment 5) hrs.
* Per g wet wt.

The marked effect of CO_2, in increasing the period of survival of *T. vulpis*, *in vitro*, and in enhancing, as well as maintaining, the rate of glucose uptake, may be due to mechanisms for CO_2 fixation which are essential to the functional integrity of the worm. The high CO_2 tension prevailing in the intestinal tract and contents of the host[26–30] insures the availability of this gas to the parasite in its natural habitat. CO_2 is known to be a growth factor for some micro-organisms.[37–45] Furthermore, highly active systems for CO_2 fixation have been demonstrated in two intestinal nematodes, *Heterakis gallinae*[44] and *Ascaris lumbricoides*.[45] In these organisms CO_2 condenses with pyruvate eventually giving rise to succinate. Unpublished observations of two of the authors (E.K. and E.B.) have indicated that from 10 to 15 per cent of the carbohydrate utilized by *T. vulpis* is converted to succinate. Should this be confirmed, CO_2 fixation may be prevalent in this nematode also. In addition, propionate is a fermentation product of *T. vulpis*. It has been demonstrated that in muscle strips of *Ascaris*, propionate is formed by the decarboxylation of succinate.[45] Should a similar reaction account for the formation of propionate by *T. vulpis*, at least 25 per cent of the carbohydrate utilized by this parasite would be metabolized through pathways involving CO_2 fixation.

An inhibition of glucose uptake by *T. vulpis* was observed with concentrations of dithiazanine which produced no change in the motility of the worms. Therefore this

biochemical effect is not secondary to, but precedes the anthelmintic action of the cyanine dye. If this decrease in the uptake of glucose was due to an inhibition of one or of several enzymes concerned with the intracellular metabolism of carbohydrate there should be an accumulation of glucose within the worm. However, the reduction of glucose uptake produced by dithiazanine was associated with a marked decrease in the concentration of free glucose in the worm. Therefore it is apparent that dithiazanine interferes with the uptake of glucose from the medium, rather than with its utilization. Reduced availability of exogenous glucose due to an inhibition of the transport of this sugar into the parasite should increase the utilization of endogenous carbohydrate reserves. This is consistent with the marked decrease in the concentration of the total carbohydrate of the worms which had been incubated with dithiazanine, indicating again that dithiazanine did not interfere with the intracellular utilization of carbohydrate. Furthermore, even high concentrations of dithiazanine had no inhibitory effect on the activity of hexokinase in homogenates of *T. vulpis*; therefore, the cyanine dye did not inhibit the phosphorylation of glucose present in the parasite. It is concluded that the depletion of the carbohydrate reserves is secondary to the decreased availability of exogenous glucose due to interference by dithiazanine with the transport of this sugar into the worm. This circumstance, in turn, leads to a decrease in the rate at which energy-rich phosphate bonds are formed, and accounts for the reduction in the concentration of ATP in the parasite. Owing to these changes in carbohydrate metabolism brought about by dithiazanine, the supply of energy required for survival is decreased and eventually should become inadequate, resulting in the death of the worm.

The biochemical effects of dithiazanine on *T. vulpis* are irreversible, since they persist and become even more pronounced after exposure of the worms to the cyanine dye has been discontinued by transfer into dithiazanine-free medium. This property is in agreement with the observed irreversibility of the chemotherapeutic action on *Trichuris trichiura* after oral administration of the drug.[1]

It is not known whether glucose enters *T. vulpis* through the cuticle or via the alimentary canal. In the latter eventuality the possibility should be considered that dithiazanine interferes with the intestinal absorption of glucose.

The biochemical effects of another cyanine dye (CC no. 863) qualitatively were similar to those of dithiazanine, but, higher concentrations were required to obtain these effects, which proved to be reversible in the case of this particular dye. The latter cyanine is one of the most potent ones with regards to its ability to inhibit oxygen uptake of *L. carinii*.[7, 8] Thus, there is no parallelism between these two types of effects of cyanines on helminths; in fact, two unrelated mechanisms may be involved. It remains to be determined which particular structural characteristics confer to a cyanine dye high activity and irreversibility for the inhibition of glucose transport in *T. vulpis*. Furthermore, the question arises whether there exists a relationship between the inhibitory effects of cyanines on glucose transport in *T. vulpis* and those on the active transport of organic bases across renal tubuli reported by Peters *et al.*[46–48]

Among helminths, considerable differences in biochemical characteristics have been encountered, even among species which are closely related to each other morphologically and taxonomically.[17, 18] Although *T. trichiura*, the whipworm invading man, and *T. vulpis* are equally susceptible to the chemotherapeutic actions of dithiazanine,[1, 49] investigations of the effects of this cyanine dye on *T. trichiura* are required

in order to ascertain more definitely whether inhibition of glucose transport accounts for the anthelmintic action of dithiazanine in human trichuriasis.

REFERENCES

1. W. W. FRYE, C. SWARTZWELDER, R. LAMPERT, S. A. ABADIE and C. B. CARSON, JR., *Amer. J. Trop. Med.* **6**, 890 (1950).
2. J. C. SWARTZWELDER *et al.*, *J. Amer. Med. Ass.* **165**, 2063 (1951).
3. E. BUEDING and C. SWARTZWELDER, *Pharm. Rev.* **9**, 329 (1957).
4. L. S. G. BROOKER and L. A. SWEET, *Science* **105**, 496 (1947).
5. A. D. WELCH, L. PETERS, E. BUEDING, A. VALK, JR. and A. HIGASHI, *Science* **105**, 486 (1947).
6. H. N. WRIGHT, R. N. BIETER, E. H. CRANSTON, D. C. CUCKLER, J. T. LICHFIELD, JR. and T. BREY, *Ann. N.Y. Acad. Sci.* **50**, 109 (1948).
7. L. PETERS, E. BUEDING, A. VALK, JR., A. HIGASHI and A. D. WELCH, *J. Pharmacol.* **95**, 212 (1949).
8. E. BUEDING, *J. Exp. Med.* **89**, 107 (1949).
9. E. BUEDING, *J. Gen. Physiol.* **33**, 475 (1950).
10. E. BUEDING, L. PETERS, S. KOLETSKY and D. MOORE, *Brit. J. Pharmacol.* **8**, 15 (1953).
11. E. BUEDING and J. OLIVER-GONZALEZ, *Brit. J. Pharmacol.* **5**, 62 (1950).
12. L. G. S. BROOKER, *Theory of the Photographic Process* (Edited by C. E. K. MEES) (Revised edition) pp. 371–429, MacMillan, New York (1954).
13. D. R. HALES and A. D. WELCH, *J. Pharmacol.* **107**, 310 (1953).
14. J. K. WESTON, P. E. THOMPSON, J. W. REINERTSON, R. A. FISKEN and T. F. REUTNER, *J. Pharmacol.* **107**, 315 (1953).
15. W. G. SAWITZ and F. E. KARPINSKI, JR., *Amer. J. Trop. Med.* **5**, 538 (1956).
16. A. ROYER, *J. Amer. Med. Ass.* **74**, 297 (1956).
17. E. BUEDING, *Physiol. Rev.* **29**, 195 (1949).
18. T. VON BRAND, *Chemical Physiology of Endoparasitic Animals*. Academic Press, New York (1952).
19. M. SOMOGYI, *J. Biol. Chem.* **100**, 61, 69 (1948).
20. S. B. BARKER and W. H. SUMMERSON, **138** 535 (1941).
21. W. Z. HASSID and S. ABRAHAM, *Methods in Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN) Vol. 3, p. 34. Academic Press, New York (1957).
22. E. BUEDING and H. W. YALE, *J. Biol. Chem.* **193**, 411 (1951).
23. A. T. JAMES and A. J. P. MARTIN, *Biochem. J.* **50**, 679 (1952).
24. M. W. SLEIN, G. T. CORI and C. F. CORI, *J. Biol. Chem.* **186**, 763 (1950).
25. S. P. COLOWICK. Personal communication.
26. E. J. DE BEER, C. G. JOHNSTON and D. W. WILSON, *J. Biol. Chem.* **108**, 113 (1935).
27. R. C. HERRIN, *J. Biol. Chem.* **118**, 459 (1937).
28. L. C. MCGEE and A. B. HASTINGS, *J. Biol. Chem.* **142**, 893 (1942).
29. C. S. ROBINSON, H. LUCKEY and H. MILLS, *J. Biol. Chem.* **147**, 715 (1943).
30. W. TURNER and V. E. HODGETTS, *Aust. J. Agric. Res.* **6**, 115 (1955).
31. W. C. SCHNEIDER and V. R. POTTER, *J. Biol. Chem.* **149**, 219 (1948).
32. J. GUIART, *Lyon. Med.* **110**, 325 (1908).
33. G. F. OTTO, *Amer. J. Trop. Med.* **15**, 693 (1935).
34. H. C. LI, *Amer. J. Trop. Med.* **47**, 1343 (1933).
35. B. G. CHITWOOD and M. B. CHITWOOD, *J. Wash. Acad. Sci.* **27**, 517 (1957).
36. E. BUEDING and B. CHARMS, *J. Biol. Chem.* **196**, 615 (1952).
37. S. J. AJL and C. H. WERKMAN, *Arch. Biochem.* **19**, 483 (1948).
38. R. E. HUNGATE, *Bacteriol Rev.* **14**, 1 (1950).
39. A. K. SIJPESTEIJN, *J. Gen. Microbiol.* **5**, 869 (1951).
40. M. P. BRYANT and L. A. BURKEY, *J. Dairy Sci.* **36**, 205 (1953).
41. P. J. GRIFFIN and E. RACKER, *J. Bact.* **71**, 717 (1956).
42. A. D. LARSON, R. W. TREIK, C. L. EDWARDS and C. D. COX, *J. Bact.* **77**, 361 (1959).
43. J. W. GILL and K. W. KING, *J. Bact.* **75**, 666 (1958).
44. D. FAIRBAIRN, *Exp. Parasitol.* **3**, 52 (1954).
45. H. J. SAZ and D. VIDRINE, JR., *J. Biol. Chem.* **234**, 2001 (1959).
46. L. PETERS, K. J. FENTON, M. L. WOLF and A. KANDEL, *J. Pharamacol.* **113**, 148 (1955).
47. B. R. RENNICK, A. KANDEL and L. PETERS, *J. Pharmacol.* **118**, 204 (1956).
48. L. PETERS, *Pharm. Rev.* **12**, 1 (1960).
49. M. C. MCCOWEN, M. E. CALLENDER and M. C. BRANDT, *Amer. J. Trop. Med.* **6**, 895 (1957).