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EFFECT OF RESPIRATORY INHIBITION ON *SCHISTOSOMA MANSONI**

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The observed survival of adult *Schistosoma mansoni in vivo* under conditions producing a marked reduction of respiration of this parasite has been reported previously from this laboratory (Bueding, Peters, and Welch, 1947). Mature schistosomes removed from the portal venous circulation of rabbits (which had previously been given repeated daily intravenous injections of a cyanine dye) exhibited a normal rate of glycolytic activity, although their oxygen consumption was greatly diminished. No evidence of a concurrent chemotherapeutic action of the dye was observed, and no differences in the appearance, motility or distribution of the parasites were detectable in comparison with parasites from untreated animals.

This observation has been extended by the experiments described below. One of these shows that repeated injections of the same compound into hamsters infected with *S. mansoni* seven weeks previously, produced no observable effect on the mature worms other than a depression of respiration. However, other experiments demonstrated that significant changes in the course of infection were associated with this respiratory depression, when treatment was begun at an earlier stage of development of the parasite. It is this latter observation and its implications which is believed to deserve publication.

METHODS

The cyanine dye, 1'-ethyl-3:6-dimethy-2-phenyl-4-pyrimido-2'-cyanine chloride, whose structural formula appears elsewhere (Welch *et al.*, 1947; Peters *et al.*, 1949; Bueding, 1949a) was administered intraperitoneally as a 0.125% (w/v) solution in 0.85% (w/v) NaCl.

Young Syrian hamsters, weighing 85 to 120 g., were exposed to freshly shed cercariae from laboratory-infected snails (*Australorbis glabratus*, Puerto Rican strain). Infection was accomplished in Exps. 1 and 2 (Tables I and IIA) by intraperitoneal injection of aqueous suspensions of 160 to 180 cercariae. The alternative procedure used in Exps. 3 and 4 (Tables IIB and III)—namely, immersions of the animals in similar suspensions—ultimately led to heavier infections with adult schistosomes.

Faecal pellets from the individually housed animals were collected on clean moist blotting-paper placed under the wire-mesh cage flooring. Single pellets were transferred to glass slides, mixed with several drops of distilled water, covered with a glass slip and examined microscopically for ova (100-fold magnification). Ova were found in untreated animals six to eight weeks after infection. Whenever repeated examination failed to reveal the presence of ova up to the time of sacrifice, mucosal scrapings from the large bowel were examined microscopically *post mortem*.

Animals were usually sacrificed for necropsy 24 hours after the last injection. Of the remainder, those found moribund as a result of the infection were sacrificed immediately; those found dead were discarded. The flukes were removed from the portal and mesenteric veins with dissecting needles, and were transferred, after counting, to a salt solution containing 200 mg. glucose per 100 ml. (Bueding, 1950). Lots of eight to ten worm pairs then were transferred to 0.8 ml. volumes of a similar solution contained in small Warburg vessels (total volume of vessels: 5 to 5.5 ml.). The oxygen uptake was measured manometrically over a period of two hours. Schistosomes from untreated animals served as controls. Comparison of the oxygen uptake in two, and at times three, vessels containing flukes from a single hamster revealed a maximum variation of only 8%.

Other worm pairs from treated and untreated animals were fixed in a solution containing 4% (w/v) formaldehyde, 50% alcohol (v/v), and 2% (v/v) acetic acid (Landowsky's mixture) by vigorous shaking in small vials. The whole worms were subsequently stained with Mayer's carmalum and were examined microscopically for morphological changes. In the

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female worms particular attention was given to the development of the ovary and vitelline glands, the presence of sperm in the oviduct, and the presence of an egg in the oötype or uterus. Male worms were examined especially for testicular development and for the presence of sperm in the seminal vesicles.

Sections of the livers of treated and untreated animals were also removed at necropsy. Several small weighed fractions (8 to 14 mg.) of each of these were immediately pressed between glass slides and examined microscopically for the presence of schistosome ova. Quantitative results of such examinations are expressed as the number of ova per mg. of tissue (wet weight). In addition, livers of treated and untreated animals were fixed by immersion in a 4% (w/v) solution of formaldehyde in saline. Histological sections of these were examined microscopically for pathological changes.

Some of the animals in each experimental group served as controls and were not treated with the cyanine. They, and the schistosomes and livers removed from them, were subjected to procedures otherwise identical with those described.

Quantitative data were analysed statistically by standard methods (for example, Burn, 1930). Such data have been expressed in the Tables as means ± the standard error of the mean. Corresponding mean values for treated and untreated experimental groups were considered to be significantly different if a value greater than 2.5 resulted on dividing the differences between the two means by the square root of the sum of the squares of the standard errors.

Results

No signs of a chemotherapeutic effect were detected in hamsters infected with *S. mansoni* when treatment with cyanine dye was deferred until seven weeks after infection. Characteristic ova, indicative of maturity of the worms, were present in the faeces of all animals at that time and persisted in approximately equal and undiminished numbers in both treated and untreated animals. Necropsy performed 24 hours after the last injection revealed no macroscopically visible differences between treated and untreated hamsters in the degree of focal fibrosis of the livers induced by deposition of and reaction to schistosome ova. As shown in Table I, there was no significant difference between the treated and untreated hamsters with respect to the average number of worms recovered per animal from the extrahepatic portal-venous system, or with respect to their distribution therein. Comparison likewise revealed no effect of therapy on the motility and appearance of the worms. In contrast, therapy with the cyanine dye produced a marked depression in the oxygen uptake of the schistosomes. As shown in Table I, the average rate of respiration of the worms removed from treated animals was less than 50% of that of worms removed from controls.

By contrast, when treatment with the cyanine dye was begun at an earlier interval, definite effects on the course of the infection were discernible. Tables II and III illustrate these effects under different experimental conditions.

Table I

THE EFFECT OF DELAYED TREATMENT WITH CYANINE DYE ON *SCHISTOSOMA MANSONI* IN HAMSTERS

Treatment begun seven weeks after cercarial infection when all animals were excreting ova; dose: 5.0 mg./kg. i.p. daily for four to eight doses, then 2.5 mg./kg. daily. No difference was observed between treated and untreated animals with respect to excretion of ova; gross pathological changes in liver of host; motility, distribution, degree of pairing, gross and microscopic appearance of worms

Group	Total Dose mg./kg.	Duration of Therapy (Days)	Necropsy—24 Hr. After Last Dose		
			Days after Infection	No. of Worm Pairs in Portal and Mes. Veins	Qo_2
Treated (11) ..	20–55	4–15	53–64	9±1.0	4.1±0.2
Untreated (12) ..	0	0	53–64	11±1.4	10.0±0.3

Table II

THE EFFECT OF EARLY TREATMENT WITH CYANINE DYE, 3 MG./KG. I.P. DAILY, ON *SCHISTOSOMA MANSONI* IN HAMSTERS

Treatment	Group	Appearance of Ova in Faeces (Days after Infection)	Necropsy				
			Days after Infection	Hepatic Involvement		No. of Worm Pairs in Portal and Mes. Veins	Qo_2
				Ova/mg. Wet Weight	Pathological Changes		
A. From 27th day after cercarial infection	Treated (18)	60±0.4	57–71	4.6±0.5	1+ to 2+	19±0.5	2.9±0.14
	Untreated (16)	50±1.25	41–56	16±1.0	3+ to 4+	31±1.6	8.5±0.35
B. From 9th day after cercarial infection	Treated (22)	58±0.5	52–64	2.7±0.5	1+ (14) 2+ (8)	7±1 portal 6±0.8 mes.	4.5±0.45
	Untreated (13)	47±0.4	54–66	7.8±1.0	2+ (3) 3+ (6) 4+ (4)	6±0.9 portal 11±0.4 mes.	6.7±0.33

In the experiment shown in Table IIA, therapy was begun approximately three weeks before ova appeared in the faeces of a group of untreated animals studied at the same time. The delay in excretion of ova, the decrease in the number of worms recoverable at necropsy, and the lesser degree of hepatic involvement in the treated animals were all highly significant statistically, and all indicate that the dye exerted a definite chemotherapeutic effect. The reason for the earlier necropsy of untreated animals was that their infection had reached a terminal stage, whereas treated animals remained healthy. Had treated animals been necropsied as early as untreated controls, the difference in the degree of infection would probably have been even more apparent. When the livers of five untreated hamsters were minced and suspended in water at 30° C., innumerable miracidia were produced by hatching of viable eggs. The low degree of viability of ova from worms of treated hamsters was illustrated by the fact that only two of eighteen livers examined yielded one miracidium each, and the remainder none. Histological sections of liver stained with haematoxylin-eosin revealed a significantly smaller degree of tissue reaction and tubercle formation around deposited ova in treated, as compared to untreated, animals. The depressant effect of the dye on the oxygen uptake of the worms was of the same order of magnitude as that which occurred in the first experiment (Table I).

TABLE III

THE EFFECT OF A SHORT PERIOD OF THERAPY WITH CYANINE DYE BEFORE MATURATION OF THE WORMS ON THE SUBSEQUENT COURSE OF SCHISTOSOMIASIS IN HAMSTERS

Treatment: 3 mg. of cyanine dye per kg., administered i.p. daily from 30th to 40th day (incl.) after cercarial infection.

Group	Appearance of Ova in Faeces (Days after Infection)	Necropsy				
		Days after Infection	Hepatic Involvement		No. of Worm Pairs in Portal and Mes. Veins	Qo_2
			Ova/mg. of Liver (wet wt.)	Pathological Change		
Treated (28)	49–56	41–56	4.0±0.6	± (5) 1+ (9) 2+ (5) 3+ (9)	51±2.8 24±1.6 portal 27±1.7 mes.	5.8±0.3
Untreated (18)	48–56	41–56	7.2±0.9	1+ (2) 2+ (3) 3+ (9) 4+ (4)	51±3.7 26±1.9 portal 31±2.4 mes.	8.6±0.4

On the basis of these findings it seemed possible that treatment begun at an even earlier interval might exert a curative effect. Therefore, in another experiment (Table IIB) treatment was begun only nine days after infection, i.e. approximately five weeks before ova appeared in the faeces of untreated controls. The treated animals showed a significant delay in the excretion of ova and a significant decrease in the degree of hepatic involvement resulting from egg deposition. A cyanine-induced decrease in the viability of ova was again illustrated by the fact that none of the livers from 22 treated hamsters yielded miracidia, when minced and suspended in water, whereas miracidia appeared rapidly in suspensions from nine of 13 livers in the untreated group. However, none of these differences was greater than those seen in animals whose treatment began on the 27th day after infection. Furthermore, the reduction in the number of parasites recovered at necropsy was much less; it was statistically significant only with respect to the number found in the mesenteric veins, but not in the portal vein. An equally surprising finding was the fact that the cyanine dye in this experimental group produced a smaller, though significant, reduction in the oxygen uptake of the worms. This suggested the possibility that an alternative pathway for metabolism, less susceptible to inhibition by cyanine dyes, had been developed as a result of exposure to this agent at a very early and possibly highly adaptive stage of development of the parasite.

In the experiment shown in Table III, treatment was begun at an intermediate interval after infection, approximately the same as that of the experiment in Table IIA. Therapy was discontinued on the tenth day so that the total dose was within the range of that given at an advanced stage of infection in the first experiment (Table I). Necropsies were begun on the day following the last dose of drug. As shown in Table III, the oxygen consumption of worms from treated animals was significantly depressed. The less marked hepatic involvement in treated animals was indicated by the fact that pathological changes were less advanced and the egg content of the livers was statistically lower than that of livers from the untreated group. The differences in Qo_2 and hepatic egg content were considerably greater when only animals necropsied before ova appeared in the faeces were compared, i.e., animals sacrificed soon after cessation of therapy. This would suggest that worms which had been exposed to the dye recovered considerably at intervals further removed from the therapy period. Statistically, however, the differences were no greater than when all animals were considered, because of the small

number of animals in the early groups. Finally, the interval at which excretion of ova began and the number of worms recovered at necropsy were not affected by treatment.

Microscopic studies failed to reveal any morphological differences between schistosomes removed from treated and untreated animals in the four experiments described.

Discussion

A high rate of aerobic metabolism is essential for some parasitic helminths. For example, the filarial nematode, *Litomosoides carinii*, survives for less than 12 hours in an atmosphere of nitrogen (Ross and Bueding, 1950), and reduction of its rate of respiration by the administration of cyanine dyes to its mammalian host results in the death of these worms (Welch *et al.*, 1947 ; Peters *et al.*, 1949).

S. mansoni is considerably more resistant to anaerobiosis and to inhibition of its oxygen uptake. It has been suggested (Bueding, 1949b) that this difference may be a quantitative rather than a qualitative one ; i.e., that this parasite has a much smaller requirement for respiratory metabolism, but, nevertheless, a very definite and ultimately essential one. Though it can survive *in vitro* in the absence of oxygen for five days, survival under aerobic conditions has been observed for as long as 16 days (Ross and Bueding, 1950). The present study shows that partial inhibition of the respiratory metabolism of *S. mansoni* in hamsters, by treatment of the host with a cyanine dye, had no noticeable effect on the worms when such therapy was of relatively short duration, and was deferred until after maturation of the worms to the egg laying stage (Table I). When therapy of comparable intensity (Table III) was begun shortly before maturation of the parasites, a mild chemotherapeutic effect was reflected in a slight inhibition of egg production. This chemotherapeutic effect was increased greatly when therapy was continued for a much longer period (Table IIA) ; under these circumstances egg production was much reduced and there was also a definite reduction in the number of worms recoverable from the portal venous system of the host. Though the criteria for appraisal of chemotherapeutic activity were not equally applicable to all stages of the infection, the results suggest that dependence on aerobic metabolism is greater just before maturation of the parasite. Finally, when intensive and prolonged therapy was begun at a still earlier stage of development of the parasites (Table IIB), the sensitivity of the worms to the cyanine dye was not as great, from either a chemotherapeutic or a metabolic standpoint, as it was when therapy was begun several weeks later (Tables II and III). It is possible that at the very early stage of development the worms may be capable of developing a pathway of respiratory metabolism which is not susceptible to inhibition by the cyanine dye.

The relatively low dependence of *S. mansoni* on oxidative metabolism indicates that a major portion of the energy derived for survival and reproduction of this organism originates from anaerobic reactions. This might explain its very high rate of glycolysis, which is not affected by oxidative metabolism (Bueding, 1950). It appears likely, therefore, that inhibitors of essential anaerobic reactions would hold forth greater promise as potential chemotherapeutic agents against *S. mansoni* than would compounds, such as the cynanine dyes, which affect its oxidative metabolism.

Summary

1. Inhibition of the oxidative metabolism of *S. mansoni* by administration of a cyanine dye to infected hamsters is not associated with the dramatic chemotherapeutic effects observed in another parasitic worm, the filarial nematode, *L. carinii*.

2. When daily parenteral injections of the dye into infected hamsters are begun two weeks before maturation of *S. mansoni* and are continued until four to six weeks after maturation, a mild though definite chemotherapeutic effect is observed.

3. Shorter periods of therapy, or therapy begun earlier or later in the cause of the infection, were much less effective. This suggests that the dependence of the parasite on oxidative metabolism for survival is of a low order, and varies to some extent with the stage of development of its life cycle.

References

Bueding, E. (1949a). *J. exp. Med.*, **39**, 107.
—— (1949b). *Physiol. Rev.*, **29**, 195.
—— (1950). *J. gen. Physiol.*, **33**, 475.
—— Peters, L., and Welch, A. D. (1947). *Fed. Proc.*, **6**, 313.
Burn, J. H. (1930). *Physiol. Rev.*, **10**, 146.
Peters, L., Bueding, E., Valk, A., Jr., Higashi, A., and Welch, A. D. (1949). *J. Pharmacol.*, **95**, 212.
Ross, O. A., and Bueding, E. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 179.
Welch, A. D., Peters, L., Bueding, E., Valk, A., Jr., and Higashi, A. (1947). *Science*, **105**, 486.