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Some Aspects of Carbohydrate Metabolism in the Cercariae of SCHISTOSOMA MANSONI Sambon, 1907

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SOME ASPECTS OF CARBOHYDRATE METABOLISM IN THE CERCARIAE OF SCHISTOSOMA MANSONI SAMBON, 1907

by

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Each person whose signature appears below certifies that he has read this thesis and that in his opinion it is adequate, in scope and quality, as a thesis for the degree of Master of Arts.

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INTRODUCTION

The metabolism of larval trematodes has been scarcely studied, and what little information there is gives a confused and somewhat contradictory picture. Large stores of glycogen occur in freshly emerged cercariae of Schistosoma mansoni, and it has been suggested that these serve as an energy reserve for activity (Axmann, 1947). The rate of glycogen utilization and the end products of metabolism are not known. At least a portion of the end products could be lactic acid, since the adults (Bueding, 1949) and probably the intramolluscan larvae of Schistosoma mansoni produce lactic acid. Although the free cercariae are generally sensitive to a lack of oxygen, some are apparently able to survive under anaerobic conditions (Olivier, von Brand, and Mehlman, 1953). This, too, would suggest the use of anaerobic metabolic pathways.

The life span of in vitro cercariae appears to be lengthened when glucose is added to the medium. The life span of the strigeid cercaria, Cotylurus brevis, is extended from a normal 8-10 hours to 30 hours when 0.3% glucose is added (Ginetsinskaya and Dobrovolski, 1963), and the half life of Schistosoma mansoni cercariae is extended from a usual 8-16 hours to 20 hours when held in Tyrode solution containing 0.1% glucose (Schreiber and Schubert, 1948). But cercariae incubated in glucose-C14 produced negative autoradiographs when taken individually, and large samples showed only slight activity (Lewert and Para, 1966).

A knowledge of cercarial metabolism could aid our understanding of the physiological continuity of the larvae between molluscan and mammalian hosts. It could also aid in realizing the goal of complete in vitro cultivation of all stages of the life cycle. As a contribution to this end, it is the purpose of this research to quantitatively measure the rate of glycogen utilization and related aspects of metabolism of the cercaria of Schistosoma mansoni.

MATERIALS AND METHODS

Maintenance and infection of snails

The snails were a cross between Puerto Rican and Brasilian strains of Australorbis glabratus, and were supplied preinfected by Dr. Henry van der Schalie of the University of Michigan.[1] They were received in our laboratory about one month after infection, were kept in darkness in an incubator at 28 C, and were maintained on romaine lettuce.

Preparation

To obtain cercariae the snails were removed from the incubator, rinsed twice to remove the debris, and placed in the "snail cage." The "snail cage," an 8-oz soft-plastic refrigerator container which had been slotted with a hot soldering iron, was used to transfer the snails more rapidly and retain most of the slime and debris produced during the shedding period. The cage was capped, drained, and transferred to about 400 ml of water (enough to cover the cage) in a clean 600-ml beaker. This unit was covered with foil and placed about 14-16 inches from a 100-watt lamp so that the water would rise from room temperature to 28 or 29 C during the 2 hours allowed for shedding. Aeration was accomplished by passing air from an aquarium pump through a gang valve (for flow-rate control), a length of plastic tubing, and a Pasteur pipette which was changed for each shedding procedure.

After two hours the plastic cage was carefully removed and drained so that the cercariae-laden water remained in the beaker and the debris remained adhering to the mass of slime coating its inside walls. The water containing the cercariae was then poured into a millipore filter unit incorporating a filter of 5.0μ pore size. Gentle suction was applied, and the water level was lowered to about 5-15 ml. Water was added to raise the level to about 50 ml, and it was again lowered to 5-15 ml. This was repeated three more times. The final level was adjusted to about 25 ml, and the suspension of cercariae was carefully poured into a 100-ml graduated cylinder. This procedure reduced the snail waste

products and bacteria, and concentrated the cercariae to a usable volume.

The water which was used in all experimental procedures, except as noted, was snail aquarium water which had been filtered through a millipore filter of 0.45μ pore size, boiled for 30 minutes to help remove lactic acid, and sealed in bottles while still hot.

The concentrated cercariae were diluted to an appropriate volume, and experimental samples were drawn alternately with 1-ml samples used for cercarial counts. To each 1-ml portion in a 60 x 15 mm petri dish was added 1 ml of 66% ethanol.

Glycogen determination

Experimental samples of 10 ml were placed in 12-ml centrifuge tubes. The 4-hour experimental samples were placed about 18 inches from a 100-watt lamp, and air was bubbled through the suspension at the rate of about 1 bubble per second. After the incubation period they were cooled. The 0-hour samples were cooled immediately after they were drawn. Cooling was accomplished by placing them in a constant-temperature chest at 1 C for 45 minutes.

After the cercariae had been inactivated by cooling, each tube was centrifuged for 2-3 minutes at about 3500 rpm. The supernatant fluid was poured into a clean tube and centrifuged again. It was then discarded and the remaining cercariae were transferred to the first tube with 3 5-drop portions of absolute methanol. Glycogen was precipitated by adding one drop of saturated Na_2SO_4 and mixing. The tubes were then covered with foil and stored at 1 C in a constant-temperature chest until analyzed.

The amount of glycogen present in the cercariae was estimated using a slight modification of van Handel's method (1965a, 1965b). Since very small amounts of tissue were used, the separation steps were omitted, as was the procedure of grinding with a glass rod. The tapered tips of the centrifuge tubes were just as efficient in handling small volumes of material as small test tubes. A radiant-wall oven set at 110 C was used to evaporate the methanol and excess water from the

cercarial concentrate. Glycogen standards and process blanks were treated in the same way.

All of the materials (acid, anthrone reagent, experimental tubes, pipettes) used in the anthrone treatment were chilled (Seifter, et al., 1950) in a constant-temperature chest at 1 C. During the processing procedures, the reagent and tubes were kept cold in a water bath at 4-6 C. Rapid mixing of reagent with sample was accomplished with a Vortex Jr. Mixer, and the tubes were covered with Kimax plastic caps.

After exactly 20 minutes in a hot water bath kept at 90 C, the tubes were plunged into cold water at 4-6 C to stop the reaction. Absorbance readings were made on a Spectronic 20 Colorimeter set at 620 mμ.

Methods used to test for glucose utilization

Experimental samples containing 0.012 mg of glucose per ml, 0.2 mg of glucose plus 0.2 mg of trehalose per ml, and 1.0 mg of glucose per ml were used. Preparation and incubation of cercariae were performed as for the glycogen determinations, except that in the latter two cases, sugar solutions (made with bottled tank water) were used in place of plain water to rinse, concentrate, and dilute the cercariae. Fifteen- and 10-ml samples were used respectively. To make the 0.012 mg per ml sugar solutions, 2 ml of glucose solution containing 0.072 mg of glucose per ml were added to each 10-ml sample.

The samples were cooled until the cercariae had settled to the bottom of the tubes. Ten ml of the supernatant fluid were withdrawn from samples containing less than 1.0 mg of glucose per ml and evaporated to dryness on a Calab Rotary Evaporator. The water bath in which the evaporator flask was rotated was 40 C.

After evaporation of the samples, the glucose residue was dissolved in 1 ml of distilled water, and a 0.1-ml fraction was withdrawn for analysis. In the case of the 1.0 mg of glucose per ml samples, a 0.1-ml fraction was withdrawn directly from each cooled experimental tube. Glucose determinations were performed using a slightly modified Glucostat[2] method 1-A (glucose in aqueous solution). To each 0.1-ml

fraction was added 0.4 ml of distilled water and 4.5 ml of reagent. In the preparation of the reagent the final volume was adjusted to 90 ml for samples containing 0.2 or 1.0 mg of glucose per ml, but it was adjusted to 45 ml for samples containing 0.012 mg of glucose per ml. The reaction was allowed to run to completion (30 minutes) before reading the absorbance on a Spectronic 20 Colorimeter set at 400 mμ.

Methods used to test for lactic acid production

Shedding, concentration, and incubation of cercariae were performed as described for the glycogen determinations. The water portion of the cercarial suspension, obtained by filtration through Whatman No. 44 filter paper, was used for lactic acid determinations. The method of Markus (1950) was employed with the following modifications. Deproteinization was accomplished by using 2 ml of the 20% trichloroacetic acid and filtering through Whatman No. 44 filter paper within 5 minutes. To the filtrate were added 3.5 ml of 10% $CuSO_4$, 30% NaOH to neutralize (10-12 drops), and 6.0 ml of 10% lime suspension. After 4 hours of standing desugarization is complete, but 24 hours were allowed. Decationization using Amberlite IR-120[3] was used in preliminary studies but was found to be unnecessary if extra care was taken not to contaminate the samples with lactic acid. Color was developed by using the method of Markus (1950) except that a 6-ml Hycel volumetric dispenser was used to dispense the H_2SO_4 instead of the apparatus suggested. A Spectronic 20 Colorimeter set at 570 mμ was used to read the percentage transmittance.

Respiration determination

Oxygen uptake and carbon dioxide evolution were measured with a Warburg constant volume respirometer. Standard manometric techniques were employed (Umbreit, Burris, and Stauffer, 1964). Shedding and concentration of cercariae were performed as described for the glycogen determinations, and 5-ml cercarial portions were used. Carbon dioxide absorption was accomplished by using 0.1 ml of 10% KOH in the center well, and buffer retention was corrected for by using 0.5 ml of 4% H_2SO_4 in the side-arm. When the acid was tipped into the cercarial

suspension, the resulting pH was about 2. The water bath temperature was 27.7 C.

Bacterial counts were made on 0.05-ml samples that had been heat-fixed, dried, and stained in crystal violet for 45 seconds.

Care of glassware

All glassware was washed in strong (60 grams per gallon of water) Alconox solution, rinsed in tap water, rinsed 4 times in distilled water, and dried in an oven at 110-130 C. Special care was employed in handling glassware for the lactic acid determinations by using washed rubber gloves. However, since rubber affects the Glucostat system, rubber gloves were not used to handle glassware intended for glucose determinations. Plastic and glass tubing were used for rinse-bottle connections.

RESULTS

Glycogen utilization

The average amount of glycogen utilized by a single cercaria in 4 hours is determined as 43.2×10^{-4} µg $\pm$ 6.6×10^{-4} µg (Table I). Although the mean of the first 6 determinations is 38.6×10^{-4} µg $\pm$ 9.9×10^{-4} µg of glycogen/cercaria/4 hours (Table II), the last set of 12 determinations are considered valid for cercariae shed during the first or prime shedding weeks of a snail. The first six determinations were made using cercariae which had been obtained from the remnant (10-15 snails) of a group of 50 snails which had been regularly stimulated to shed for a period of about 2 months. The last 12 determinations were made using cercariae from 2 separate groups of snails which had been shedding for only 2 weeks.

Glucose utilization

Glucose was not observed to have been utilized (Table III). It has been reported (Chernin, 1964) that the emergence of cercariae from *in vitro* snail tissue is elevated when both glucose and trehalose are added to the basic salt solution. But when trehalose was added to glucose solution in an attempt to effect glucose utilization, negative results were again encountered.

Lactic acid production

No lactic acid was observed to have been produced, even when 30,000 cercariae were incubated in a 10-ml sample (Table IV). Five- and 10-µg amounts of lactic acid added to experimental samples were recovered during the determinations. The results indicate that lactic acid is not excreted in an amount in excess of about 5×10^{-4} µg/cercaria/4 hours.

Respiration

The average amount of oxygen utilized by a single cercaria in 4 hours is determined as 29.0×10^{-4} µl $\pm$ 3.4×10^{-4} µl (Table V, Fig. 1).

The average amount of carbon dioxide produced by a single cercaria in 4 hours is determined as 34.6×10^{-4} µl $\pm$ 4.6×10^{-4} µl (Table V). These figures give a respiratory quotient of 1.19 ± 0.08.

Bacterial counts indicated that the increase in bacterial numbers between 0 and 4 hours was negligible; but between 0 and 21 hours there was over a thousand-fold increase. Estimates of bacterial volumes were $2.18 \times 10^4 \mu^3$ at 0 hours, $2.16 \times 10^4 \mu^3$ after 4 hours, and $2,252 \times 10^4 \mu^3$ after 21 hours. Cercarial volume was estimated to average $1,004.17 \times 10^4 \mu^3$. The ratio of bacterial volume to cercarial volume at both 0 and 4 hours would be 0.002:1. The ratio would be 2.24:1 after 21 hours.

Table I. Glycogen Utilization of Cercariae From Recently Infected Snails

ABSORBANCE 620 mμ					μg OF GLYCOGEN					
Experiment Number	40 μg Std.	Exp. 0 Hr	Exp. 4 Hr	No. of Cercariae	Total 0 Hr	Total 4 Hr	Total/ Cercaria 0 Hr	Total/ Cercaria 4 Hr	Total Diff.	Per Cercaria Diff.
7	0.63	1.30	0.76	6270	82.40	48.40	0.01314	0.00772	34.00	0.00542
8		1.10	0.66	6290	70.00	42.00	0.01113	0.00668	28.00	0.00445
9		1.00	0.72	4410	63.60	45.60	0.01442	0.01034	18.00	0.00408
10		0.89	0.62	3840	56.40	39.20	0.01469	0.01021	17.20	0.00448
11		0.74	0.64	3740	47.20	40.80	0.01262	0.01091	6.40	0.00171
12		0.77	0.46	3850	48.80	29.20	0.01268	0.00758	19.60	0.00509
13	0.62	0.46	0.26	3030	29.60	16.80	0.00977	0.00554	12.80	0.00422
14		0.20	0.12	980	12.80	7.60	0.00306	0.00776	5.20	0.00530
15		0.40	0.285	1700	25.60	21.40	0.01505	0.01082	4.20	0.00424
16		0.27	0.21	1330	17.60	13.60	0.01321	0.01023	4.00	0.00301
17		0.51	0.35	2160	32.80	22.40	0.01518	0.01037	10.40	0.00482
18		0.34	0.26	1370	22.00	16.80	0.01606	0.01226	5.20	0.00380
						TOTAL				0.05185
						MEAN				0.00432
						95% CONFIDENCE INTERVAL				±0.00066

Table II. Glycogen Utilization of Cercariae from Snails of Long-Standing Infection

ABSORBANCE 620 mμ					μg OF GLYCOGEN					
Experiment Number	40 μg Std.	Exp 0 Hr	Exp 4 Hr	No. of Cercariae	Total 0 Hr	Total 4 Hr	Total/ Cercaria 0 Hr	Total/ Cercaria 4 Hr	Total Diff.	Per Cercaria Diff.
1	0.62	0.67	0.47	4740	43.20	30.40	0.00911	0.00641	12.80	0.00170
2		0.62	0.40	3140	40.00	25.60	0.01274	0.00815	14.40	0.00459
3	0.59	0.42	0.30	3320	28.40	20.40	0.00855	0.00614	8.00	0.00241
4		0.40	0.305	2900	27.20	20.80	0.00938	0.00717	6.40	0.00221
5	0.60	0.395	0.28	2000	26.40	18.80	0.01210	0.00940	7.60	0.00380
6	0.655	0.45	0.29	3180	27.60	17.60	0.00858	0.00554	10.00	0.00314
						TOTAL				0.006947
						MEAN				0.00386
						95% CONFIDENCE INTERVAL				±0.00099

Table III. Glucose Utilization

ABSORBANCE

	Exp. No.	0 Hour	4 Hour	.1 mg/ml standard*
0.012 mg/ml GLUCOSE	I	0.24	0.23	0.20
	II	0.24	0.25	0.20
0.2 mg/ml GLUCOSE + TREHALOSE	I	0.14	0.14	0.07
	II	0.135	0.14	0.07
1.0 mg/ml GLUCOSE	I	0.69	0.70	0.07
	II	0.70	0.69	0.07
	III	—	0.68	0.07

*Differences in absorbance readings are due to variations in color of the Glucostat reagent. This does not affect the analysis.

Table IV. Lactic Acid Production

% TRANSMITTANCE

Experiment Number	0 Hours	4 Hours	Bottled Tank Water
1	100	100	
2	100	100	
3	100	100	100
4	100	99	
5	100	100	
6	100	100	
10 μg lactic acid added	93	93	
5 μg lactic acid added	97	97	100
10 μg standard	93	93	
5 μg standard	97	97	

Table V. Respiration of Cercariae

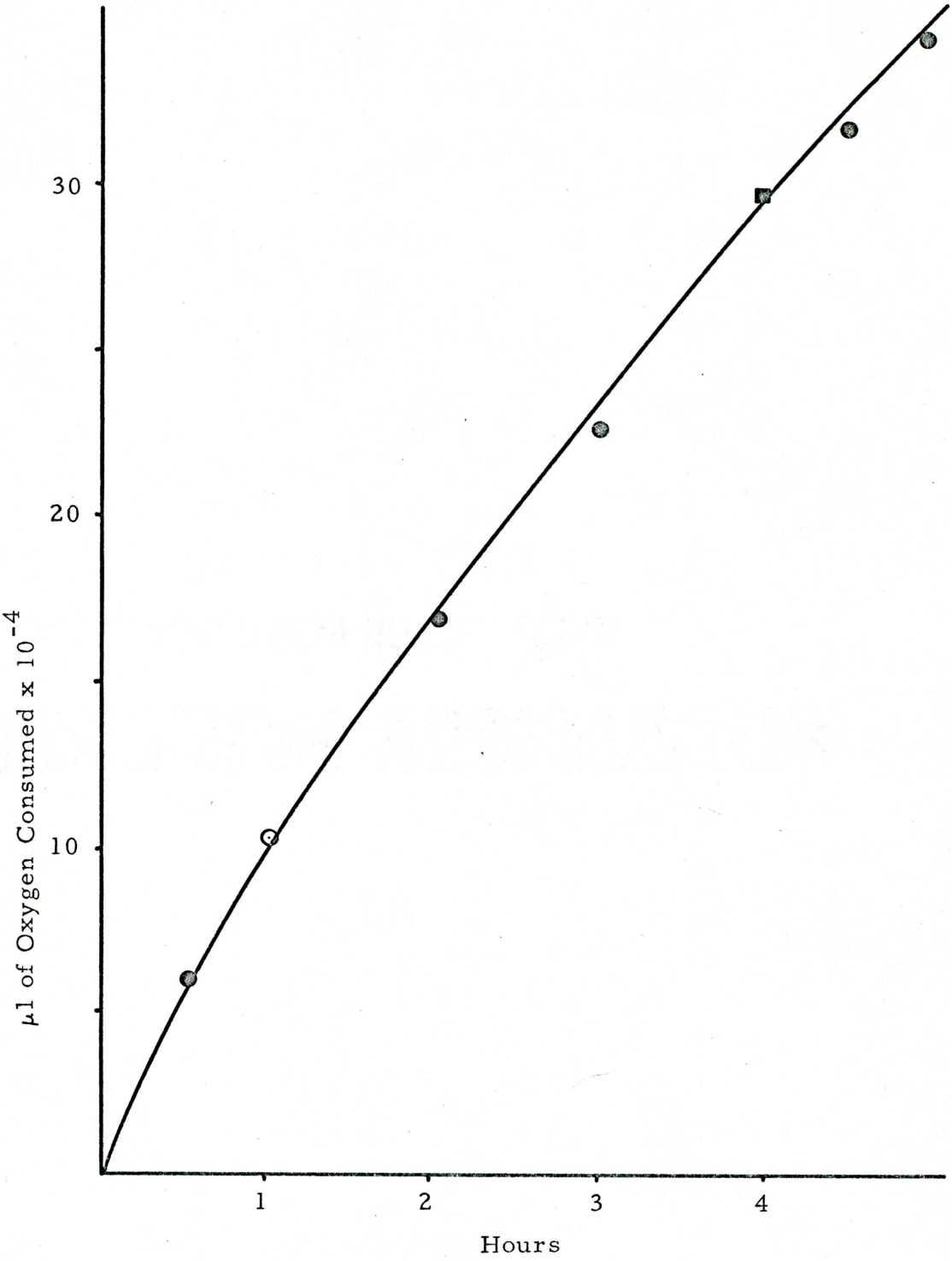
Exp. No.	No. of Cercariae	Q_{O_2} x 10^{-4}/Cercaria										Q_{CO_2} x 10^{-4}/Cercaria
		Hours of Incubation										Hours of Incubation
		0.5	1.0	1.5	2.0	3.0	4.0	4.5	5.0	6.0	21.0	4.0
1	10,865	4.5	8.5	13.0	16.4	20.9	29.2	31.8	35.2	-	188.0	34.6
2	12,260	4.3	7.7	10.3	12.2	17.0	22.8	23.9	26.3	30.6	142.4	29.5
3	4,550	6.6	11.2	15.2	19.6	26.6	35.4	37.6	41.5	48.1	-	44.0
4	10,000	6.5	11.0	-	17.5	23.8	27.6	30.7	32.6	36.4	97.3	33.3
5	6,996	-	9.3	-	-	-	31.2	-	-	-	-	37.3
6	10,865	-	-	-	-	-	27.8	-	-	-	-	28.0
	Mean						29.0					34.4

Figure 1. Time Course of Oxygen Consumption

● - Average of 4 Readings

⊙ - Average of 5 Readings

■ - Average of 6 Readings


30
20
10
μl of Oxygen Consumed x 10^{-4}
1
2
3
4
Hours

DISCUSSION

These studies indicate that glycogen is the main energy supply for cercarial activity and that carbon dioxide is the main end product of metabolism. A number of inherent sources of error must be considered. In the first place, individual cercariae are not being studied, but, rather, large numbers. What variations may exist between cercariae in metabolism, glycogen supply, and general physiology can only be guessed. It has been suggested that variations in snail physiology may be a factor in the degree of cercarial infectivity for the mammalian host (Evans and Stirewalt, 1952). If this is true, the overall physiology of any given cercarial population could vary according to which snails happen to be shedding on that particular day. In addition to the physiological differences between snails, there is the factor of decreased polysaccharide availability in infected snails (von Brand and Files, 1947). During the early stages of infection, the snail can compensate for its polysaccharide losses by increasing its food intake. But as the course of infection progresses, it appears that either the polysaccharide levels are being depleted faster than the snail can replace them or damage due to the parasite's presence causes the snail to lower its food intake. Infected snails which are near death are frequently observed to rest motionless on the walls of the aquarium for several days before death actually occurs, and the glycogen levels of cercariae from such snails appear to be lower. All of the snails used in determinations 1-6 were dead within a week of the last determination. It is hoped that by using large numbers of snails which had just begun to shed, many of these variable factors were compensated for.

Another, but less critical problem in using large numbers of cercariae is estimating the numbers present in a sample and keeping those numbers constant in each sample taken. Cercariae tend to aggregate if not adequately mixed. But even with adequate mixing, significant differences may exist between samples on the basis of chance alone.

Using such small volumes as those employed increases the chance of error. The slightest variation in technique can alter the outcome of a determination. In an attempt to eliminate such variations, samples were handled as little as possible. In the glycogen determinations, for instance, the cercariae were retained in the same centrifuge tube from the time the sample was taken until the time the readings were made on the colorimeter. Only after the color had been completely developed was the liquid finally transferred to a colorimeter tube for reading. Process standards and blanks were also used as control measures against variations in technique. The elimination of the cation exchange column in the lactic acid determinations removed one of the prime areas of inherent error. Several experimental samples needed to be combined in order to produce a volume of fluid great enough to run through the column. Although the column may be recommended for greater quantities of material, the samples used in these experiments were too small to be handled in this manner. The elimination of the column was justified by performing biuret tests on fractions of the deproteinized-desugarized lactic acid samples. Proteins created no problem, for all of the tests were negative.

Glycogen estimation is not affected by low values of amino acids, purine bases, and proteins (Seifter, et al., 1950). Since the glycogen-to-protein ratio was high and the total amount of protein was low, little or no interference would be expected.

The effect of glucose on cercarial longevity is at present somewhat obscure. It has been shown that glycogen reserves are not replaced in Cotylurus brevis by the addition of glucose; only the life span is lengthened (Ginetsinskaya and Dobrovolski, 1963). Apparently the same principle applies in the cercariae of S. mansoni. They do not give positive autoradiographs after having been placed in a glucose-C14 solution for 4 hours (Lewert and Para, 1966), but they survive for a longer period of time in Tyrode solution containing 0.1% glucose than they do in Tyrode solution alone (Schreiber and Schubert, 1947). Since cercariae do not appear to use glucose for metabolic purposes, this may represent an osmotic effect. Although cercariae survive for a

longer period of time in Tyrode-sugar solution than they do in Tyrode solution alone, they also live longer in Tyrode solution than they do in spring water. The use of sugar to maintain osmotic balance during the process of mitochondrial extraction is a well-known technique. The effect of glucose could be that of slowing down the process of active transport of water in the cercaria, thus leaving more energy reserve for other life processes.

Pijoan, Kozloff, and Kuntz (1945) reported that during an incubation period of 22 hours cercariae of S. mansoni consumed an average of 41 μl of oxygen. If the cercariae were incubated in 0.1% glucose solution for the same length of time, an average of 90 μl of oxygen was consumed. However, the period of rapid oxygen consumption (the sigmoid portion of the respiratory curve) fell within the half-life period (8-16 hours) of a cercaria (Schreiber and Schubert, 1947), and the control flasks did not contain dead cercariae. Because of the effect that dead and dying cercariae have on bacterial growth, these data are not conclusive. Since senescent cercariae in a bacteria-rich environment are probably not a very reliable source of information, the experiments described in this study were confined to the first 5 or 6 hours of cercarial activity.

When oxygen uptake was correlated with carbon dioxide output, it was found that a respiratory quotient of greater than 1 was obtained. On the basis of glucose being the substrate for metabolism, one would expect a respiratory quotient of about 1. The value of greater than 1 could suggest the use of some Krebscycle intermediate such as succinate as a substrate.

Kloetzel (1967) has demonstrated that cercariae are quite resistant to mechanical stress. Cercarial damage during the processes of rinsing and concentration probably amounts to less than 1%.

Cooling the glycogen samples and the anthrone reagent during addition and mixing procedures was found to be imperative. Erratic results were obtained from standard solutions when this precaution was not adhered to.

There seems to be little doubt that cercariae of S. mansoni are aerobic in their metabolism, but it still remains a curious point that a few of them are able to get along quite well under totally anaerobic conditions (Olivier, et al., 1953). This investigation showed similar results when cercariae were placed in an atmosphere of cyanide gas. Most died, but several continued to wriggle slowly for more than 4 hours after a 30-minute exposure to the gas. The same results were obtained when KCN was added directly to the water. The passage of a cercaria from the snail host to the mammalian host probably involves a double triggering mechanism——one which adapts the cercaria from a tissue stage to a free, water stage and another which adapts it back to a tissue stage. It is therefore possible that the free cercaria has latent capabilities for anaerobic metabolism. Perhaps certain situations of stress help to trigger anaerobic metabolism in some of the cercariae. Despite these speculations, lactic dehydrogenase is found in the adult worms, but not in the cercariae (Conde-del Pino, et al., 1966).

FOOTNOTES

[1]U. S. Panel on Parasitic Diseases of the U. S.-Japan Cooperative Medical Science Program, Contract No. PH 43-67-736, National Institute of Allergy and Infectious Diseases, N.I.H., U. S. Public Health Service.

[2]Worthington Biochemical Corporation, Freehold, New Jersey. Glucostat Special was used.

[3]The Amberlite IR-120 was supplied courtesy of the Rohm and Haas Company, Philadelphia. This product replaces the former Amberlite IR-100.

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SOME ASPECTS OF CARBOHYDRATE METABOLISM IN THE CERCARIAE OF SCHISTOSOMA MANSONI SAMBON, 1907

by

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ABSTRACT

Cercariae of Schistosoma mansoni were studied for glycogen utilization, uptake of glucose from solution, lactic acid production, and respiration.

Using the anthrone method of determination of carbohydrate, the amount of glycogen used by a single cercaria in 4 hours is determined as 43.2×10^{-4} µg $\pm$ 6.6×10^{-4} µg.

Glucose was not observed to have been used from solution under the conditions of the experiment, even in the presence of trehalose. The determinations were made by the Glucostat method.

After deproteinization and desugarization, aliquots of supernatant from cercarial suspensions were analyzed for lactic acid using p-phenylphenol and sulfuric acid. The production of significant amounts of lactic acid by the cercariae was not observed.

Respiration was measured on a Warburg constant volume respirometer. The amount of oxygen used by a single cercaria in 4 hours is determined as 29.0×10^{-4} µl $\pm$ 3.4×10^{-4} µl. The amount of carbon dioxide produced by a single cercaria in 4 hours is determined as 34.6×10^{-4} µl $\pm$ 4.6×10^{-4} µl. A respiratory quotient of 1.19 $\pm$ 0.08 was obtained.