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Carbon Dioxide Fixation in Schistosoma Mansoni

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CARBON DIOXIDE FIXATION IN SCHISTOSOMA MANSONI

by

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Master of Arts in the Field of Biology

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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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Dedicated to my Wife, Patty,

who has contributed so much to my education.

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INTRODUCTION

Carbon dioxide fixation has been found to be important in carbohydrate metabolism in many helminths (von Brand, 1966). Bueding et al. (1961) indicated that twenty-five percent of the carbohydrate utilized by Trichuris vulpis might involve metabolic pathways that include carbon dioxide fixation. Glucose utilization in Hymenolepis diminuta is markedly stimulated by the presence of carbon dioxide (Fairbairn et al. 1961). Fairbairn (1954) noted a large amount of carbon dioxide fixation into propionic and succinic acid in the nematode Hetarakis gallinae. To the writer's knowledge no information has been published on the role of carbon dioxide in the metabolism of Schistosoma mansoni, a blood fluke which spends its adult life in the hepatic portal system of the mammalian host. This habitat is rich in carbon dioxide, glucose, and free amino acids. These experiments were performed to shed some light on the part that carbon dioxide may play in schistosome metabolism by comparing the relative significance of carbon dioxide in relation to the utilization of glucose and aspartate. Glucose was used in the experiments because it has been well established as an important compound in the metabolism of S. mansoni (Bueding, 1950). Aspartic acid-4-C^{14} was used in the experiment to compare results of glucose and carbon dioxide with an amino acid. The use of aspartic acid would also give some indication of the feasibility of comparing the efficiency of utilization of this free amino acid with aspartic acid obtained through carbon dioxide-

C^{14} fixation onto pyruvate, which would thus be also labelled in the number four position. This pathway has been demonstrated by Hammen and Lum (1962) as the main pathway for some flatworms.

MATERIALS AND METHODS

CF1 female mice infected with S. mansoni were obtained from Dr. van der Schalie of the University of Michigan. Adult S. mansoni were perfused from these mice 60-70 days post infection using warm beef serum under pressure as described by Roth and Heidtke (1966).

Experiments comparing the relative value of the carbon sources were conducted in vitro. Radiochemicals serving as carbon sources were d-glucose-UL-C^{14}, S. A. 216 mc/mM; aspartate-4C^{14}, S. A. 3.16mc/mM; sodium bicarbonate-C^{14}, S. A. 48 mc/mM; obtained from International Chemical and Nuclear Corporation, City of Industry, California. The sodium bicarbonate served as a source for carbon dioxide. The incubation medium was Eagle's minimal essential medium (Eagle, 1959) with glutamate and 0.03 mg/100 ml nonlabelled aspartate. The aspartate was added so as to more nearly approximate the conditions in man, one of the worm's natural hosts. A total of 26 samples was used. Each sample contained 20 pairs of S. mansoni in 1.9 ml of incubation medium and 10 μc of one of the labelled compounds suspended in 0.01 ml of water. For each radiochemical source employed, five to seven experimental and three control samples were used. The worms serving as controls were killed with acetone prior to the addition of the labelled compounds and incubation. All samples were incubated at 37.5°C for two hours in a Warburg respirometer after which metabolism was stopped rapidly by the addition of 2 ml of cold acetone. The incubation medium was then decanted and the worms were

washed three times; each wash consisting of 2 ml of water. The worms were then homogenized in an all glass tissue grinder with 5 ml of water and centrifuged. The water soluble phase was pipetted off. The precipitate was washed three times with ether (2 ml). The ether soluble fraction was then removed and the remaining precipitate was washed in hot tri-chloroacetic acid (2 ml). The T.C.A. fraction was removed and the remaining precipitate was washed three times with 80% ethanol (2 ml) and decanted, leaving the protein precipitate. The supernatants resulting from the washings were placed directly into glass liquid scintillation counting vials and the amount of radioactivity present was determined. Bray's solution (Bray, 1960) was employed as the scintillation fluid for all the liquid phases. The protein phase was pipetted onto dry Cab-O-Sil obtained from Packard Instruments Company, Inc. in a counting vial and the moisture removed by heating in an oven at 200°C for three hours. To the dried material toluene with scintillators was added in sufficient quantities to make a 3-5% concentration of Cab-O-Sil (Ott et al. 1959).

Counting efficiency of each sample was determined by the channel ratio method (Bush, 1964) and all counts were adjusted to 100% efficiency.

A *t* test comparing the means of the experimental with control groups based on the number of disintegrations per minute was performed on the chemical fractions separated from worms incubated in each of the three radiochemicals. Due to the large differences in the specific activity of the radiochemicals used, the disintegration rate of each fraction was converted to micromoles of material for comparative purposes.

RESULTS

Carbon-14 from sodium bicarbonate, glucose and aspartate was recovered in each of the five chemical fractions separated from *S. mansoni*. These fractions are: (1) the water soluble, mostly soluble sugars, free amino acids and glycogen; (2) the ether soluble, mostly lipids; (3) the tri-chloroacetic acid soluble, mostly nucleic acids; (4) the ethanol soluble, nature unknown; (5) the remaining residue, mostly protein.

Results are reported in Table I to IV. The greatest amount of assimilation of both the aspartate and sodium bicarbonate was found to be in the protein fraction. The least amount of radioactivity recorded from worms incubated in aspartic acid or sodium bicarbonate was in the ether soluble fraction. No significant differences between the experimental and control samples were found in the ethanol soluble material from these worms.

Worms incubated in labelled glucose showed the greatest amount of radioactivity in the water soluble fraction. About one-third the amount of radioactivity recovered from the water soluble fraction was found in the protein fraction. The ethanol soluble material showed highly significant differences between experimental and control samples from these worms (P 0.0037).

Table IV gives t values and levels of significance for comparisons between control and experimental groups. Comparisons are made for each fraction separated from worms incubated with each of the three different

labelled sources of carbon. The protein fractions gave the highest level of significance in each case.

Comparisons of results between the various substrates used as carbon sources should be done with caution. The figures give some basis for comparison but probably do not represent comparable quantitative values and under the experimental conditions employed may not represent a normal pattern. Factors which may alter the quantitative significance are: incomplete separation of fractions, variables in liquid scintillation counting techniques, and changes in the medium during the course of the experiments.

Table I. Disintegrations per minute recorded from various fractions after two hours incubation for experimental group.

	Water Soluble	Ethanol Soluble	Ether Soluble	T.C.A. Soluble	Protein
Glucose	53051.10	1029.63	2403.35	987.92	10165.54
	20001.00	1364.09	2350.85	1329.95	12485.36
	35591.39	2044.32	2160.01	6364.80	12578.15
	29680.02	1103.40	1198.15	4651.10	12848.22
	30235.95	2100.63	2688.77	6243.38	10028.40
Aspartate	2335.87	59.83	162.37	212.52	5233.96
	1474.53	106.42	229.54	63.98	4794.63
	2152.87	500.68	227.27	244.80	5831.31
	1203.53	373.54	42.17	110.16	4475.15
	1462.93	562.57	81.67	146.88	3977.59
	1761.37	118.76	66.75	157.23	4969.00
	2158.01	138.59	116.33	265.38	4627.25
Sodium Bicarbonate	150.67	285.00	18.67	24.48	512.28
	138.89	180.24	8.78	293.70	443.48
	143.94	47.10	12.81	24.82	574.78
	193.91	59.90	13.55	27.29	512.01
	242.76	30.92	23.33	25.96	690.09
	127.26	34.59	22.66	59.82	518.16
	177.62	29.45	15.50	42.18	417.53

Table II. Disintegrations per minute recorded from various fractions after two hours incubation for control group.

	Water Soluble	Ethanol Soluble	Ether Soluble	T.C.A. Soluble	Protein
Glucose	1234.54	31.18	16.65	74.28	124.12
	951.08	46.40	19.41	120.06	78.30
	1258.18	199.95	49.55	42.95	349.34
Aspartate	129.52	29.97	11.55	122.24	64.79
	190.58	582.65	8.44	21.30	152.80
	183.00	50.40	14.55	52.92	31.60
Sodium Bicarbonate	30.31	8.68	6.68	11.02	5.83
	78.33	203.64	4.97	0.00	15.35
	42.67	46.85	4.93	19.94	12.65

Table III. Equivalent amounts of labelled compounds in micromoles recovered from various fractions after two hours incubation.

Fraction	Glucose	Aspartate	Sodium Bicarbonate
Water soluble	15572.0×10^{-6}	2.31×10^{-6}	417.4×10^{-6}
Ethanol soluble	568.1×10^{-6}	0.63×10^{-6}	31.6×10^{-6}
Ether soluble	62.7×10^{-6}	0.17×10^{-6}	38.9×10^{-6}
T.C.A. soluble	1783.9×10^{-6}	0.15×10^{-6}	215.0×10^{-6}
Protein	5450.2×10^{-6}	6.8×10^{-6}	1836.7×10^{-6}
Total label recovered	0.024369	0.000010	0.0025396
Total substrate available per flask	10.60	3.17	52.61

Table IV. Degrees of freedom, t value, and level of significance for each of the fractions recovered from worms incubated for two hours in each of the labelled sources of carbon as compared to controls.

Source of carbon and fractions	Degrees of freedom	t value	Level of significance
Glucose Source			
Water soluble	4	5.974	0.00394
Ethanol soluble	4	6.100	0.00365
Ether soluble	4	8.352	0.00112
TCA soluble	4	3.288	0.03026
Protein	4	18.124	0.00005
Bicarbonate Source			
Water soluble	9	5.603	0.00033
Ethanol soluble	5	0.126	0.90411
Ether soluble	7	5.198	0.00125
TCA soluble	6	1.607	0.15902
Protein	6	15.068	0.00000
Aspartate Source			
Water soluble	6	9.899	0.00006
Ethanol soluble	3	0.226	0.83511
Ether soluble	6	4.199	0.00569
TCA soluble	8	2.611	0.03107
Protein	6	21.124	0.00000

DISCUSSION AND CONCLUSIONS

Under the experimental conditions employed, carbon dioxide fixation is a definite factor in the metabolism of S. mansoni. Its significance remains to be determined. Carbon-14 from CO_2 was recovered in the protein fraction in larger quantities than in any other fraction. Flickinger (1959) demonstrated carbon dioxide fixation into the protein fraction of the planarian, Dugesia tigrina. Hammen and Lum (1962) demonstrated $C^{14}O_2$ fixation into glutamic acid, aspartic acid, and threonine. These authors suggested that the protein fraction in Flickinger's investigations may have originated from these amino acids which could have been synthesized as a result of CO_2 fixation onto pyruvate followed by a transamination of oxaloacetic acid and α-ketoglutaric acid. The tracer recovered in the proteins of S. mansoni incubated in $NaHC^{14}O_3$ could have originated in the same way.

A quantitative determination of the labelled amino acids will be necessary before this idea can be supported. Only a small amount of activity was recorded from the water soluble fraction of S. mansoni using $NaHC^{14}O_3$ as the label. This activity could be a result of the presence of labelled free amino acids, possibly aspartic and glutamic acid.

Glucose was incorporated much more rapidly than either aspartate or bicarbonate into both the water soluble fraction and the protein fraction. Male schistosomes have a large gynecophoric canal in which lie the females. It is quite possible that the activity recorded in the

water soluble fraction may be partially due to unused label trapped in this canal. If this were the case, the activity recorded from the water soluble fraction, using sodium bicarbonate, aspartate or glucose as the label, could be partially due to this contamination. Part of the activity recovered from this fraction is undoubtedly due to free labelled substrate taken up by the worms and not utilized by the time of killing.

Sneft (1965) suggested that aspartate is possibly used in schistosome metabolism *in vitro*, although his data were not conclusive. My data indicate that aspartic acid is definitely incorporated by *S. mansoni* into the proteins in significant quantities. This is evident by the consistency in the amount of tracer recovered in this fraction. These data are consistent with the findings of Roth (unpublished data) that *S. mansoni* utilizes large quantities of aspartate *in vitro*.

Glucose was incorporated into all fractions separated. Bueding (1950) demonstrated that glucose was utilized by *S. mansoni* at a rate equivalent to 15-26% of the dry weight per hour and that from 81-91% of the glucose taken up is converted to lactic acid indicating that the amounts recovered in the present experiments represent a small fraction of the actual amounts absorbed.

It is interesting to note that most organisms which fix CO_2 usually have succinate or propionate as the major end product of fermentation. Kalnitsky et al. (1943) have shown that *Escherichia coli* fixes CO_2 into pyruvate and produces succinate. Similarly, the results of Bowmen et al. (1963) indicate that succinate production is completely dependent on CO_2 fixation onto pyruvate in *Trypanosoma cruzi* under anaerobic conditions.

Saz and Vidrine (1959) demonstrated CO_2 fixation onto pyruvate to form succinate, which is then decarboxylated to form propionate in muscle strips of Ascaris lumbricoides. Succinic acid had already been shown to be a major end product in Ascaris lumbricoides by Bueding and Farrow (1956). Hammen and Lum (1962) discovered that free-living flatworms fix CO_2 into pyruvate to form malic and succinic acid. Heterakis gallinae fixes $C^{14}O_2$ with most of the label being recovered in propionic and succinic acid (Fairbairn, 1954).

Various pathways for CO_2 fixation exist. Hammen and Wilbur (1959), showed that the main pathway in the oyster is propionate to succinate. Two pathways frequently described in the literature are carboxylation of pyruvate or phosphoenolpyruvate forming either malic or oxaloacetic acid.

In contrast to those animals that fix CO_2 and form succinic and propionic acids, S. mansoni, which also fixes CO_2, produces lactic acid as its main end product of metabolism (Bueding, 1950). Trichomonas vaginalis fixes carbon dioxide and has been found to incorporate the C^{14} label into the carboxyl group of lactic acid which is a major end product produced by that organism (Kupferberg et al., 1953 and Wellerson et al., 1960). The latter authors suggest another pathway of CO_2 fixation which is carboxylation of ribose diphosphate producing 3 phosphoglyceric acid which would result in a carboxyl labelled lactic acid when metabolism is accomplished through glycolysis. A similar pathway might be present in S. mansoni. Further investigation is needed to determine this.

Bueding (1962) proposed a pathway for CO_2 fixation which explains its value to anaerobic animals. It consists of fumaric acid acting as an electron and hydrogen acceptor in the reoxidation of reduced NADH produced in glycolysis from oxidation of 3 phosphoglyceraldehyde. This scheme would be particularly useful in anaerobic organisms that have succinate as the major end product of fermentation. Since S. mansoni produces large quantities of lactic acid it does not appear to have any problem of reoxidizing reduced NADH from glycolysis. Thus, the importance of CO_2 fixation in this organism remains an enigma. Bueding et al. (1961) have shown that the presence of CO_2 significantly enhances the rate of glucose uptake and the survival time of Trichuris vulpis in vitro. Carbon dioxide fixation may be important here. A. J. MacInnis, UCLA, (personal communication) indicates that his preliminary investigations on S. mansoni show that the absence of CO_2 in the medium decreases the rate of glycogen deposition. This suggests a useful function for CO_2 fixation in this organism.

SUMMARY

Many parasitic protozoans, cestodes, and nematodes have been found to fix carbon dioxide. To the author's knowledge no information has been published on carbon dioxide fixation in a parasitic trematode. This study was made to determine the significance of carbon dioxide as a carbon source in S. mansoni. Two compounds, glucose and aspartate, which are known to have physiological significance in S. mansoni, were used for a comparison. Similarities in the utilization of carbon dioxide and aspartate were noticed. The label from both of these compounds was recovered in the largest quantity in the proteins while the least amount of label from both was recovered in an ether soluble fraction. No significant differences between the experimental and control samples were noted with either of these compounds in the ethanol soluble fraction. These similarities may indicate similar pathways for metabolism.

Carbon dioxide was found to be less important as a carbon source than glucose in S. mansoni under the experimental conditions employed. Further studies will have to be performed to determine the pathway of carbon dioxide in this parasite.

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ABSTRACT

Adults of the blood fluke Schistosoma mansoni were perfused from the hepatic portal system of CF1 female mice sixty to seventy days post infection. The worms were then incubated in Eagle's minimal essential medium with glutamate and 0.03 mg/100 ml of nonlabelled aspartic acid. To this medium was added 10 μc of either $NaHC^{14}O_3$, glucose-UC^{14} or aspartate-4-C^{14}. The worms were found to fix carbon from the $NaHC^{14}O_3$ and to incorporate the glucose and aspartate. The amount of activity recovered in five different chemical fractions after two hours incubation was determined for each radiochemical used and compared to determine the relative significance of carbon dioxide in Schistosoma mansoni metabolism.

All five fractions which were separated from worms incubated in glucose were significantly different from the control samples. The worms incubated in aspartate and sodium bicarbonate incorporated these compounds into every fraction except the ethanol fraction. Worms incubated in both aspartate and sodium bicarbonate had the greatest amount of incorporation of label in the protein fraction with the least amount in the ethanol soluble fraction.

Under the experimental conditions employed glucose appears to be the most important source of carbon when compared with either aspartate or bicarbonate.