71-27,636

PAPPAS, Peter William, 1944-
UPTAKE, LOCALIZATION, AND FATE OF TRITIATED ARGININE IN HAEMATOLOECHUS MEDIOPLEXUS (TREMATODA).

The University of Oklahoma, Ph.D., 1971
Biochemistry

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

UPTAKE, LOCALIZATION, AND FATE OF TRITIATED ARGININE IN *HAEMATOLOECHUS MEDIOPLEXUS* (TREMATODA)

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Norman, Oklahoma

1971

UPTAKE, LOCALIZATION, AND FATE OF TRITIATED ARGININE IN *HAEMATOLOECHUS MEDIOPLEXUS* (TREMATODA)

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

Without the help of many people, the completion of this investigation would not have been possible. To Dr. J. Teague Self go my greatest thanks for his continual assistance, direction, and reassuring attitude throughout my tenure at The University of Oklahoma. As chairman of my Dissertation Committee, Dr. Self also gave substantial time and effort by way of directing my research, and my academic progress, in a manner best suited to attain the goals I had set forth.

I am also indebted to Dr. Eddie C. Smith, Department of Chemistry, for the assistance he rendered in helping me solve the biochemical problems encountered in this research, and for serving as a member of my Dissertation Committee in the absence of Dr. Leon Ciereszko. I also wish to thank Drs. Howard Haines and Donald Cox for their generous loans of time and equipment while I was involved in the liquid scintillation counting procedures.

The continual assistance provided by Dr. Paul M. Nollen, Western Illinois University, is gratefully appreciated. Dr. Nollen was instrumental in helping me

with the autoradiographic techniques and was also kind enough to read and comment on this dissertation.

Drs. Jack E. Young and Hubert Frings, who as members of my Dissertation Committee, provided helpful comments and suggestions as to the writing of this dissertation and deserve special thanks.

The suggestive criticisms of Messrs. John Ezra Trainer, Jr., and Gary Eugene Rodrick are acknowledged for, in many instances, these suggestions helped make this investigation more comprehensive and understandable.

Lastly, to my wife Carolyn go my special thanks for her continual encouragement and understanding, and financial assistance during my three years at The University of Oklahoma.

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UPTAKE, LOCALIZATION, AND FATE OF TRITIATED ARGININE IN *HAEMATOLOECHUS MEDIOPLEXUS* (TREMATODA)

CHAPTER I

INTRODUCTION

The ability of parasitic helminths to absorb small, water soluble, organic molecules when incubated *in vitro* is well documented. In much of the definitive work dealing with the uptake of solutes by helminths, cestodes have been employed as experimental models. Cestodes represent unusually favorable material for absorption studies as they lack both a digestive tract and oral opening; thus all materials entering these animals must enter through the external surface (see Read and Simmons, 1963; Read *et al.*, 1963; von Brand, 1966; Read, 1966; Smyth, 1969, for reviews). The Acanthocephala, like the Cestoda, lack a gut and oral opening and studies have shown that these helminths absorb amino acids in much the same manner as cestodes when incubated *in vitro* (see Rodgers, 1968; Rodgers and Sommerville, 1968, for reviews). Trematodes, as opposed to cestodes and acanthocephala, possess both a gut and tegument, both of which could possibly serve as absorptive surfaces.

Until Mansour (1959) demonstrated that Fasciola hepatica absorbed glucose via the tegument, it was thought that the trematode tegument was a non-cellular layer secreted by an underlying hypodermis. Subsequent electron microscope studies of a number of flukes have shown that the tegument is a syncytium connected to underlying cells by cytoplasmic processes (see Lee, 1966; Smyth, 1966, for reviews). Other morphological studies dealing with the luminal surface of the trematode gut have revealed the presence of filamentous structures suggestive of an absorptive surface (Senft et al., 1961; Dike, 1967, 1969; Davis et al., 1968; Shannon and Bogitsh, 1969a).

Experimental investigations cited above have yielded direct evidence that both the tegument and gut epithelium of trematodes are absorptive in nature, and several of these investigations have shown that the tegument and gut epithelium may be independently selective of the types of compounds absorbed. F. hepatica absorbs glucose via the tegument (Mansour, 1959), and amino acids via both the tegument and gut epithelium (Thorsell and Bjőrkman, 1965; Thorsell et al., 1966; Isseroff and Read, 1969). Transtegumental glucose absorption also occurs in the trematodes Philophthalmus megalurus (Nollen, 1968) and Haematoloechus medioplexus (Parkening and Johnson, 1969). In addition, P. megalurus absorbs leucine (Nollen, 1968; Cain, 1969) and tyrosine (Nollen, 1968) via the tegument. Schistosoma

mansoni has also been reported to absorb numerous amino acids when incubated in vitro, however, in this parasite the tegument and gut epithelium have both been postulated to be functional in absorption (Senft et al., 1961; Robinson, 1961; Senft, 1963, 1966). There has, however, been some question as to whether S. mansoni can absorb amino acids through both the tegument and gut epithelium as previously suggested (Read, 1970, personal communication).

It therefore appears that in P. megalurus, and possibly H. medioplexus, the gut and tegument function quite differently in absorption of glucose and amino acids.

In order to elucidate more clearly the functional role of the tegument and gut epithelium in trematodes, this research was undertaken to characterize some aspects of amino acid absorption and metabolism in H. medioplexus, the frog lung-fluke, by studying tritiated arginine absorption and assimilation (incorporation) in vitro. This characterization included determining (1) the route (i.e., trans-tegumental or trans-epithelial) via which arginine enters this parasite, (2) whether arginine is preferentially assimilated into any organs or tissues, (3) into which class of macromolecules (i.e., proteins, carbohydrates, lipids) the arginine is assimilated, (4) whether the end products of arginine metabolism are excreted, and (5) the effects of specific inhibitors

(i.e., 2,4-dinitrophenol (DNP), acti-dione (cycloheximide), and iodoacetate) on the uptake and assimilation of arginine.

CHAPTER II

MATERIALS AND METHODS

Gravid adults of H. medioplexus were obtained from naturally infected Leopard frogs (Rana pipiens) which were purchased from commercial sources or collected in Norman, Oklahoma. After removal from the lungs and prior to experimentation, all parasites were rinsed in two changes of phosphate buffered (pH 7.1) amphibian Ringer's solution.

For initial autoradiographic studies, ligated (see below) and non-ligated worms were incubated individually in 15 μc of ^{3}H-arginine (L-arginine-T(G) monohydrochloride, 151 mc/mM, Amersham/Searle Co., final arginine concentration = 0.132 mM) for varying time periods. All experimental incubations were carried out at 25 C. Parasites were then removed and rinsed in two changes of buffered Ringer's, killed and fixed in warm (60 C) alcohol-formalin-acetic acid (AFA) fixative, and embedded in Para-plast. Sections were cut at six micra and floated onto subbed (chrome alum-gelatin coated) microscope slides (Pappas, 1971a), deparaffinized, and hydrated. Slides were then coated with NTB-2 Nuclear Track Emulsion (Eastman-Kodak)

under #2 safelight conditions, exposed for 28 days at 4 C, and developed in Dektol Developer (1:1) (Eastman Kodak) for two minutes at 15 C. Sections were post-stained in Harris' hematoxylin and eosin Y and mounted in Permount. This same technique, with the exception of differences in the incubating medium, was used for subsequent autoradiographic studies of paraffin sections.

The oral openings of parasites were ligated by placing the parasites in warm (30 C) buffered Ringer's and, with the aid of a dissecting microscope, placing a small loop of surgical silk around the anterior end. In most instances it was impossible to attach the ligature to the most anterior portion of the worm but in all cases the ligature was confined to the anterior quarter of the worm. After ligation, parasites were placed in 25 C buffered Ringer's for at least ten minutes prior to incubation in labeled arginine.

To localize soluble isotope (unassimilated arginine washed out during the above procedure), a modification of the method suggested by Rodgers (1967) was employed. Unligated worms and worms that had been pre-incubated in cycloheximide (see below) were incubated in ^{3}H-arginine as described above, rinsed in two changes of buffered Ringer's and placed in small plastic "boats" filled with Tissue-Tek O.C.T. Medium (Ames C., Elkhart, Indiana). The boats were frozen using liquid nitrogen and the

frozen blocks sectioned at 15 micra, under #2 safelight conditions, on an AO Cryo-Cut cryostat. Frozen sections were picked up on frozen, emulsion coated microscope slides and exposed for 28 days at -15 C. After exposure, the slides were allowed to slowly thaw, passed through buffered formalin (pH 7.0) to fix the tissue, and developed in Dektol as described above. Sections were post-stained in Harris' hematoxylin and eosin Y and mounted in Permount.

An eyepiece micrometer grid, in conjunction with a 12.5X eyepiece and 97X oil immersion objective, was used to make counts of developed silver grains over various organs and tissues, and adjacent areas for background determination. The average count of 50 areas of 60 μ^2 was determined for each tissue or organ, and the average count of 200 areas of 60 μ^2 was determined, in background areas where the emulsion did not cover the tissue sections, for background determination. The significance of differences between mean grain counts over tissues (organs) and background was determined by the Student's $\underline{t}$ test.

Only developed silver grains associated with the following organs and tissues were counted: Oral sucker, pharynx, anterior parenchyma (at the level of the pharynx), median parenchyma (at the level of the ovary), posterior parenchyma, sperm in the seminal receptacle and cirrus (directly posterior to the genital pore), anterior and

posterior testis, and ovary. Other organs and tissues were not counted, due either to the small amount of tissue present, or, their highly refractive quality (vitellaria) which made silver grains indistinguishable in many instances, even under dark-field illumination. However, heavy concentrations of silver grains were noted when associated with other organs and tissues.

Because autoradiographic studies do not give reliable indications of total uptake of labeled materials, liquid scintillation counting was also used. For this, groups of ligated and non-ligated worms (three worms to a group), and worms treated with various inhibitors, were incubated in 80 µc of ^{3}H-arginine (final arginine concentration = 0.132 mM) for varying time periods, rinsed in two changes of buffered Ringer's and hydrolyzed in 1 ml of 1 N NaOH for 12 hours at 25 C. The hydrolysate was neutralized with 0.2 ml of 5 N HCl and a 50 µl aliquot added to 15 ml of Beckman TLA-Fluoralloy Scintillation Fluid with 7% (v/v) BBS-3 Bio-Solv Solubilizer added. Counting was done on a Beckman DPM-100 Liquid Scintillation Counter using an external standard. Three 50 µl aliquots of each hydrolysate were counted (for 20 minutes or to 2% accuracy) and averaged. Where applicable, quench curves were constructed and counts corrected accordingly. This same technique was used for all subsequent liquid scintillation counting. The protein content of three

duplicate aliquots (0.2 ml each) of each hydrolysate was determined by the method of Lowry et al. (1951) and averaged. Spectrophotometric values thus obtained were compared with a standard bovine serum albumin curve.

To determine the fate of the absorbed arginine, parasites were incubated, in groups of ten (to insure sufficient absorption and to decrease the chances of variation due to individual differences among worms) in 250 μc of ^{3}H-arginine (final arginine concentration = 0.662 mM) for varying time periods. Worms were then removed and rinsed in two changes of buffered Ringer's, quenched in liquid nitrogen and fractionated as follows: Worms were extracted overnight in 1 ml of 70% ethanol. The ethanol was decanted and saved and the worms homogenized in 2 ml of fresh 70% ethanol. After centrifugation (3,000 x g), the ethanol was decanted and combined with the first fraction (these two fractions contained the "pool" components which consist mainly of free amino acids), and 5 ml of 70% ethanol-diethyl ether (3:1, v/v) was added to the remaining tissue pellet. After mixing and centrifugation, the ethanol-diethyl ether supernatant was decanted and evaporated, and a known volume of toluene added (this sample contained the lipids). To the remaining tissue pellet, 3 ml of a phosphate buffer, 90% saturated with ammonium sulfate ($(NH_4)_2SO_4$) was added, mixed and centrifuged, and the supernatant

decanted and saved (this supernatant contained the carbohydrate fraction and a small amount of protein). The remaining tissue pellet was hydrolyzed in 6 N HCl for 12 hours at 60 C and the hydrolysate then neutralized with an equivalent of 6 N NaOH. Aliquots of 50 µl of each of the above fractions, with the exception of the lipid fraction, were then added to scintillation fluid and counted as described above. Because the lipid fraction was dissolved in toluene, the entire fraction was divided into thirds and each third was added to a separate counting vial and counted. The protein content of each protein hydrolysate was determined as previously described, and the amino acid content of three duplicate aliquots (0.4 ml each) of the pool fractions was determined using a ninhydrin spectrophotometric assay (Clark, 1964). Amino acid content was expressed in leucine equivalents. In addition, a Lowry's test was run on each of the carbohydrate fractions, and these all resulted in slight color reactions. However, due to some turbidity in these tests, the protein content could not be determined.

The effect of cycloheximide (Nutritional Biochemical Corp.) was determined by pre-incubating parasites in a solution of 1 mg cycloheximide/ml buffered Ringer's for two hours and then incubating the parasites for varying time periods in 15 µc (for autoradiographic studies) or 80 µc (for liquid scintillation studies) of ^{3}H-arginine

as described above. Worms were then processed for autoradiography of paraffin and frozen sections and liquid scintillation counting as described above.

The effects of DNP and iodoacetate were determined by two experiments. The first experiment consisted of incubating parasites (in groups of three) in 80 μc of ^{3}H-arginine to which either DNP (final DNP concentration = 0.2 nM) or iodoacetate (final iodoacetate concentration = 1.0 mM) had been added. The second experiment consisted of pre-incubating parasites in either DNP (0.2 nM) or iodoacetate (1.0 mM) for five minutes and then incubating them in the labeled arginine plus inhibitor as described above. Varying periods of incubation in the labeled arginine were used, and worms were processed for liquid scintillation counting as described above.

To determine whether end products of arginine metabolism are excreted, worms were incubated in groups of six in 40 μc of ^{3}H-arginine (final arginine concentration = 0.264 mM) for one hour and rinsed in two changes of buffered Ringer's. Parasites were then treated as follows: One group was placed in 3 ml of buffered Ringer's solution while those in the other group were ligated and placed in a separate 3 ml aliquot of buffered Ringer's. Aliquots (0.2 ml each) of each of the above Ringer's solutions were taken at various time

intervals and added to scintillation fluid and counted as previously described. At the end of the experimental period, the remaining Ringer's was analyzed for protein and amino acid content by previously described methods, the parasites were hydrolyzed and counted, and the protein content of each hydrolysate was determined.

All experiments were duplicated to determine reproducibility of results. In almost all cases, exact grain counts for autoradiographs or counts per minute for liquid scintillation studies could not be duplicated. However, basic trends (i.e., which organs or tissues were most heavily labeled in autoradiographs, or which treatment had the most significant effect on arginine absorption and assimilation) were reproducible. Therefore, only one set of data is presented for each experiment, each set representing an experiment where all parasites were collected from a minimum number of hosts, and all hosts were collected over the shortest period of time. This was done in an attempt to keep uncontrollable variables to a minimum.

All tabular data were analyzed using a two-way analysis of variance without replication and a Student-Newman-Keuls (a posteriori) test (using $P=0.05$ as Type I error) to determine whether significant differences existed among various grain counts or counts per minute when compared with length of incubation, biochemical fraction, or treatment of parasites.

CHAPTER III

RESULTS

Grain counts of autoradiographs of paraffin sections (Table I) indicate that all organs and tissues studied assimilate significant amounts of ^{3}H-arginine in a minimum of 15 minutes, and during all subsequent time periods (Fig. 1-5). There are no significant differences between grain counts of various organs and tissues studied, and there is no correlation between total amount of amino acid assimilated (total grain count/worm) and length of incubation period. There was a significant increase in grain counts per worm (Table I) from 30 to 60 minutes.

Although not quantitated, organs and tissues other than those listed in Table I accumulated measurable amounts of radioactivity. This was especially true of the vitellaria of some specimens, as is evidenced by the high number of silver grains associated with these organs (Fig. 6). However, conclusions can not be drawn from these few observations because of the inability to quantitate silver grains associated with the vitellaria in all specimens. In addition, the tegument of some parasites assimilated significant amounts of radioactivity

Table 1. Mean grain counts (± S.E.) over 60 μ^2 for organs and tissues of worms maintained in tritiated arginine for varying time periods. All counts are corrected for background.

Tissue or organ	15 min.	30 min.	60 min.	90 min.	120 min.
oral sucker	1.10±0.13	1.41±0.17	1.88±0.19	1.49±0.16	0.78±0.11
pharynx	0.44±0.10	0.99±0.14	0.84±0.14	1.01±0.13	0.54±0.09
anterior parenchyma	0.95±0.14	1.11±0.13	1.42±0.18	1.97±0.15	1.88±0.21
median parenchyma	1.15±0.11	0.24±0.16	1.84±0.16	1.16±0.11	0.86±0.11
posterior parenchyma	0.84±0.11	0.83±0.09	1.01±0.18	1.73±0.15	0.84±0.10
cirrus	0.87±0.14	(0.10±0.03)*	0.74±0.11	0.73±0.11	0.60±0.12
seminal receptacle	1.07±0.14	0.52±0.13	1.00±0.13	1.01±0.14	0.96±0.12
anterior testis	1.46±0.13	0.63±0.12	2.22±0.24	1.67±0.21	2.24±0.20
posterior testis	1.42±0.08	0.71±0.10	0.94±0.11	3.31±0.28	3.78±0.24
ovary	0.43±0.08	0.59±0.10	4.12±0.25	0.89±0.13	2.18±0.14

*values in parentheses are not significantly greater than background ($P < 0.05$) when compared with the Student's t test.

(Fig. 3), however, this only occurred in parasites that had been incubated for one hour or longer. The tegument of specimens incubated for less than one hour did not assimilate measurable amounts of radioactivity (Fig. 7).

If parasites are first ligated and then incubated in ^{3}H-arginine, grain counts over many organs and tissues are insignificant when compared with background (Table 2). These data show that only organs and tissues that were anterior to the ligature (oral sucker, pharynx, anterior parenchyma, sperm in the cirrus) had significantly higher grain counts than background , while all organs and tissues posterior to the ligature had insignificant grain counts. Also, the grain counts associated with the posterior parenchyma, anterior and posterior testes, ovary, and sperm in the seminal receptacle (organs posterior to the ligature) are significantly lower than grain counts associated with the oral sucker and anterior parenchyma (organs anterior to the ligature). These data therefore demonstrate that only the gut epithelium is active in ^{3}H-arginine absorption. This observation is supported by autoradiographs of frozen sections which show that the labeled arginine is localized in the gut epithelium after only two minutes incubation (Fig. 8), indicating absorption through this surface. The tegument, however, did not become labeled even after ten minutes

Table 2. Mean grain counts (± S.E.) over 60 μ^2 for organs and tissues of worms maintained in tritiated arginine after ligation of their oral openings. All counts are corrected for background.

Tissue or organ	15 min.	30 min.	60 min.
oral sucker	0.95 ± 0.09	3.12 ± 0.24	0.90 ± 0.12
pharynx	1.10 ± 0.14	1.49 ± 0.20	0.59 ± 0.10
anterior parenchyma	1.25 ± 0.11	3.24 ± 0.25	0.97 ± 0.14
median parenchyma	(0.10 ± 0.03)*	(0.18 ± 0.05)	(0.18 ± 0.04)
posterior parenchyma	(0)	(0.10 ± 0.02)	(0)
cirrus	0.96 ± 0.07	1.98 ± 0.14	0.85 ± 0.11
seminal receptacle	(0.11 ± 0.05)	(0.07 ± 0.01)	(0.08 ± 0.01)
anterior testis	(0.13 ± 0.02)	(0.11 ± 0.03)	(0.04 ± 0.01)
posterior testis	(0)	(0.09 ± 0.01)	(0.06 ± 0.00)
ovary	(0.01 ± 0.02)	(0.13 ± 0.02)	(0)

*values in parentheses are not significantly greater ($P < 0.05$) than background when compared with the Student's t test.

incubation (Fig. 9), indicating the non-permeability of the tegument to this amino acid.

As is the case with the data in Table 1, there is no correlation between grain counts and length of incubation period for ligated worms (Table 2). The only significant difference noted between total counts per worm was that the total number of grains at 30 minutes was significantly greater than at 15 and 60 minutes.

Pre-incubation of parasites in cycloheximide inhibits the initial assimilation and/or absorption of the labeled arginine. This is shown by the insignificant number of grains as compared to background over all organs and tissues, except the oral sucker, pharynx, and anterior parenchyma, at 15 and 30 minutes incubation in ^{3}H-arginine (Table 3). At 60 and 90 minutes incubation, the effect of the cycloheximide ceases, as is evidenced by the increased grain counts over almost all organs and tissues, and the fact that the total grain count at 90 minutes is significantly greater than that at 15, 30, and 60 minutes incubation. However, there is no significant difference between grain counts over various organs and tissues. Therefore, even though the cycloheximide does inhibit assimilation of the labeled arginine in many organs and tissues during the first hour of incubation, once the effects of the inhibitor cease

Table 3. Mean grain counts (± S.E.) over 60 μ^2 for organs and tissues of worms pre-incubated in cycloheximide for two hours and then maintained in tritiated arginine for varying time periods. All counts are corrected for background.

Tissue or organ	15 min.	30 min.	60 min.	90 min.
oral sucker	0.57 ± 0.11	0.20 ± 0.06	0.34 ± 0.07	0.65 ± 0.18
pharynx	0.43 ± 0.09	(0)*	0.18 ± 0.09	3.08 ± 0.18
anterior parenchyma	0.43 ± 0.07	0.36 ± 0.08	0.23 ± 0.06	3.30 ± 0.24
median parenchyma	(0)	(0)	0.58 ± 0.11	1.78 ± 0.21
posterior parenchyma	(0)	(0.25 ± 0.03)	0.33 ± 0.06	1.18 ± 0.14
cirrus	(0.01 ± 0.00)	(0.01 ± 0.00)	(0.11 ± 0.02)	2.86 ± 0.17
seminal receptacle	(0.12 ± 0.03)	(0.02 ± 0.00)	0.28 ± 0.07	0.78 ± 0.10
anterior testis	(0.29 ± 0.04)	(0.06 ± 0.01)	1.67 ± 0.20	2.78 ± 0.28
posterior testis	(0)	(0)	0.63 ± 0.10	2.54 ± 0.19
ovary	(0)	(0)	0.90 ± 0.14	2.14 ± 0.15

*values in parentheses are not significantly greater than background ($P < 0.05$) when compared with the Student's t test.

(between 60 and 90 minutes), the organs and tissues that are initially inhibited can rapidly assimilate large quantities of it.

Autoradiographs of frozen sections of worms pre-incubated in cycloheximide and then incubated in labeled arginine show that the absorption of the latter occurs through the gut epithelium (Fig. 10), as was the case with the worms not pre-incubated in cycloheximide (Fig. 8). Therefore, the action of cycloheximide is that of inhibiting the assimilation, and not the absorption, of the labeled amino acid.

Data from the liquid scintillation studies are presented in Table 4, and are reported as counts per minute per microgram of protein (CPM/µg protein). Because accurate weights of parasites were difficult to obtain, the protein content of worms hydrolysates was used as a standard with which to compare the various experimental groups.

Compared to the control worms incubated in ^{3}H-arginine only (Table 4, Group 1), all experimental groups (Groups 2-7) showed significantly lower CPM/µg protein values. However, as was the case with the autoradiographic studies, there is no correlation between length of incubation period and amount of absorbed and/or assimilated amino acid (this method, using liquid scintillation, will not differentiate between absorbed and assimilated arginine).

Table 4. Total counts per minute per microgram of protein (CPM/µg protein) for hydrolysates of H. medioplexus which had been treated in various ways or incubated in different inhibitors and then maintained in tritiated arginine for varying time periods. All counts are corrected for background and quenching.

	5 min.	10 min.	15 min.	30 min.	60 min.
Incubated in arginine only (GROUP 1).	214.3	264.6	180.6	197.1	165.5
Incubated in arginine after ligation of oral openings (GROUP 2)	17.9	40.1	31.5	40.3	33.3
Pre-incubated in cyclo-heximide then incubated in arginine (GROUP 3)	30.1	15.6	26.1	38.5	54.4
Incubated in DNP + arginine (GROUP 4)	13.8	9.4	16.3	13.3	10.9
Pre-incubated in DNP then incubated in DNP + arginine (GROUP 5)	8.2	6.8	7.6	3.7	8.0
Incubated in iodo-acetate + arginine (GROUP 6)	84.9	67.4	60.2	41.9	38.6
Pre-incubated in iodo-acetate then incubated in iodoacetate + arginine (GROUP 7)	19.4	24.4	10.8	12.6	16.1

The fact that CPM/µg protein values for ligated worms (Group 2) are significantly lower than those for the controls again indicates that the gut epithelium is responsible for arginine absorption. One may suspect that the data for ligated worms should approach the values obtained using DNP and iodoacetate as inhibitors (Groups 5 and 7), but the fact that the CPM/µg protein values were not lower than reported can be accounted for by the ability of organs anterior to the ligature to continue to assimilate the amino acid even after ligation. The absorption of the labeled arginine by cycloheximide treated worms (Group 3) was not significantly different from that of the ligated worms. Thus, the cycloheximide treatment is not completely effective in terminating ^{3}H-arginine absorption and assimilation, as was previously indicated by the autoradiographic studies (Table 3). These data (Table 4) also show that amino acid absorption in the cycloheximide treated worms ceases after an initial period of absorption. This is indicated by the observation that CPM/µg protein values do not approach those of the control worms (Group 1) as would be expected if amino acid absoprtion continued throughout the experimental period.

Iodoacetate is an effective inhibitor of ^{3}H-arginine absorption, as is shown by the decreased CPM/µg protein

values for worms incubated in iodoacetate plus labeled arginine (Group 6). It is interesting to note that pre-incubation of worms in iodoacetate (Group 7), or incubation of parasites in DNP plus ^{3}H-arginine (Groups 4 and 5), yields significantly lower CPM/µg protein values than worms incubated in iodoacetate plus arginine (Group 6). Also, values for worms incubated in DNP plus arginine (Group 4) or pre-incubated in DNP and then incubated in DNP plus arginine (Group 5) are not significantly different. These data indicate a distinct difference between the modes of action of these two inhibitors in that the iodoacetate must first be absorbed into the cell before it is effective (causing a "lag" period between exposure to the inhibitor and complete inhibition), while the effects of DNP are instantaneous upon reaching the gut (resulting in no "lag" period).

The arginine that is actually assimilated by the parasites was recovered mainly from the protein fraction of these worms (Table 5), while only small amounts were recovered in the carbohydrate and lipid fractions. The counts obtained from the amino acid (unassimilated radioactivity) and protein fractions are not significantly different from each other, however, the counts from both the lipid and carbohydrate fractions are significantly less than the former two fractions. Considering the

Table 5. Total counts per minute for various chemical fractions of worms maintained in tritiated arginine for varying time periods. Data also include counts for "total absorbed" and "total assimilated" amino acid, and counts from the combined carbohydrate and lipid fractions for each time period. All counts are corrected for background and quenching.

	5 min.	10 min.	15 min.	30 min.	60 min.
Amino acid fraction	46,100 (4,610)*	58,300 (4,941)	61,500 (3,844)	65,600 (4,860)	57,700 (3,394)
Protein fraction	47,100 (23.0)**	49,000 (21.4)	48,800 (23.2)	57,800 (25.6)	91,200 (27.8)
Carbohydrate fraction	2,980	2,370	3,470	3,890	3,260
Lipid fraction	232	608	590	437	491
Total absorbed amino acid (sum all fractions)	96,412	110,278	114,360	127,727	152,651
Total assimilated amino acid (protein + carbohydrate + lipid)	50,312	51,978	52,860	62,127	94,951
Carbohydrate + lipid	3,212	2,978	4,060	4,327	3,751

*values in parentheses reported as CPM/leucine equivalent
**values in parentheses reported as in Table 4.

total CPM data of all fractions for each time period (assimilated and unassimilated amino acid), and the total CPM data from the carbohydrate, lipid, and protein fractions (assimilated amino acid only), the combined lipid and carbohydrate fractions account for an average of 3% of the total radioactivity and 5.9% of the assimilated radioactivity. Therefore, most of the absorbed amino acid is assimilated into the protein fraction of these worms.

Because these data (Table 5) do not differentiate between extracorporeal amino acid (that found free in the gut) and intracorporeal amino acid (that found free in the intra- and intercellular fluid), it is impossible to determine the fraction of the total amino acid count that is contributed by amino acid in the gut and body, respectively. Also, the fact that the carbohydrate fraction was contaminated with small amounts of protein suggests that the radioactivity in this fraction may actually be from labeled protein and not labeled carbohydrate. Whether or not this was the case was not determined.

If parasites are incubated in labeled arginine for one hour and placed, either ligated or unligated, in unlabeled buffered Ringer's, they excrete, or regurgitate, radioactive materials representing either the original

tritiated arginine or end products of arginine metabolism (Table 6). If parasites are ligated, after pulse labeling in ^{3}H-arginine and prior to being placed in unlabeled Ringer's, the amount of radioactive material liberated is significantly less than that by parasites not ligated.

At the end of the experimental period, the Ringer's of both ligated and non-ligated worms contained no protein and approximately equal amounts of "ninhydrin positive compounds" (Table 6). These latter compounds are referred to as ninhydrin positive compounds for chemical classification of them was unsuccessful. They are, however, almost certainly amino acids. This indicates that these ninhydrin positive compounds are excreted through either the tegument or excretory pore, but not regurgitated from the gut lumen. The fact that ligated worms excreted significantly less radioactive material, as is indicated by the lower CPM in the buffered Ringer's and higher CPM/μg protein values for worm hydrolysates at the end of the experimental period, does show that radioactive compounds were regurgitated from the gut of unligated worms. Because the experimental amino acid was tritiated on carbons 2, 3, 4, and 5 with no functional groups (carboxyl, amino, guanidino) labeled, the significantly higher radioactivity in the Ringer's of the unligated worms must have been brought about by

Table 6. Total counts per minute, at various time intervals, of buffered Ringer's solution in which pulse labeled parasites had been placed. Data also include protein and "ninhydrin positive compounds" content of the Ringer's at the end of the experimental period, and CPM/µg protein values for hydrolysates of worms at the end of the experimental period.

	5 min.	10 min.	15 min.	30 min.	60 min.
Worms not ligated after pulse labeling	8,400	9,120	9,200	9,860	9,650
Worms ligated after pulse labeling	748	821	840	970	1,003

	protein content	ninhydrin positive	CPM / µg protein
Worms not ligated after pulse labeling	-0-	144 ug*	73.0
Worms ligated after pulse labeling	-0-	140 ug	151.0

*data reported as leucine equivalents

regurgitation of the carbon skeleton after removal of the amino, and possibly guanidino, groups. The loss of these two functional groups is indicated by the almost identical quantities of ninhydrin positive compounds in the Ringer's of both experimental groups. Therefore, the materials excreted from the gut lumen are end products of arginine metabolism while the material excreted through the tegument and/or excretory pore is almost certainly an amino acid (or small peptide), both compounds apparently being radioactive and therefore corresponding, at least in part, to the original ^{3}H-arginine.

PLATE I

Figures 1-4. Autoradiographs of paraffin sections of H. medioplexus after incubation in ^{3}H-arginine.

1. Section of the oral sucker after 30 minutes incubation (710x).
2. Section of the anterior parenchyma after 15 minutes incubation (1635x).
3. Section of the median parenchyma and tegument (asterisk) after two hours incubation (867x).
4. Section of the posterior testis after 60 minutes incubation (544x).

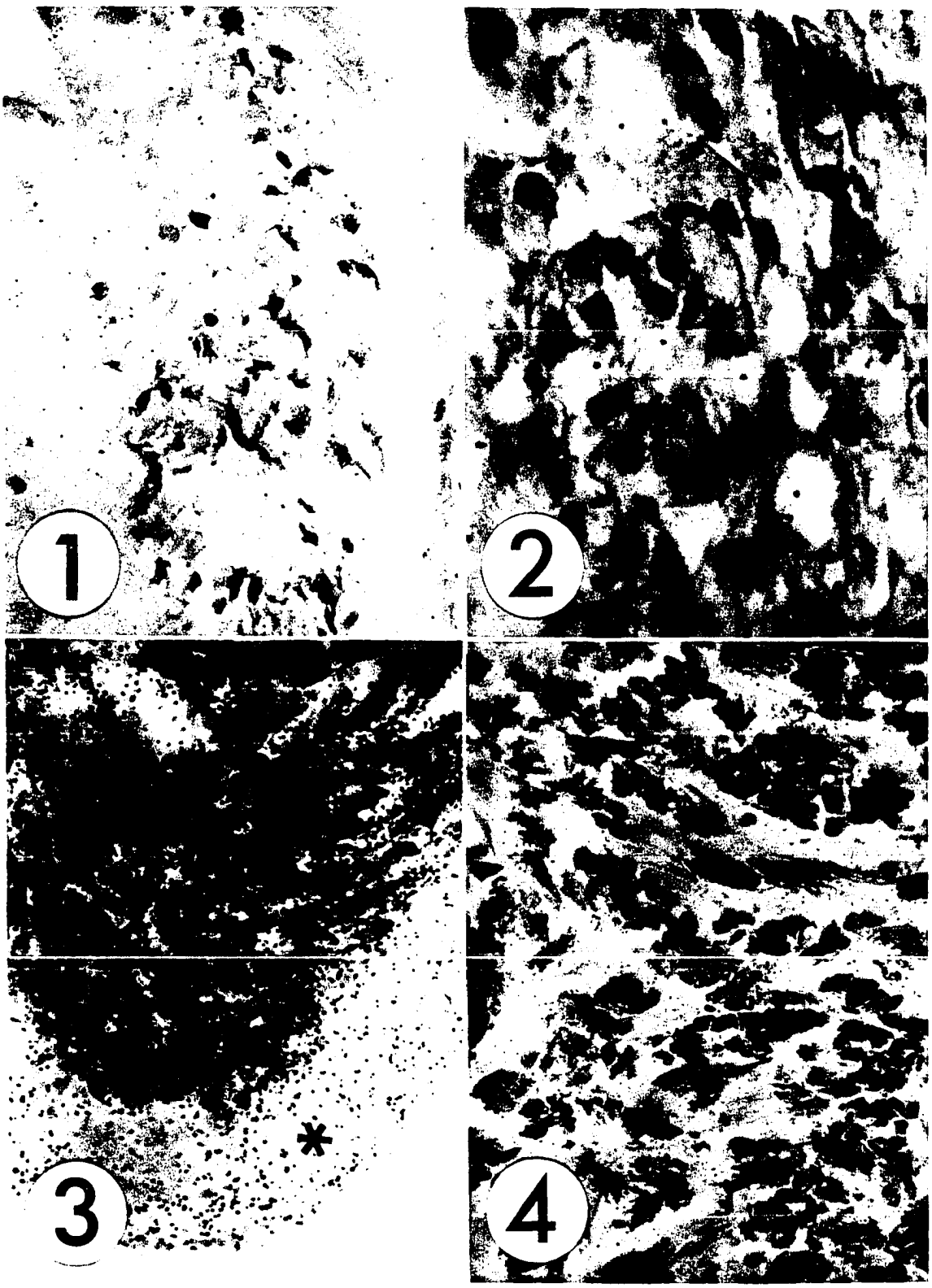

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PLATE II

Figures 5-7. Autoradiographs of paraffin sections of H. medioplexus after incubation in ^{3}H-arginine.

5. Section of the ovary after two hours incubation (710x).
6. Section of the vitellaria (dark staining material) after 60 minutes incubation (867x).
7. Section of the tegument (asterisk) after 30 minutes incubation (867x).

Figure 8. Autoradiograph of a frozen section of the gut lining after two minutes incubation in ^{3}H-arginine (867x).

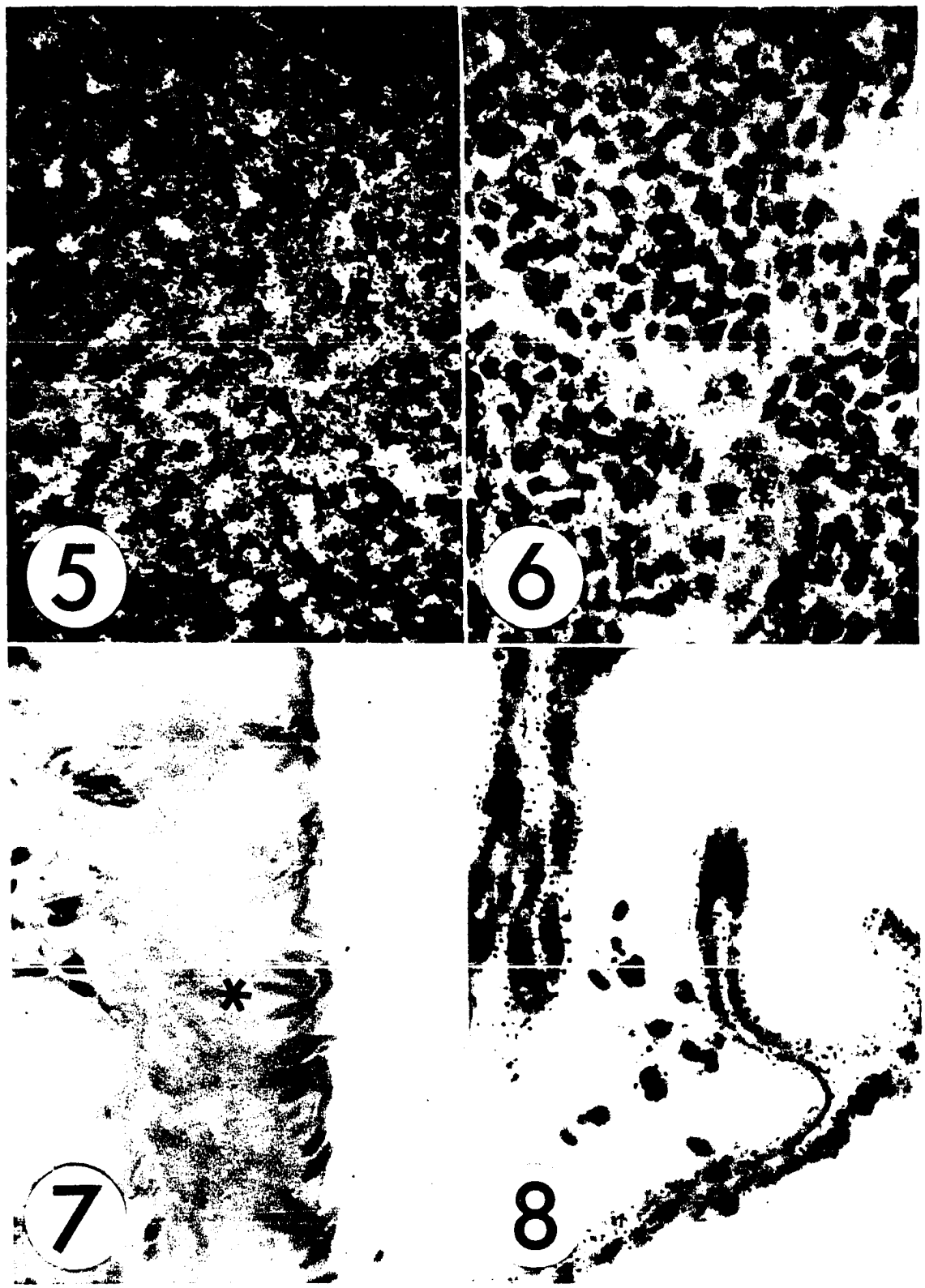

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PLATE III

Figures 9-10. Autoradiographs of frozen sections of H. medioplexus after incubation in ^{3}H-arginine.

9. Section of the tegument (asterisk) after ten minutes incubation (867x).

10. Section of the gut lining after two hours pre-incubation in cycloheximide and ten minutes incubation in labeled arginine (867x).


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CHAPTER IV

DISCUSSION

H. medioplexus can rapidly absorb and assimilate arginine, and it is not localized in significant amounts in any one organ or tissue. Other researchers have demonstrated that parasites, including H. medioplexus, can preferentially assimilate some amino acids into specific organs. Thorsell et al. (1966) showed that F. hepatica assimilates methionine, glycine, and tyrosine into the ovary, intestinal lining, and vitellaria (tyrosine only), while Thorsell and Bjőrkman (1965) demonstrated that this parasite assimilates leucine, methionine, phenylalanine, and tryptophane into the gut lining. In addition, Pantelouris and Gresson (1960) and Pantelouris (1964) demonstrated that F. hepatica assimilates phenylalanine and methionine into the tegument and gut lining. S. mansoni has been shown to assimilate proline into the gut lining and tegument of males and tegument and vitellaria of females by Senft (1968). Nollen (1968) demonstrated that P. megalurus concentrates leucine and tyrosine in the gut and vitellaria (tyrosine only) and Burton (1963) showed that H. medioplexus

concentrates tyrosine in the vitellaria when exposed *in vivo*. From these previous investigations, many of which are incomplete in that only a few organs and tissues were studied, there is substantial evidence that digenetic trematodes assimilate many amino acids, but that of all the internal organs only the vitellaria consistently concentrates any one amino acid. The reason for this consistent accumulation of tyrosine in the vitellaria of trematodes has been discussed by Burton (1963) and Nollen (1968). The fact that arginine is not preferentially assimilated into any one organ is not surprising for arginine, unlike tyrosine, does not appear to play any specialized role in the metabolism of *H. medioplexus*.

The role of the tegument and gut epithelium in absorption of small molecular weight compounds by trematodes remains uncertain. Some studies on *F. hepatica* show that amino acids absorbed from an external medium are generally localized in, and absorbed through, the gut epithelium (Pantelouris and Gresson, 1960; Pantelouris, 1964; Thorsell and Björkman, 1965; Thorsell *et al.*, 1966), while the tegument appears solely responsible for glucose absorption (Mansour, 1959). The tegument of *F. hepatica* is permeable however to some amino acids for Pantelouris and Gresson (1960) localized phenylalanine in the tegument. In addition, Kurelec

and Ehrlich (1963) demonstrated that alanine, as well as alpha-ketoglutaric acid, pyruvic acid, and glutamic acid, can permeate the tegument of this worm via transamination reactions, Isseroff and Read (1969) showed absorption of cycloleucine (1-aminopentane-1-carboxylic acid), proline, arginine, and methionine via the tegument of both F. hepatica and Fascioloides magna, and Senft (1968) suggested that both the tegument and gut epithelium of S. mansoni are responsible for proline absorption.

As was originally postulated by Burton (1962) and later demonstrated experimentally by Parkening and Johnson (1969), only the tegument of H. medioplexus is known to be permeable to glucose, while my study demonstrates that only the gut epithelium is functional in arginine absorption. P. megalurus has also been shown to absorb glucose via the tegument (Nollen, 1968) and amino acids via the gut epithelium (Nollen, 1968; Cain, 1969). Therefore, in the case of H. medioplexus and P. megalurus, there is evidence that the tegument and gut epithelium are selective in amino acid and glucose absorption. However, Nollen (1968) also showed that both the tegument and gut epithelium of P. megalurus are functional in thymidine absorption, while the tegument and gut epithelium of H. medioplexus have been shown to be permeable to ferritin (Rothman, 1968; Dike,

1969) and $FeCl_3$ (Shannon and Bogitsh, 1969b). This selective absorption may therefore not exist with all compounds in these two species.

The action of cycloheximide has been extensively studied in isolated mammalian tissue and yeast cultures and has been shown to be an inhibitor of DNA synthesis and protein synthesis (Bennett et al., 1964, 1965; Siegel and Sisler, 1964a, b). The fact that arginine is assimilated mainly into protein is demonstrated by the relatively low grain counts and CPM/µg protein values (Tables 3 and 4) obtained from the worms pre-incubated in cycloheximide. These results are consistent with those of Nollen (1968) who demonstrated that cycloheximide decreased assimilation of leucine and tyrosine in P. megalurus. However, Nollen (1968) did not demonstrate conclusively that the action of cycloheximide in P. megalurus was that of protein synthesis inhibition for his results could have been obtained if absorption of the radioactive materials had been inhibited. In my work, the frozen sections of cycloheximide treated worms demonstrate that absorption continues to occur and therefore the action of this inhibitor is that of inhibiting protein synthesis and not amino acid absorption.

After the effects of the cycloheximide have ceased, the worms can readily absorb and assimilate significant amounts of the labeled arginine into all organs and

tissues. This suggests that the inhibitory action of cycloheximide is transitory and readily reversible in the absence of the inhibitor, a fact not noted by Nollen (1968).

From the results of my investigation, it is obvious that the cycloheximide treatment is not inhibiting amino acid assimilation in all organs and tissues of the worm, the reason for this being uncertain. Nollen (1970, personal communication) suggested that the highly muscular nature of the pharynx and oral sucker impedes the absorption of the cycloheximide in these two organs. He also indicated that, in the case of P. megalurus, the anterior parenchyma displays higher metabolic activity than other tissues. The ability of the oral sucker, anterior parenchyma, and pharynx to continue assimilating labeled arginine (Table 3) may therefore result from the two hour pre-incubation period not being long enough to completely inhibit protein synthesis in these organs and tissue.

Although liquid scintillation studies indicate that the initial absorption of arginine is not inhibited by cycloheximide, absorption over longer periods of time is partially inhibited. Though one can not be certain, this could be brought about by an increased intracorporeal arginine concentration (due to a lack of assimilation) which causes a cessation of net absorption by one of the

following mechanisms: If absorption is due to a physical process, then an increased intracorporeal arginine concentration may cause a decrease in the concentration gradient to such a point that the absorption is inhibited. The other possibility is that as the concentration of arginine increases in the inter- and intracellular fluids, its loss through excretion, secretion, or leakage increases to a point where its rate of loss equals that of absorption.

The results of past researches indicate that either of these mechanisms may be functional. Isseroff and Read (1969) demonstrated that several amino acids enter through the tegument of F. hepatica and Fascioloides magna by simply diffusion over short periods of time. These same authors suggested that simple diffusion may not be the only means by which amino acids permeate the tegument of these parasites. Although the results of Isseroff and Read (1969) do not mention the gut epithelium of these worms, there remains the possibility that both the tegument and gut epithelium of parasites absorb amino acids via simple diffusion. The gut of F. hepatica has also been shown at times to be secretory (Thorsell and Bjőrkman, 1965), and this may be the case with the gut of H. medioplexus for the morphology of the two gut linings resemble each other in many respects (Thorsell

and Björkman, 1965; Dike, 1967, 1969, Davis et al., 1968). In addition, the end products of arginine metabolism and/or the arginine itself are excreted by H. medioplexus. Therefore, the lack of continual net absorption may simply be due to the lack of a large enough concentration gradient to cause diffusion, or to the excretion, secretion, or leakage of the arginine and/or end products of arginine metabolism at a rate equalling that of arginine absorption.

Very little is known of the kinetics or mechanisms involved in the absorptive processes of trematodes (see Isseroff and Read, 1969, for the one exception), so it is not known whether absorption is affected by intracorporeal amino acid concentrations. If the absorption of amino acids in H. medioplexus takes place by simple diffusion then an increase in the intracorporeal amino acid concentration could definitely limit the amount of arginine absorbed. If, however, the absorptive process is found to be mediated, as is the case in cestodes and acanthocephala (Read and Simmons, 1963; Rothman and Fisher, 1964), then the intracorporeal amino acid concentration would be expected to have little effect. Therefore, to determine accurately the explanation of the cessation of arginine absorption following cycloheximide treatment, the kinetics and mechanisms of amino acid absorption in trematodes must be elucidated.

Determining whether a compound is rapidly metabolized is extremely important in an uptake study for rapid metabolism of the absorbed compound may cause rapid changes in the intracorporeal concentration, and the amount of assimilated radioactivity, through the formation of compounds that are liable to metabolism and/or excretion. My data show that H. medioplexus absorbs and assimilates large quantities of arginine in very short periods (five minutes), demonstrating the extreme rapidity with which this amino acid is metabolized. The fact that this amino acid is rapidly metabolized, and also incorporated into excretory products, or excreted directly, may explain the lack of a correlation between time and amount of arginine absorbed and assimilated in all experiments. This lack of a time correlation was also noted for glucose absorption by H. medioplexus (Parkening and Johnson, 1969); however, these investigators did not determine whether the glucose was being metabolized (as was most certainly the case), a fact which likely affected their results.

It is not surprising to find that 94% of the assimilated arginine is found in the protein fraction for arginine is not readily metabolized through other intermediate pathways (TCA cycle, glycolysis, fatty acid metabolism). In addition, chemical analysis of several

species of trematodes, including H. medioplexus, has demonstrated that they are composed mainly of protein (49-67 percent dry wt.), with only small amounts of carbohydrate and lipid present (Flury and Leeb, 1926; Weinland and von Brand, 1926; Goil, 1958a, b; Smyth, 1966).

There is evidence that arginine may play a role in the excretory processes of trematodes by being directly involved in the formation of excretory products. Previous investigations have suggested that a functional ornithine-urea cycle is present in many trematodes, although a complete cycle has yet to be demonstrated in any one trematode (Campbell and Lee, 1963; Senft, 1966; Janssens and Bryant, 1969). The results of this investigation demonstrate that H. medioplexus can excrete, through the tegument and/or excretory pore, a radioactive compound, which is probably an amino acid, while this same species seems to regurgitate a different radioactive compound. The exact nature of the product excreted from the ligated worms is uncertain, but appears to be an amino acid or small peptide for it is reactive with ninhydrin. Several trematodes have been shown to excrete amino acids or small peptides. F. hepatica excretes peptones (Weinland and von Brand, 1926) and numerous amino acids including arginine (Moss, 1970), Cephalogonimus americanus excretes peptides which contain

arginine (Pappas, 1971b), and S. mansoni excretes several amino acids including arginine (Senft, 1963). Among trematodes, therefore, H. medioplexus is not exceptional in its apparent ability to excrete peptides which contain arginine, or to excrete the free amino acid itself.

The material regurgitated from the gut of non-ligated worms is radioactive and not reactive with ninhydrin so it must be an end product of arginine metabolism. It is interesting to note that the products liberated from the tegument and/or excretory pore are different from those regurgitated from the gut lumen.

The effects of DNP and iodoacetate on amino acid absorption are most interesting in that they are quite different. The failure of iodoacetate to inhibit arginine absorption, unless the worms are first pre-incubated in the inhibotor, indicates that the iodoacetate must first be absorbed before being effective as an inhibitor. The data show that if worms are not pre-incubated in iodoacetate prior to incubation in the labeled arginine, only a minimal amount of the inhibitor is absorbed during the first five minutes of incubation and that the amino acid enters faster than the inhibitor. If the inhibitor were absorbed faster, pre-incubation would not be a prerequisite for inhibition. This same "lag" period in the effects of iodoacetate has been demonstrated in the

cestode Calliobothrium and this supports the hypothesis that this inhibitor is affecting some internal cellular process rather than the permeability of the cell membranes (Read et al., 1960).

The results using DNP as an inhibitor are different from those produced by iodoacetate, and from those of Read et al. (1960) using Calliobothrium. The action of DNP in immediately inhibiting arginine absorption indicates that this inhibitor is either penetrating the cell membranes more rapidly than the amino acid and inhibiting some internal process rapidly enough to inhibit amino acid absorption, or the DNP may be altering the permeability of the membranes to the amino acid. Although little is known of the metabolic processes in many trematodes, previous investigators (Read et al., 1960) indicate that results of this sort could be caused by an alteration in the permeability of the membranes, so the action of DNP appears to be that of altering membrane permeability in the cells bordering the gut lumen.

CHAPTER V

SUMMARY

The uptake, localization, and fate of tritiated arginine in Haematoloechus medioplexus was studied using autoradiography of paraffin and frozen sections and liquid scintillation counting techniques. The purposes of this research were to determine (1) the route via which arginine enters this parasite, (2) if arginine is preferentially assimilated into any specific organs, (3) into which class of macromolecules (protein, lipid, carbohydrate) the arginine is assimilated, (4) if the arginine, or end products of arginine metabolism, is excreted by this worm, and (5) the effects of 2,4-dinitrophenol, iodoacetate, and cycloheximide on arginine absorption and assimilation.

Autoradiographic and liquid scintillation studies on ligated and unligated parasites demonstrated that only the gut epithelium is active in arginine absorption. Autoradiographic studies also showed that the arginine is not preferentially assimilated into any organ or tissue studied.

Pre-incubation of parasites in cycloheximide for two hours inhibits assimilation of arginine but not the initial absorption of the amino acid. Iodoacetate and dinitrophenol are both effective inhibitors of arginine absorption. Liquid scintillation experiments demonstrated that parasites must first be pre-incubated in iodoacetate for five minutes for this inhibitor to be totally effective. Dinitrophenol is instantaneous in inhibiting absorption; no pre-incubation is necessary. Apparently, iodoacetate must be absorbed to inhibit absorption, and is therefore inhibiting some internal cellular process, while dinitrophenol affects the permeability of the cell membranes bordering the gut lumen.

Of the arginine assimilated, 94% is incorporated into the protein fraction of these parasites, the remaining arginine being found in the carbohydrate and lipid fractions.

Using parasites which had been pulse labeled in arginine, it was found that these worms could excrete radioactive materials. The tegument and/or excretory pore is responsible for the excretion of a ninhydrin positive, radioactive compound, this probably being the original arginine or a small peptide containing the original arginine. These worms also regurgitate a radioactive compound from the gut which is not reactive with ninhydrin.

No correlation between total uptake of labeled arginine and length of incubation period could be found. This is likely due to the ability of these worms to absorb, as well as excrete, arginine and the end products of arginine metabolism during short term incubations.

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