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GRADUATE COLLEGE

THE NITROGEN AND AMINO ACID COMPOSITION OF THREE SPECIES OF ANOPLOCEPHALID CESTODES: *MONIEZIA EXPANSA*, *THYSANOSOMA ACTINIOIDES*, AND *CITTOTAENIA PERPLEXA*

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THE NITROGEN AND AMINO ACID COMPOSITION OF THREE SPECIES OF ANOPLOCEPHALID CESTODES: *MONIEZIA EXPANSA*, *THYSANOSOMA ACTINIOIDES*, AND *CITTOTAENIA PERPLEXA*

CHAPTER I

INTRODUCTION

One of the characteristics of cestode physiology is a high rate of protein synthesis. The ability of cestodes to synthesize large amounts of protein is manifested by their high rate of egg production, which begins with maturation, and by the formation of proglottids, which is more or less continuous throughout the life of apolytic species. Although the ability to synthesize large amounts of protein has been known for quite some time, it is only recently that there have been investigations of the intermediary protein metabolism of these animals.

Early studies of this aspect of tapeworm physiology were concerned with the source of protein for these parasites. These studies were initiated by Chandler (1), who presented evidence that the rat tapeworm, *Hymenolepis diminuta*, is independent of the dietary protein of its host. The evidence presented by Chandler was based upon the effects of alterations of the host diet upon the cestodes. Independence from the host

diet for several vitamins was also shown for this species. On the other hand, H. diminuta was found to be very sensitive even to partial restriction of carbohydrate in the host diet. Chandler postulated that H. diminuta is capable of obtaining nitrogenous material by direct absorption from the host's intestinal mucosa. Earlier work by Reid (2) on Raillietina cesticillus in chickens had strongly suggested this idea. Reid found that this tapeworm is able to maintain its nitrogen content in hosts which are starved for sixteen to twenty hours. More substantiating evidence for this concept was presented by Chandler, et al. (3), who showed that H. diminuta is able to incorporate S^{35}-labeled thiamine administered parenterally to its host. The incorporation of radioactivity by the cestodes was of the same magnitude as that of the host's intestinal mucosa.

A more recent attempt to elucidate the relationship between the amino acids of the host and those of the parasite was made by Goodchild and Wells (4). Several stages of the life cycle of H. diminuta and the tissues of the hosts, with which these parasites are normally in contact, were analyzed for amino acids. A parallel was shown to exist between the qualitative distribution of the amino acids in the parasite and the tissue with which it was associated. The quantitative distribution of the amino acids was also roughly parallel between parasite and host tissue. It was found that there is a quantitative decrease in the amino acids present in specimens kept in Thiry-Vella fistulas when they are compared to worms taken from a normal intestine. Assuming there is a normal blood supply to these fistulas, this is contrary to what one would expect if these cestodes are capable of obtaining their amino acids from the tissues of

the host. In way of explanation, Goodchild and Wells suggest that "protein" is not as available from the fistular mucosa as from the normal gut and therefore, these fistulas are an atypical environment for the helminths. An alternative suggestion, not made by these authors, is that the glucogenic amino acids are utilized once the glycogen deposits are depleted. These amino acids cannot then be replaced in the absence of sufficient energy sources, in the form of glucose, for active absorption. However, the quantitative aspects of this work and the results of the acid hydrolyses, specifically the long times required to liberate certain amino acids and the destruction of the free amino acids, some of which are normally very stable to acid hydrolysis, are not made clear.

The *in vitro* uptake of amino acids by cestodes from their surrounding environment has been established. Daugherty (5) found that the rat tapeworm, *H. diminuta*, can actively absorb S^{35}-labeled methionine when incubated *in vitro*. Absorption of methionine is higher in the scolex region than in the strobilar region. This latter fact is consistent with Chandler's hypothesis that cestodes obtain their amino acids, as well as other nutritional requirements, from the host's intestinal mucosa. Since one would assume the scolex to be in more intimate contact with the intestinal mucosa than is the rest of the worm, its greater absorptive capacity is not surprising. However, Read (6) has pointed out that there may be a more intimate contact of strobila and intestinal mucosa than has previously been thought to exist. The study of the *in vitro* absorption of amino acids has been extended by Daugherty and Foster (7,8) to include *R. cesticillus* from chickens. In a comparative study of *R. cesticillus* and *H. diminuta*, it was shown that the former absorbs neutral amino acids

at a rate four to six times that of the latter. Both cestodes absorb dicarboxylic amino acids by a different mechanism than that involved in the absorption of neutral amino acids. The nature of these two mechanisms has not been explained.

Some of the different metabolic roles of these amino acids, once they are obtained by the helminths, have been elucidated in several studies on the intermediary amino acid metabolism of cestodes. Transamination in H. diminuta was first investigated by Aldrich, et al. (9). These workers demonstrated the presence of two active transaminase systems, alanine $\rightleftharpoons$ glutamate and aspartate $\rightleftharpoons$ glutamate, the latter system being the more active of the two. These enzyme systems are dependent upon an intact host endocrine system for their normal rate of activity, host orchidectomy resulting in a marked decrease in transaminase activity. Foster and Daugherty (10) recently made a comparative study of transamination in R. cesticillus and H. diminuta and showed the alanine-glutamate and aspartate-glutamate systems to be present in both species. R. cesticillus had the lower transaminase activity of the two species. The synthesis of amino nitrogen in H. diminuta was studied by Daugherty (11). This species was found to synthesize amino nitrogen in the presence of either alpha-ketoglutaric acid, pyruvic acid, or oxaloacetic acid and ammonium ions. Glucose, through its degradation to intermediary keto acids, serves as a substrate for the synthesis of amino nitrogen from ammonium nitrogen. In a survey of the amino acid oxidases of H. diminuta, Daugherty (12) showed the glutamic acid dehydrogenase system to be the most active. This enzyme system is not affected by alteration of the host's endocrine system nor by the absence of protein in the diet of the

host. Adaptation of the glutamic acid dehydrogenase enzyme system to a high-protein host diet was manifest by an increase in its activity.

Of the three species of cestodes used in the present study, Moniezia expansa is the only one that has been previously submitted to any biochemical analyses. Quantitative and qualitative analyses of the glycogen in M. expansa were made by Weinland (13). Von Brand (14) reported the chemical composition of M. expansa in terms of dry matter, glycogen, ether-soluble material, and nitrogen and qualitatively determined lactic acid, succinic acid, saturated and unsaturated fatty acids, hydroxy fatty acids, phosphatides, and glycerol. Osterlin and von Brand (15) continued the qualitative study of the hydroxy fatty acids and glycogen. Wardel (16, 17) reported the general chemical composition of M. expansa in terms of dry matter, glycogen, and nitrogen. More recently, Abdel-Akher and Smith (18) determined the number of glucose residues per unit chain length in glycogen isolated from M. expansa. The results of these researches are briefly summarized in the succeeding statements. The glycogen which occurs in M. expansa is similar to mammalian glycogen, both in its optical activity, which is strongly dextrorotatory, and in the number of glucose residues per unit chain length. This glycogen makes up 24 to 32% of the solid substance. However, since there is a marked correlation between the carbohydrate content of the host diet and the percentage glycogen in the parasites, these values are not too significant unless the experimental conditions are defined. Due to the linear decrease in the glycogen content of worms surviving in vitro and the rapid depletion of glycogen stores in worms when carbohydrate is absent from the host diet, this polysaccharide apparently serves as the energy

reserve for these animals. Dry matter accounts for approximately 10% of the weight of the worm. Of this dry matter, lipid accounts for approximately 30% and protein for around 35%. Von Brand (14) hypothesized that the high percentage of lipid is due to its being the end product of the anaerobic metabolism of carbohydrates. Kent (19, 20) and Kent and Macheboeuf (21, 22, 23, 24) made a rather extensive study of the protein complexes which occur in M. expansa and characterized several of these as protein-bile acid, protein-glycogen, and protein-cerebroside-glycogen complexes.

The general chemical composition of cestodes is reviewed by von Brand (25) and will not be discussed here. However, for comparative purposes, the results of previous analyses of the general protein and amino acid composition of cestodes warrant mention. In the works cited here, the protein content has been calculated from the data presented by the investigator(s) for the total nitrogen of the worms. The standard factor 6.25, based upon 16% nitrogen for most proteins, was used in the calculations. Smorodinzew and co-workers (26, 27, 28, 29) determined the nitrogen content of Taenia saginata, T. solium, and Diphyllobothrium latum. On the basis of their data, protein accounts for 33, 47, and 60%, respectively, of the solid matter of these three species. Smorodinzew and Pawlowa (30) studied these same cestodes relative to the amount of nitrogen in the following protein fractions: albumin, globulin, nucleoprotein, keratin, elastin, collagen, and reticulin. The percentage nitrogen in these fractions of T. saginata and T. solium was characteristically very constant and approximately equal for both species. D. latum is characterized by a greater percentage of nitrogen generally,

as well as in the albumin, globulin, and reticulin fractions, when compared to the other two species. Salisbury and Anderson (31) found 32% of the dry weight of lipid-extracted Cysticercus fasciolaris (=larvae of T. taeniaformis) to be protein. Hopkins (32) showed 36% of the dry weight of Schistocephalus solidus larvae to be protein. In preliminary work on the metabolism of Echinococcus granulosus, Agosin et al. (33) found 62% of the solid material of the hydatid scolices to be protein. Recently, Kent (34, 35) extended his studies of the protein complexes of cestodes to include R. cesticillus and H. diminuta. Four complexes were isolated from each of these cestodes. These differed in their composition for each species. The quantitative aspects of these analyses of the protein composition of cestodes are summarized in the discussion section.

Several amino acids have been identified in cestode tissue. Kent (19) reported the presence of ten or more amino acids in one of the protein-bile acid complexes of M. expansa. Of these ten, eight were identified by paper partition chromatography to be aspartic acid, glutamic acid, valine, serine, alanine, leucine, histidine, and arginine. In later work, Kent (20) indirectly confirmed the presence of glycine when glycocholic acid was identified as the bile acid of the protein complex. The amino acid composition of H. diminuta has been examined by several investigators. Aldrich et al. (9) identified eighteen amino acids and related substances in the free amino acid extracts of this parasite. They are alanine, arginine, aspartic acid, citrulline, ethanolamine phosphoric acid, glutamic acid, glutamine, glutathione, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine,

taurine, tyrosine, and valine. Goodchild and Wells (4) reported these amino acids in whole worm hydrolysates of adult and larval stages of H. diminuta with the exceptions of citrulline, ethanolamine phosphoric acid, glutamine, glutathione, and taurine. In addition to those reported by Aldrich et al., they reported cystine, cysteine, histidine, hydroxyproline, methionine, threonine, and tryptophan. Kent (34) identified alanine, arginine, glutamic acid, glycine, histidine, leucine, methionine, serine, threonine, tyrosine, and valine in four of the protein complexes of H. diminuta. Foster and Daugherty report that H. diminuta contains five freely occurring amino acids in addition to those found in R. cesticillus but do not identify them.

Two things are evident from this brief review of the present knowledge of protein metabolism in cestodes. First of all, the exact source of amino acids for these animals remains obscure. The failure of the absence of protein in the diet of the host to affect these parasites indicates that they can obtain their amino acids from the host's tissues. On the other hand, the in vitro absorption of amino acids by these helminths indicates that they are potentially capable of utilizing the hydrolysis products of the protein in the host diet. Secondly, investigations of the intermediary protein metabolism in cestodes have been limited to two species, H. diminuta and R. cesticillus. Both of these factors, the lack of specific knowledge of the source of amino acids utilized by cestodes and the limitation of studies of protein metabolism to two species, led to the present study. One of the main reasons for suspecting the independence of cestodes from the dietary protein of their hosts, given by Chandler (1), was the fact that mature tapeworms are not

always confined to the lumen of the intestine. Chandler postulated that these forms must obtain their nourishment from the tissues with which they are in contact since they are not in direct contact with the blood or tissue fluids.

On the basis of the above postulate, it was felt that a comparison of a form, which normally occurs outside the lumen of the intestine, with intestinal forms might prove of interest and might perhaps demonstrate significant differences in their nitrogen metabolism, thus reflecting differences between the availability of nitrogenous materials in the two environments. The three cestodes studied were selected first of all because they are considered to be closely related taxonomically; secondly, because they occur in herbivorous animals; and thirdly, because two of the species have never been subjected to any nitrogen or amino acid analyses. Since they occur in herbivorous animals, it was felt that the intestinal forms would be exposed to a somewhat similar host diet. The three species are: Moniezia expansa Rudolphi, 1810, from the small intestine of sheep, Thysanosoma actinioides Diesing, 1835, from the biliary passages and gall bladder of sheep, and Cittotaenia perplexa Stiles, 1895, from the small intestine of Sylvilagus floridanus, the cottontail rabbit.

CHAPTER II

MATERIALS AND METHODS

Specimens of M. expansa and T. actinioides were collected at a local slaughter house immediately after the death of their sheep host and were placed in warm (approximately 37° C) Tyrode's solution. This saline solution was contained in Thermos bottles in which the worms were transported back to the laboratory. Tyrode's solution was used by Wardle (16) in a study of the in vitro survival of M. expansa in different saline media. Although Wardle adjudged all saline solutions as unsatisfactory for long term experiments, Tyrode's solution gave good results for twenty-four hours and is approximately isotonic to M. expansa. Due to the findings of Wardle (16) and Smyth (36) that glucose added to the culture media decreases the longevity of cestodes in vitro, it was not added to the Tyrode's solution in the present work. Upon arrival at the laboratory, single worms were removed from the saline solution, washed quickly with three changes of distilled water, and blotted dry. Each worm was weighed, wrapped in aluminum foil and quickly frozen by exposure to solid carbon dioxide for approximately thirty minutes. The maximum time elapsed between collection from the host and freezing was not more than four hours, usually between two and three. The frozen specimens were kept at -20° C until analyzed. Positive species identification was made from slides

prepared of specimens taken from the same collection as those taken for the analyses. The specimens of C. perplexa were obtained from cottontail rabbits, Sylvilagus floridanus, which were shot in the field and brought back to the laboratory for autopsy. They were placed in Tyrode's solution after removal from the small intestine. Since infestations of these rabbits by C. perplexa and C. variabilis occur concurrently, one or two mature proglottids and one gravid proglottid from each worm were prepared for identification purposes. Identification was based on the taxonomic characters used by Arnold (37). The remainder of each worm was washed free of Tyrode's solution with three changes of distilled water and was frozen for storage as described for M. expansa and T. actinioides. All of the worms used in the analyses contained sexually mature proglottids.

Weights of M. expansa used in the quantitative work ranged from 3.638 to 12.821 grams (average = 6.130), T. actinioides from 0.421 to 0.909 grams (average = 0.587), and C. perplexa from 0.435 to 0.684 grams (average = 0.560). Dry weights of these specimens were determined by drying at 120° C for eighteen hours. Determinations of the dry weight of four individuals of each species were made. Solid matter ranged from 7.42 to 10.82% (average = 9.20%) of the wet weight of M. expansa, from 15.16 to 17.93% (average = 16.36%) of T. actinioides, and from 25.46 to 28.66% (average = 27.15%) of C. perplexa.

The following quantitative determinations were made on individual worms: total nitrogen (TN), non-protein nitrogen (NPN), and protein nitrogen (PN) by the Kjeldahl method; non-protein carboxyl nitrogen (NPCN) and protein carboxyl nitrogen (PCN) by the method of Van Slyke, MacFadyen, and Hamilton (38, 39, 40, 41).

Each worm was homogenized, while still frozen, in five or ten volumes of distilled water; five volumes were used for M. expansa and ten volumes each for T. actinioides and C. perplexa. Homogenization was effected in TenBroeck homogenizers. These homogenizers were fitted to such clearances that two to three minutes homogenization by hand gave an essentially cell-free homogenate as determined by routine microscopical examinations. Aliquots of the homogenate were taken for duplicate analyses of the total nitrogen. In initial trials on the determination of the total nitrogen of the homogenates, a linear relationship was obtained between amount of nitrogen and volume of homogenate. There was, however, some settling in the homogenates and, in cases where the two aliquots were not taken rapidly after homogenization, high duplicate differences resulted.

After the aliquots were taken for Kjeldahl determinations, four volumes of ice-cold absolute ethyl alcohol were added to the homogenate and it was placed in the refrigerator for two hours at 4° C. Ethyl alcohol was the protein precipitant of choice first of all, because it was found by Pollak and Fairbairn (42) to be just as effective as the other commonly used precipitants in separating the protein and non-protein fractions and, secondly, because Ägren and Nilsson (43) found it to give better results when the solutions are to be used for chromatography.

When precipitation was complete, the ethanolic homogenate was centrifuged at 4000 G for fifteen minutes. The crystal-clear supernate was decanted and the residue was washed twice with two ml portions of 80% (v/v) ethyl alcohol. After the final centrifugation, the washings were decanted and combined with the supernate. Aliquots of the combined

extract were taken for duplicate analyses of non-protein nitrogen and non-protein carboxyl nitrogen. The remainder of the solution was partitioned against three volumes of chloroform for approximately eight hours at 4° C, according to the method of Awapara (44). The aqueous layer was separated from the chloroform layer and the latter was washed with 0.01 N hydrochloric acid and repartitioned. Chromatography of the concentrated chloroform-ethyl alcohol layer showed that washing this layer with a solution of high ionic strength resulted in an essentially complete recovery of the amino acids present in the original solution. The aqueous layer and the washings from the chloroform-ethyl alcohol layer were combined and evaporated to dryness over steam. The residue from the evaporation was taken up in 10% (v/v) isopropyl alcohol to give approximately one mg nitrogen per ml of solution. The amino acids in this fraction are termed the non-protein amino acids and were determined qualitatively by paper partition chromatography.

The proteinaceous residue from the centrifugation was dried *in vacuo* over sulfuric acid and duplicate portions of this residue were taken for determinations of protein nitrogen. The remainder of the residue was refluxed for twenty-four hours at 120° C in 100 volumes peroxide-free 6 N hydrochloric acid prepared according to Ingram and Salton (45). Pilot experiments on the proteins of *M. expansa* indicated that the maximal amount of carboxyl nitrogen of the protein is released by eighteen hours hydrolysis under these conditions. The hydrolysate was filtered free of humin, decolorized with charcoal (Darco, Eastman), and repeatedly evaporated to dryness over steam to remove the hydrochloric acid. The residue from the final evaporation was taken up in 10%

isopropyl alcohol to give approximately one mg nitrogen per ml of solution. Duplicate aliquots of this solution were taken for both Kjeldahl nitrogen and Van Slyke carboxyl nitrogen determinations. The amino acids in this fraction are termed the protein amino acids and were identified by paper chromatography.

Alkaline hydrolyses for tryptophan were carried out by refluxing the protein residue for twenty hours at 120° C in 100 volumes of 0.3$ N barium hydroxide. The barium was removed from the hydrolysates by neutralization with either 2 N sulfuric acid or gaseous carbon dioxide. After filtering off the barium sulfate or barium carbonate, the hydrolysate was evaporated to dryness and the residue was taken up in 10% isopropyl alcohol to give approximately one mg nitrogen per ml of solution. Carboxyl nitrogen determinations and chromatography revealed the destruction of several of the amino acids by this method.

For comparative purposes, several specimens of Dugesia sp., a free-living planarian, were submitted to analysis. They were homogenized in ten volumes of water while still fresh and were otherwise treated as described for the cestodes. Four determinations of TN, NPN, NPCN, PN, and PCN were carried out on two batches of these planaria. One batch consisted of thirty individuals whose pooled weights totaled 0.924 grams and the other batch of twenty-five individuals weighed 0.783 grams. Four determinations of dry weight were made. The dry matter ranged from 15.97 to 16.98% (average = 16.30%) of the wet weight of the worms. These planaria had been starved for five days prior to the analyses to allow any food material in the gut to be completely digested.

For the determination of the free amino acids in sheep bile, four

volumes of ice-cold ethyl alcohol were added to forty-six ml of freshly collected bile. This was allowed to stand at 4° C for three hours and was then filtered. The filtrate was partitioned against three volumes of chloroform in the cold for twelve hours. The aqueous layer was collected and, the chloroform-ethyl alcohol layer was washed with 0.01 N hydrochloric acid. The washings were combined with the aqueous layer and, the combined extract was passed through a cation exchange resin (Dowex 50 H^+) column. The column was washed several times with water. After washing, the amino acids were eluted from the column with N ammonium hydroxide. The excess ammonia was removed from the eluate by repeated evaporation in vacuo. The residue was taken up in five ml of 10% isopropyl alcohol and was used for the qualitative determination of the free amino acids by paper chromatography.

Acid hydrolysis was carried out on sixty ml of the fresh sheep bile by refluxing it for twenty-four hours at 120° C with ten volumes of peroxide-free 6 N hydrochloric acid. The hydrolysate was filtered, decolorized with charcoal, and evaporated to dryness over steam. The residue was taken up in water and passed through a cation exchange column. After washing the column, the amino acids were eluted with N ammonium hydroxide and the excess ammonia was removed from the eluate by evaporation in vacuo. The residue was taken up in ten ml of 10% isopropyl alcohol and used for chromatography. This solution contained both the free and combined amino acids of the bile.

The amino acids of the non-protein and protein fractions were determined by ascending chromatography based on the method of Williams and Kirby (46). Whatman No. 1 chromatography paper was used in the form

of squares thirty by thirty centimeter. From 30 to 90 microliters of the protein amino acid solutions and from 60 to 180 microliters of the non-protein amino acid solutions of the cestodes and planarian were spotted for two-dimensional chromatograms. For the bile, 60 to 120 microliters of the free amino acid solution and 30 to 60 microliters of the hydrolysate were used. Approximately 30 to 50 microliters of each solution were used for one-dimensional chromatography. Development was carried out at room temperature (24-28° C). For routine two-dimensional chromatograms, the first dimensional solvent was the sec-butyl alcohol:formic acid:water (75:15:10 v/v) (BFW) solvent system of Hausman (47). Maximal separation of the amino acids was obtained by repeating the development in the first dimension once. After development in the first dimension, the papers were air-dried at room temperature. They were then sprayed with 0.1 M boric acid-0.1 N sodium hydroxide buffer, pH 8.3, except for the area of the paper which contained the amino acids. The papers were again air-dried and developed in the second dimension with the m-cresol: phenol:borate buffer (300:150:75 v/v) (CPB) solvent system of Levy and Chung (48). After development in this system, they were dried for two hours in a forced-air drying oven at 60° C to remove the phenolic solvent. The color reaction was carried out with the ninhydrin-acetic acid-collidine reagent of Levy and Chung (48) at 85 to 90° C. The above solvent systems were chosen for routine chromatography because they are relatively insensitive to small temperature fluctuations and can stand overloading to a considerable extent. A chart of the movement of some of the common amino acids in these two solvent systems is given in Figure 1. In place of the usual R_f values, which were found to fluctuate

considerably, the movement of these amino acids relative to one amino acid, in this case leucine, is used. This was found to be much more constant than their movement relative to the solvent front. The following abbreviations are used in Figure 1, and in all subsequent figures where applicable:

Alanine	ALA	Lysine	LYS
Alpha-aminobutyric acid	AAB	Methionine	MET
Alpha-aminoisobutyric acid	AAIB	Methionine sulfone	MET-SO_2
Arginine	ARG	Methionine sulfoxide	MET-SO
Asparagine	ASP-NH_2	Methylhistidine	HIS-CH_3
Aspartic acid	ASP	Norvaline	NORV
Beta-alanine	BALA	Ornithine	ORN
Beta-aminoisobutyric acid	BAIB	Phenylalanine	PHE
Citrulline	CIT	Proline	PRO
Cysteine	CYSTE	Sarcosine	SAR
Cystine	CYSTI	Serine	SER
Gamma-aminobutyric acid	GAB	Taurine	TAU
Glutamic acid	GLU	Threonine	THR
Glycine	GLY	Tryptophan	TRY
Histidine	HIS	Tyrosine	TYR
Hydroxyproline	HYD	3, 5-Diiodotyrosine	TYR-I_2
Isoleucine	ISO	Valine	VAL
Leucine	LEU		

Due to the low concentrations of the basic amino acids in the non-protein fractions, they were not well-separated on the routine two-dimensional chromatograms. Good separation of these amino acids in the non-protein fractions was obtained by using a phenol:water (3:1 v/v) (PW) system in the second dimension in place of the CPB system. For confirmatory experiments, both the n-butyl alcohol:acetic acid:water (4:1:5 v/v) (BAW) system of Partridge (49) and the n-butyl alcohol:propionic acid: water (BPW) system of Benson et al. (50) were used in the first dimension, followed by either the PW or CPB system in the second dimension. One-dimensional chromatography was carried out with either the BAW system or water-saturated gamma-collidine (2,4,6-trimethylpyridine).

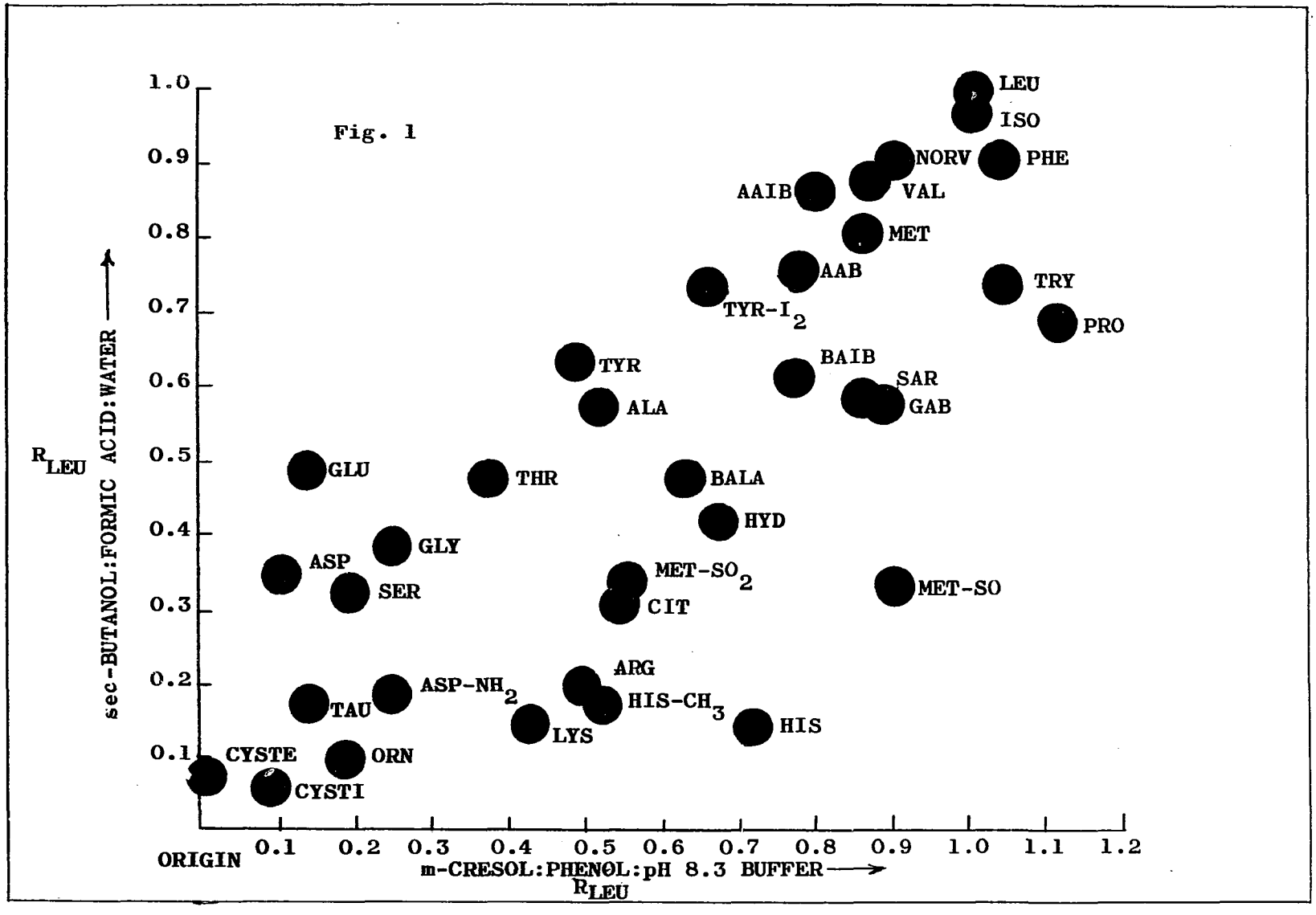


Fig. 1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R LEU
sec-BUTANOL:FORMIC ACID:WATER
LEU
ISO
NORV
PHE
AAIB
VAL
MET
AAB
TRY
TYR-I2
PRO
TYR
BAIB
SAR
ALA
GAB
GLU
THR
BALA
HYD
GLY
ASP
MET-SO2
SER
MET-SO
CIT
ARG
ASP-NH2
HIS-CH3
TAU
HIS
LYS
CYSTE
ORN
CYSTI
ORIGIN
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
m-CRESOL:PHENOL:pH 8.3 BUFFER
R LEU

Water-saturated collidine was first used by Consden et al. (51) as a solvent system in chromatography. This collidine solvent was also used for rechromatography after elution from two-dimensional chromatograms. Phenol (Baker Analyzed Reagent or Mallinckrodt, AR) was repurified by distillation from alkaline aluminum turnings. m-Cresol (Eastman, technical) was also purified by distillation. Gamma-collidine (Eastman, technical) was purified according to the method of Partridge (49). The butyl alcohols, n-butyl alcohol (Fisher, reagent grade) and sec-butyl alcohol (Matheson Coleman and Bell, B.P. 98-100° C), were used without additional purification.

Characterization and identification of several of the amino acids were made using specific color reactions which were carried out on the papers. Arginine was determined by the Sakaguchi reaction according to the method of Roche et al. (52); creatine was determined by the diacetyl reaction also according to Roche et al. (52); cystine and methionine by the reduction of the chloroplatinate reagent of Toennies and Kolb (53); histidine by diazotized p-anisidine after Sanger and Tuppy (54); ornithine by its reaction with vanillin as described by Curzon and Giltrow (55); proline and hydroxyproline by isatin as given by Smith (56); tryptophan and citrulline by Ehrlich's reagent (1% (w/v) p-dimethylaminobenzaldehyde in N HCl) as described by Block et al. (57); and tyrosine by alpha-nitroso-beta-naphthol according to the method of Acher and Crocker (58). The taurine spot was identified by elution from two-dimensional chromatograms and the subsequent cochromatography of the eluate with known taurine in water-saturated gamma-collidine. (See Figure 8.)

For the remainder of the amino acids, several methods of identification were used. The technique of elution and cochromatography as described above was used in some cases. The acidic ninhydrin-collidine reagent employed for the general color reaction gives very characteristic colors with the different amino acids and these colors were found useful in their identification. After carrying out the ninhydrin color reaction, the chromatograms were treated with 1%(w/v) copper nitrate in acetone to give a semi-permanent record. This technique is based upon the findings of Kawerau and Wieland (59). The colors formed after this treatment were also helpful in identifying certain amino acids, especially the beta- and gamma-amino acids.

Confirmation of the identity of all of the amino acids was made by the addition of 0.01 M standard solutions of known amino acids (Shandon Scientific Company) to the solutions which were being analyzed. If, upon chromatography, the added amino acid and the spot in question behaved as one spot, the identity of this spot was taken to be that of the added acid. This procedure was carried out with at least four solvent systems for each amino acid identified in the different fractions.

CHAPTER III

RESULTS

The results of the five nitrogen determinations carried out on the three species of cestodes studied are presented in Tables 1, 2, and 3. In these tables the following abbreviations are used: TN for total nitrogen, NPN for non-protein nitrogen, NPCN for non-protein carboxyl nitrogen, PN for protein nitrogen, and PCN for protein carboxyl nitrogen.

TABLE 1

NITROGEN DISTRIBUTION IN *MONIEZIA EXPANSA*
(MG NITROGEN PER 100 GRAMS TISSUE)

Specimen	TN	NPN	NPCN	PN	PCN
M-12	529	93	50	375	302
	532	89	56	383	322
	531[a]	91[a]	53[a]	379[a]	312[a]
M-13	474	91	36	347	281
	488	92	33	388	302
	481	92	35	368	292
M-14	425	70	23	258	194
	425	70	29	250	201
	425	70	26	254	198
M-15	523	75	31	412	329
	523	86	33	409	323
	523	81	32	411	326

TABLE 1 - Continued

Specimen	TN	NPN	NPCN	PN	PCN
M-16	476	78	28	377	329
	478	90	32	388	319
	477	84	30	383	324
M-17	776	109	32	628	512
	758	115	29	614	490
	767	112	31	621	501
M-18	507	92	22	368	302
	495	74	25	366	292
	501	83	24	367	297
M-19	650	98	37	522	356[b]
	653	98	39	509	329[b]
	652	98	38	516	343[b]
M-20	470	87	45	366	299[b]
	494	81	45	370	300[b]
	482	84	45	368	300[b]
M-21	600	85	36	474	280[b]
	605	86	33	476	279[b]
	603	86	35	475	280[b]
Mean	544	88	35	414	321

[a]Mean of the duplicate analyses.

[b]Determined from barium hydroxide hydrolysates; not included in statistical analyses nor calculation of mean.

TABLE 2

NITROGEN DISTRIBUTION IN THYSANOSOMA ACTINIOIDES
(MG NITROGEN PER 100 GRAMS TISSUE)

Specimen	TN	NPN	NPCN	PN	PCN
T-12	1185	142	56	630	517
	1132	137	59	630	543
	1159[a]	140[a]	58[a]	630[a]	530[a]

TABLE 2 - Continued

Specimen	TN	NPN	NPCN	PN	PCN
T-13	1545	173	65	1047	983
	1456	167	65	1114	939
	1501	170	65	1081	961
T-14	1284	167	93	871	756
	1276	159	93	893	746
	1280	163	93	882	751
T-15	1223	148	77	761	656
	1236	143	98	761	624
	1230	146	88	761	640
T-16	...[b]	175	101	904	821
	...[b]	178	88	958	771
	...[b]	177	95	931	796
T-17	1218	182	82	912	...[b]
	1214	169	82	927	...[b]
	1216	176	82	920	...[b]
T-18	1244	171	85	930	911
	1274	180	76	1043	808
	1259	176	81	987	860
T-19	1207	168	62	860	483[c]
	1229	163	68	848	460[c]
	1218	166	65	854	472[c]
T-20	1264	170	110	894	558[c]
	1314	165	100	862	544[c]
	1289	168	105	878	551[c]
T-21	1476	163	75	1075	624[c]
	1470	156	75	989	568[c]
	1473	160	75	1032	596[c]
Mean	1296	164	81	896	756

[a]Mean of the duplicate analyses.

[b]Determinations lost.

[c]Determined from barium hydroxide hydrolysates: not included in statistical analyses nor calculation of mean.

TABLE 3

NITROGEN DISTRIBUTION IN CITTOTAENIA PERPLEXA
(MG NITROGEN PER 100 GRAMS TISSUE)

Specimen	TN	NPN	NPCN	PN	PCN
C-9	1173	113	67	879	714
	1336	118	70	812	656
	1255[a]	116[a]	69[a]	846[a]	685[a]
C-10	1297	107	54	922	766
	1280	112	50	1003	806
	1289	110	52	963	786
C-11	1229	140	58	1009	903
	1204	135	65	986	887
	1217	138	62	998	895
C-12	1290	132	58	985	894
	1290	124	61	982	904
	1290	128	60	984	899
C-13	1236	130	76	1011	884
	1383	127	66	993	886
	1310	129	71	1002	885
C-14/15[b]	1358	119	53	1034	538[c]
	1414	127	53	1120	590[c]
	1386	123	53	1077	564[c]
Mean	1291	124	61	978	830

[a]Mean of the duplicate analyses.

[b]Two specimens combined.

[c]Determined from a barium hydroxide hydrolysate: not included in statistical analyses nor calculation of mean.

The main question concerning these data is whether there are significant differences between the three species in these nitrogen determinations. The criterion used is the variability between the means of each species as compared with the individual variability within each species. The most expeditious means of testing for this is by hierarchical analyses of variance. These analyses have been computed for each fraction and are presented in Tables 4, 5, 6, 7, and 8.

TABLE 4

ANALYSIS OF VARIANCE OF TOTAL NITROGEN

Source of Variation	D.F.	Mean Square	F
Between Species	2	3,349,699.201	167.2[a]
Individuals (total)	22	20,032.096	14.6[a]
C. perplexa	5	6,502.733	1.5 n.s.
T. actinioides	8	27,613.375	33.4[a]
M. expansa	9	20,809.494	323.4[a]
Duplicates (total)	25	1,368.280	
C. perplexa	6	4,352.333	
T. actinioides	9	827.722	
M. expansa	10	64.350	

[a] $P \ll 0.0005$

TABLE 5

ANALYSIS OF VARIANCE OF NON-PROTEIN NITROGEN

Source of Variation	D.F.	Mean Square	F
Between Species	2	12,443.482	46.5[a]
Individuals (total)	23	267.536	9.9[a]
C. perplexa	5	200.533	11.4[b]
T. actinioides	9	318.800	12.6[a]
M. expansa	9	253.494	7.5[b]
Duplicates (total)	26	26.904	
C. perplexa	6	17.667	
T. actinioides	10	25.400	
M. expansa	10	33.950	

[a] $P \ll 0.0005$

[b] $P \ll 0.01$

TABLE 6

ANALYSIS OF VARIANCE OF NON-PROTEIN CARBOXYL NITROGEN

Source of Variation	D.F.	Mean Square	F
Between Species	2	10,538.971	39.8[a]
Individuals (total)	23	264.983	12.0[a]
C. perplexa	5	121.483	8.0[b]
T. actinioides	9	454.111	10.9[a]
M. expansa	9	155.578	23.6[a]
Duplicates (total)	26	22.135	
C. perplexa	6	15.250	
T. actinioides	10	41.800	
M. expansa	10	6.600	

[a] $P \ll 0.0005$

[b] $P \ll 0.05$

TABLE 7

ANALYSIS OF VARIANCE OF PROTEIN NITROGEN

Source of Variation	D.F.	Mean Square	F
Between Species	2	1,641,309.25	69.0
Individuals (total)	23	23,785.98	24.2[a]
C. perplexa	5	11,433.60	7.1[b]
T. actinioides	9	34,170.16	23.2[a]
M. expansa	9	20,264.22	174.1[a]
Duplicates (total)	26	982.37	
C. perplexa	6	1,609.00	
T. actinioides	10	1,472.35	
M. expansa	10	116.40	

[a] $P \ll 0.0005$

[b] $P \ll 0.05$

TABLE 8

ANALYSIS OF VARIANCE OF PROTEIN CARBOXYL NITROGEN

Source of Variation	D.F.	Mean Square	F
Between Species	2	953,720.822	35.2[a]
Individuals (total)	15	27,077.174	41.0[a]
C. perplexa	4	17,486.000	32.8[b]
T. actinioides	5	47,557.550	33.9[a]
M. expansa	6	16,404.310	142.6[a]
Duplicates (total)	18	660.528	
C. perplexa	5	532.400	
T. actinioides	6	1,403.750	
M. expansa	7	115.000	

[a] $P \ll 0.0005$

[b] $P \ll 0.01$

In each statistical table, the mean square for total variability among individuals within each species gives an estimate of error for testing the variability between species: the five F values range from 35.2 to 167.2 and are significant at the 0.0005 level. The total variability of duplicate measurements on individual worms supplies an estimate of error for testing the variability among species: the F values range from 9.9 to 41.0 and, again, are all significant at the 0.0005 level.

The validity of the assumption that the technical variability is the same for all species must be established before conclusions can be drawn from these analyses of variance. To examine this aspect of the analyses, the contributions of each species to estimates of both the "total individuals" and "total duplicates" variability have been isolated and recorded in the tables. The mean squares give a strong impression of a tendency to vary from species to species. This tendency which appears in breakdowns of both the "duplicates" and "individuals" errors makes the assumption of homogeneity of variance somewhat dubious. Since the variances do not show a tendency to increase with the means, it is difficult to find a transformation which will stabilize the variance.

Every effort was made to treat all three species alike and, it is felt that the variability between duplicate measurements is not related to species differences nor to the manner in which each species was treated. A possible source of species differences exists, however, in regards to the technical aspects of the analyses, in the larger amount of tissue available for M. expansa analyses in comparison to T. actinioides and C. perplexa, both of which were of about equal weight. Taking the conservative viewpoint that there may be genuine heterogeneity among

the species with respect to technical ("duplicates") variability, the observations can be treated as three separate experiments, one for each species. The "individuals" mean squares can be tested against the "duplicates" mean squares within each species. Treatment in this manner gives a range of from 1.5 to 323.4 for the F values. The F value of 1.5 for total nitrogen in C. perplexa is not significant, but the other fourteen F values are significant to the 5% level or lower and, in those cases, substantiate the rejection of the null hypothesis that there is negligible variability about the species means.

From an examination of the raw data, the standard deviations for the three species would be expected to differ. In both the analysis of variance and ordinary t-tests, significance can be produced either by differences in variability or central tendency. However, if the individual mean measurements are ranked from high to low within each type of nitrogen determination, it can be determined whether or not species groupings occur. If M stands for M. expansa, T for T. actinioides, and C for C. perplexa, then the sequence of individual means is as follows:

Total nitrogen:

T-T-C-C-C-T-C-T-T-C-T-T-C-T-T-M-M-M-M-M-M-M-M-M-M

Non-protein nitrogen:

T-T-T-T-T-T-T-T-T-T-C-C-C-C-C-M-C-M-M-M-M-M-M-M-M-M

Non-protein carboxyl nitrogen:

T-T-T-T-T-T-T-C-C-T-T-C-C-T-(M and C)-C-M-M-M-M-M-M-M-M-M

Protein nitrogen:

T-C-T-C-C-T-C-C-T-T-T-T-T-C-T-T-M-M-M-M-M-M-M-M-M-M

Protein carboxyl nitrogen:

T-C-C-C-T-T-C-T-C-T-T-M-M-M-M-M-M-M

The one test of significance that can be applied here and which disengages the question of central tendency from other considerations is the extensions of the median test as described by Siegel (60). This procedure tests tendencies for groups to fall above and below the median common to all samples. When this test is applied, the following values of chi square, each with two degrees of freedom, are observed: 15.5 (TN), 20.0 (NPN), 17.1 (NPCN), 16.3 (PN), and 12.7 (PCN). All are significant at the 10% level. In all these determinations, the individuals of M. expansa form a more or less distinct group from T. actinioides and C. perplexa. The three essentially separate groups, one for each species, found in the non-protein nitrogen fraction, may be a reflection of three distinctive metabolic turnover rates for nitrogen in these three species. This fraction contains, in addition to the free amino acid pool, the nitrogenous end products of intermediary protein metabolism.

Thus, in these statistical treatments, even the most conservative tests lead to the conclusion that there is considerable variation within each species with respect to all five determinations of nitrogen. In regard to species differences, M. expansa is distinct as a species from both T. actinioides and C. perplexa in the amount of nitrogen in the different fractions. T. actinioides and C. perplexa do not differ significantly from each other. There does, however, appear to be three distinct groups, one for each species, in the amount of Kjeldahl nitrogen in the non-protein fractions.

Paper chromatography revealed the presence of nineteen amino acids common to the protein fraction of all three species of anoplocephalid cestodes studied. Eighteen of these have been positively identified.

From seventeen to twenty-two amino acids were identified in the non-protein fractions of these species of worms. It was within these non-protein fractions that qualitative differences between the three species were found. Table 9 lists the amino acids identified in the non-protein and protein fractions of the three cestodes. In this table, and subsequent tables, PAA is used to designate the protein amino acids and NPAA is used to designate the non-protein amino acids. Composite drawings of chromatograms of these two fractions are depicted in Figures 2-7 for each species. In these figures, broken lines representing an amino acid indicate that with the designated volume of solution used for spotting the chromatograms, this amino acid would not show up with the ninhydrin color reaction but, was present when tested for with a specific color reagent or when larger quantities of the solution were applied to the chromatograms.

The identity of some of the amino acids is still questionable and needs further confirmation. Citrulline, or the spot represented as such, was not consistent in its occurrence in different individuals of the same species. Since citrulline and methionine sulfone have very similar R_{Leu} values (movement relative to leucine) in all the solvent systems used and, since there is no appreciable difference in the color reaction of citrulline and methionine sulfone with the ninhydrin-collidine reagent, definite conclusions cannot be made concerning the identity of this spot. The spot is designated as citrulline because of two observations: first of all, it has a slightly lower R_{Leu} value in BFW than methionine sulfone and secondly, a very faint reaction was sometimes noted at this spot when the papers were treated with Ehrlich's reagent. This reagent gives

TABLE 9

COMPARATIVE AMINO ACID COMPOSITION OF *MONIEZIA EXPANSA*, *THYSANOSOMA ACTINIOIDES*, AND *CITTOTAENIA PERPLEXA*

Amino Acid	*M. expansa*		*T. actinioides*		*C. perplexa*	
	PAA	NPAA	PAA	NPAA	PAA	NPAA
alpha-alanine	+	+	+	+	+	+
beta-alanine	-	+	-	+	-	+
gamma-aminobutyric	-	+	-	-	-	+
beta-aminoisobutyric	-	+	-	+	-	+
arginine	+	+	+	-	+	-
aspartic acid	+	+	+	+	+	+
citrulline	+?	+?	-	-	-	+?
creatine	-	-	-	-	-	-
cysteine/cystine	+	+	+	+	+	+
glutamic acid	+	+	+	+	+	+
glycine	+	+	+	+	+	+
histidine	+	+	+	+	+	+
leucine/isoleucine	+	+	+	+	+	+
lysine	+	+	+	+	+	+
methionine	+	+	+	+	+	+
ornithine	d	+v	d	+v	d	-
phenylalanine	+	+	+	-	+	-
proline	+	+	+	+	+	+
serine	+	+	+	+	+	+
taurine	+	+v	+	+v	+	-
threonine	+	+	+	+	+	+
tryptophan	+	-	+	-	+	-
tyrosine	+	+	+	+	+	-
valine	+	+	+	+	+	+
unidentified	1	0	1	0	1	0

\+ presence of the amino acid
\- absence of the amino acid
d see text for discussion
v occurrence variable from individual to individual
? identity not confirmed

Fig. 2

Moniezia expansa
Non-protein amino acids
160µl extract
Ninhydrin color reaction

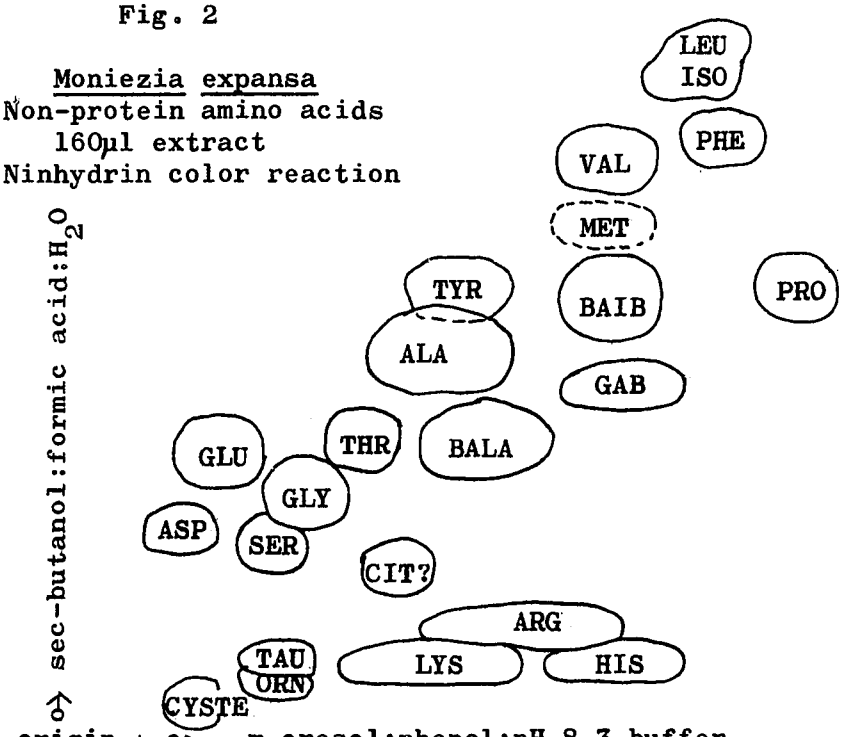


Fig. 3

Moniezia expansa
Protein amino acids
60µl extract
Ninhydrin color reaction

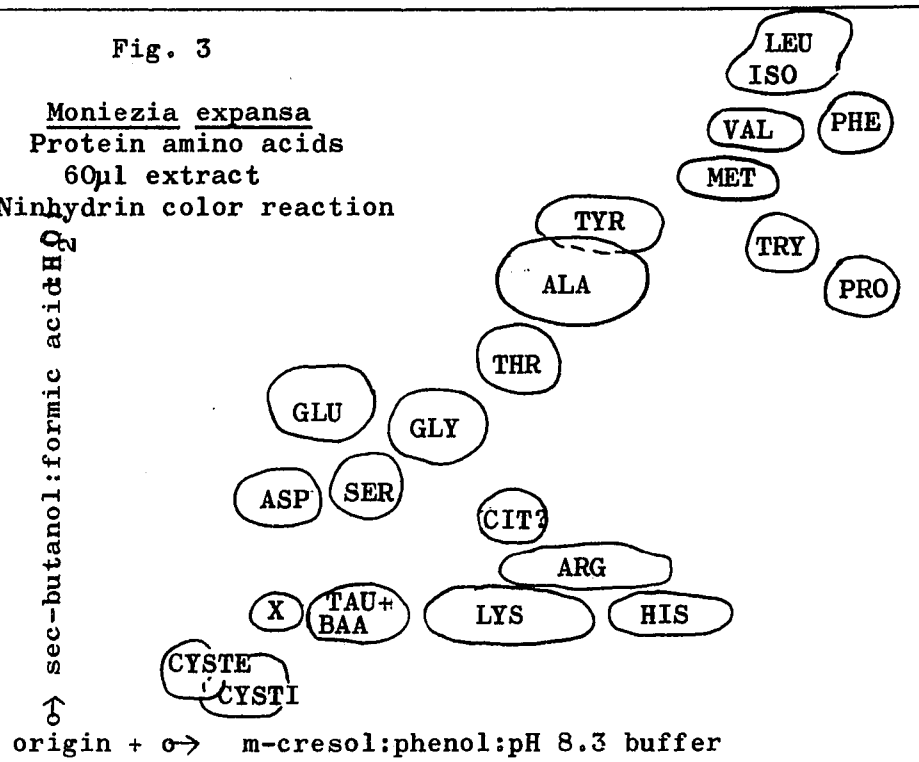


Fig. 4

Thysanosoma actinioides
Non-protein amino acids
160µl extract
Ninhydrin color reaction

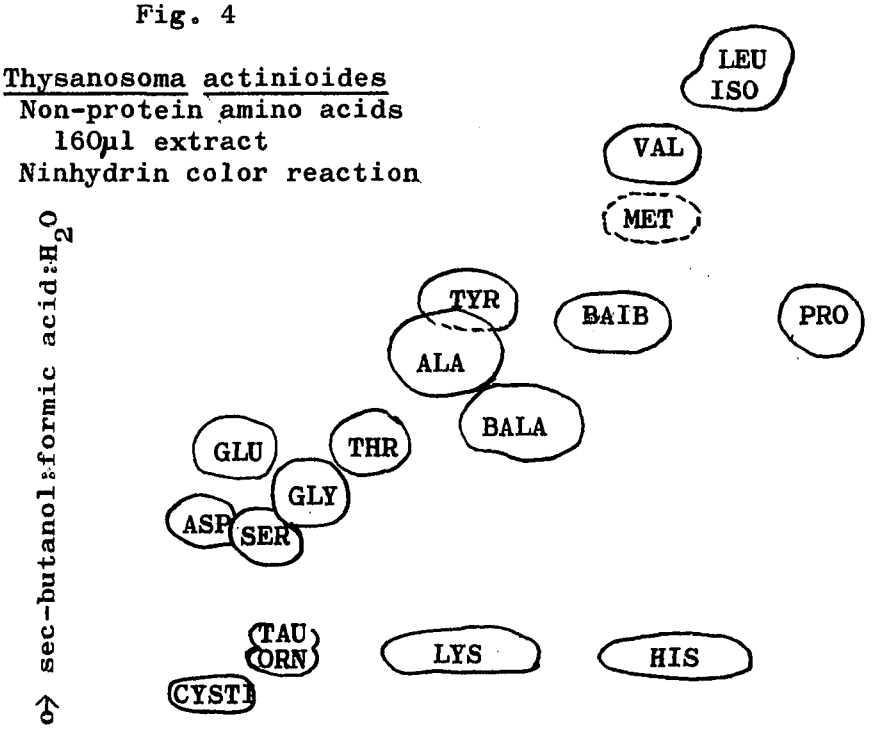


Fig. 5

Thysanosoma actinioides
Protein amino acids
60µl extract
Ninhydrin color reaction

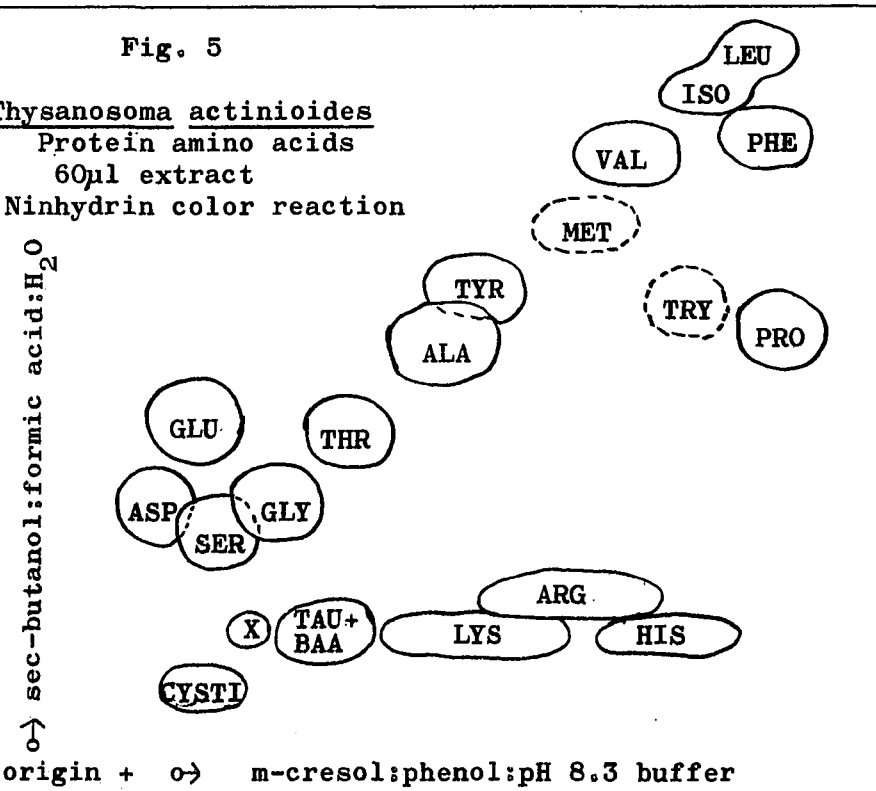


Fig. 6

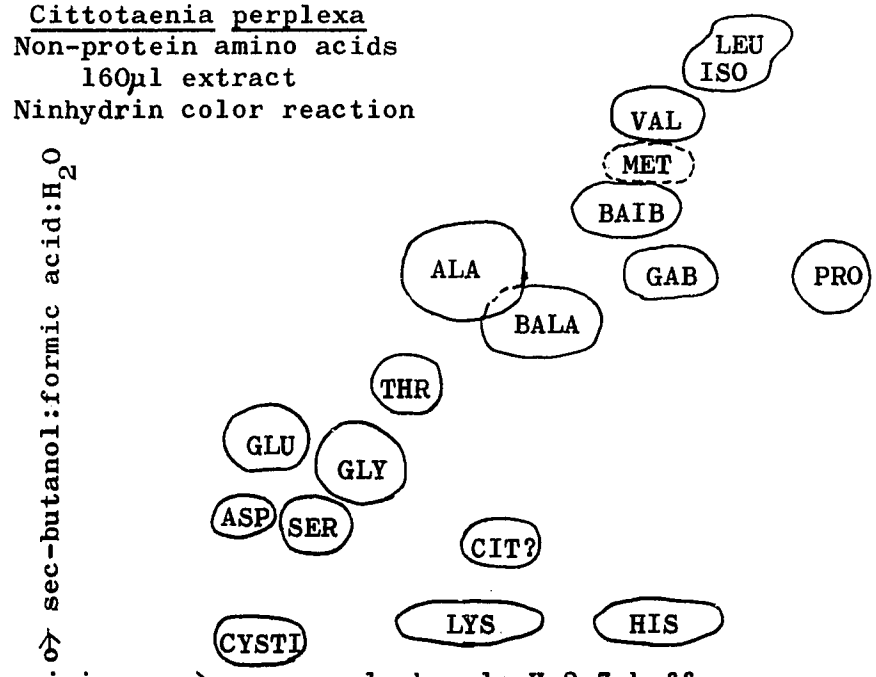
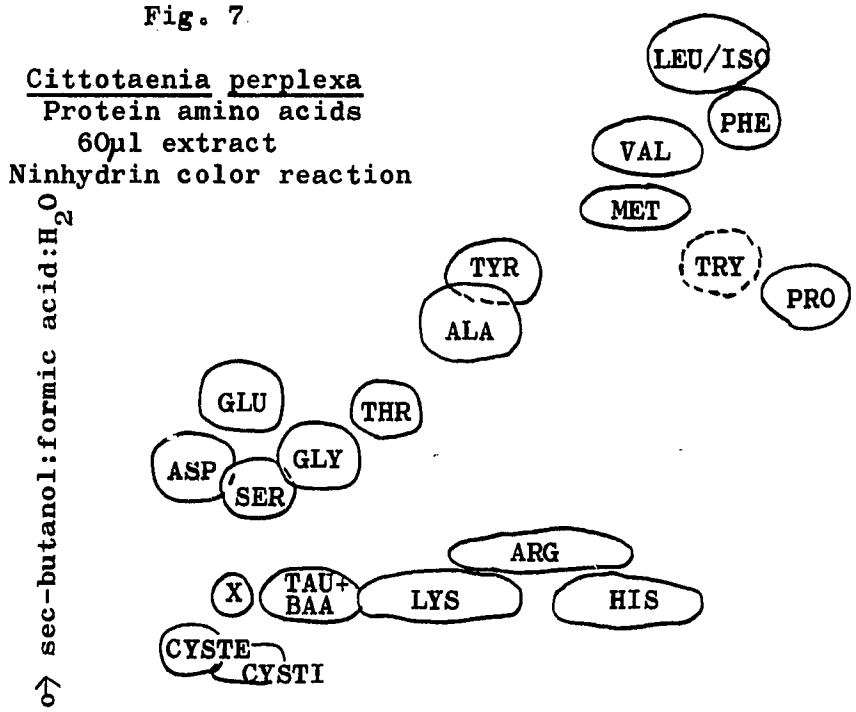

Cittotaenia perplexa
Non-protein amino acids
160µl extract
Ninhydrin color reaction
LEU
ISO
VAL
MET
BAIB
ALA
GAB
PRO
BALA
THR
GLU
GLY
ASP
SER
CIT?
LYS
HIS
CYSTI
o→ sec-butanol:formic acid:H2O
origin + o→ m-cresol:phenol:pH 8.3 buffer

Fig. 7


Cittotaenia perplexa
Protein amino acids
60µl extract
Ninhydrin color reaction
LEU/ISO
PHE
VAL
MET
TRY
PRO
TYR
ALA
GLU
THR
ASP
GLY
SER
ARG
X
TAU+
BAA
LYS
HIS
CYSTE
CYSTI
o→ sec-butanol:formic acid:H2O
origin + o→ m-cresol:phenol:pH 8.3 buffer

a yellow color with citrulline. The possibility that this is methionine sulfone, an oxidation product of the methionine present in these extracts, does exist.

According to Meister (61), ornithine has never been reported as a constituent of protein although it has been reported in peptides. Its presence in the protein fractions was established by the following procedure. The spot designated as "TAU + BAA" was eluted from two-dimensional chromatograms of the hydrolysates and, the eluates were rechromatographed one-dimensionally in water-saturated gamma-collidine along with pure lysine, arginine, ornithine, and taurine (Figure 8). Treatment of the resulting chromatogram with ninhydrin revealed the presence of three distinct spots in each eluate; one unknown, one identical in R_f to taurine, and the other a basic amino acid (BAA). The unknown spot with the highest R_f value may be identical to the spot designated as "X" on the two-dimensional chromatograms of the hydrolysates. Since the eluted spot is well-separated from the other basic amino acids and corresponds in position to ornithine, the spot with almost negligible movement in the collidine solvent is assumed to be ornithine. Another basis for this assumption is the fact that one-dimensional chromatograms of these hydrolysates developed in BAW give a strong reaction with vanillin at the spot corresponding in R_f to added ornithine. The occurrence of ornithine in the protein fraction, as well as in the non-protein fraction, may be due to the hydrolysis of arginine either during the preparation of the solutions for chromatography or during the process of chromatography. As judged from the density of the ninhydrin reaction, the concentration of ornithine in the hydrolysates is approximately

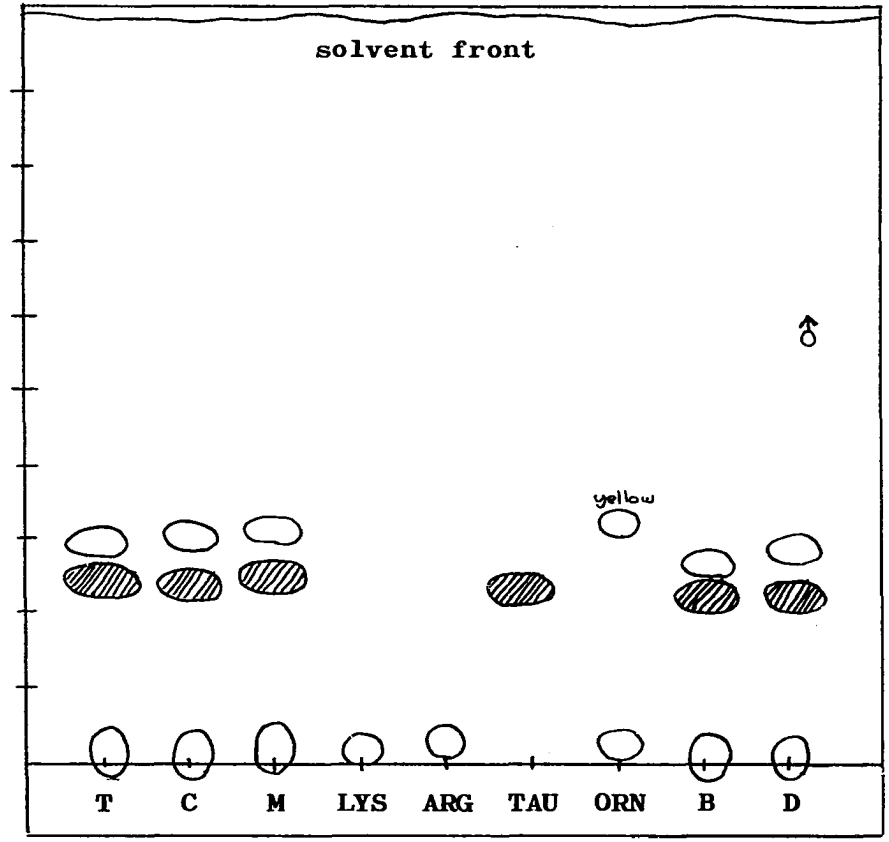


Fig. 8 One-dimensional chromatogram of "TAU+BAA" spots eluted from two-dimensional chromatograms of protein hydrolysates. M = M. expansa, C=C. perplexa, T=T. actinioides, B=Bile, and D=Dugesia sp. Development with water-saturated gamma-collidine.

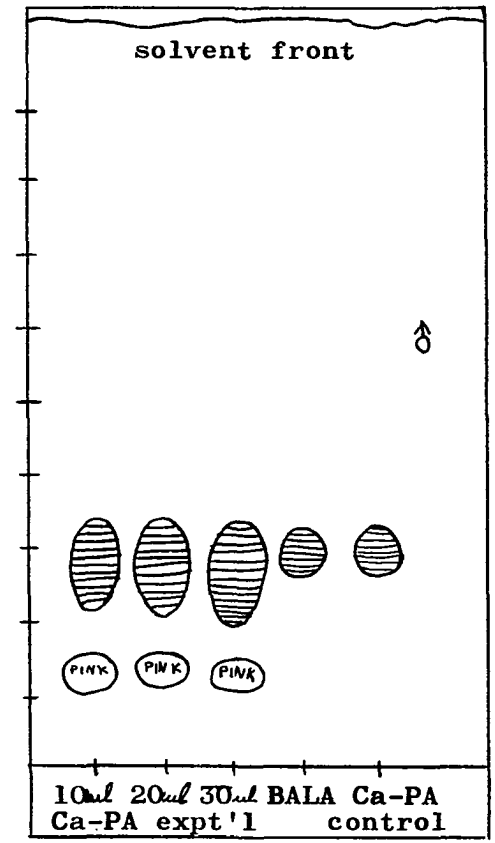


Fig. 9 One-dimensional chromatogram of calcium pantothenate. Development with BAW. See text for discussion.

equal to the concentration of either arginine or lysine. Ornithine occurs in low concentration in the non-protein fraction of M. expansa and T. actinioides as indicated by its weak reaction with ninhydrin. At these low concentrations, it separates from taurine on the two-dimensional chromatograms. When either taurine or ornithine are present in higher concentration, they do not separate on the two-dimensional chromatograms. The occurrence of ornithine in the non-protein fraction is variable from individual to individual within the two species.

Taurine is also variable in its occurrence in the non-protein fractions of M. expansa and T. actinioides. It occurs consistently in the protein fractions of all three species of cestodes as well as in the protein fraction of Dugesia sp. and in the bile hydrolysate. Its presence in the protein fractions may be due to its limited solubility in the concentration of ethyl alcohol used to separate the protein from the non-protein fraction. The findings of Kent and Macheboeuf (19-24) that bile acids are associated with some of the proteins of M. expansa may indicate a possible origin of taurine in this fraction. However, Kent (20) found glycocholic acid to be the bile acid of these complexes. Sulfur was not present in detectable quantities which would indicate that taurocholic acid is absent.

Cystine and cysteine are listed together in Table 9 since they, too, are variable in the individual worms and also, because it was found by Dent (62) that cystine is converted to cysteine in certain instances during chromatography. Leucine and isoleucine were not clearly separable with any of the solvent systems tried, including systems specifically described for their separation by Block and Weiss (63). Indications

that both of these isomers are present were obtained in some of the chromatograms (see figures). The small size of papers used in this work may account for failure to resolve these two isomers.

Beta-alanine occurs in the non-protein fractions of all cestodes studied. Since it may be associated with lipid metabolism in these worms, its possible origin from pantothenate was investigated. A 0.01 M solution of calcium pantothenate (Nutritional Biochemicals) was treated in the same manner as the non-protein fractions of the helminths. Ten to thirty microliters of the solution (CA-PA expt'l.) were spotted on a chromatogram. Five microliters each of an untreated 0.01 M solution of calcium pantothenate (CA-PA control) and 0.01 M beta-alanine were also spotted. After development in BAW and treatment with ninhydrin, the results depicted in Figure 9 were obtained. There was extensive hydrolysis of the pantothenate during the preparation procedure used for the non-protein fractions of the helminths and also during chromatography.

The quantitative data for the nitrogen determinations in Dugesia sp. are presented in Table 10. When these data are ranked with the data from the cestodes (see page 29), they are all higher with the exception of the protein carboxyl nitrogen group. In this group, one of the observations for Dugesia sp. is only slightly lower than the highest value found for T. actinioides.

In Table 11 are listed the amino acids found to occur in the non-protein and protein fractions of Dugesia sp. A fairly high concentration of asparagine, as indicated by the intensity of the ninhydrin reaction, occurs in the non-protein fraction of this free-living planarian. This

TABLE 10

NITROGEN DISTRIBUTION IN *DUGESIA* SP.
(ML NITROGEN PER 100 GRAMS TISSUE)

Specimen	TN	NPN	NPCN	PN	PCN
P-1	1733	218	121	1348	1086
	1662	247	131	1352	1089
	1698[a]	233[a]	126[a]	1350[a]	1088[a]
P-2	1674	225	126	1272	968
	1678	227	119	1241	945
	1676	226	123	1257	957
Mean	1687	230	125	1304	1023

[a]Mean of the duplicate analyses.

TABLE 11

AMINO ACID COMPOSITION OF *DUGESIA* SP.

Amino Acid	Protein Amino Acids	Non-Protein Amino Acids
alpha-alanine	+	+
arginine	+	+
asparagine	-	+
citrulline	+?	-
creatine	-	-
cystine/cysteine	+	+
glutamic acid	+	+
glycine	+	+
histidine	+	+
leucine/isoleucine	+	+
lysine	+	+
methionine	+	+
ornithine	d	-
phenylalanine	+	+
proline	+	+
serine	+	+
taurine	+	-
threonine	+	+
tryptophan	+	-
valine	+	+
unidentified	1	0

\+ presence of the amino acid
\- absence of the amino acid
d see text for discussion
? identity not confirmed

amide was not detected in the cestodes. Composite drawings of chromatograms of the non-protein and protein fractions of the planarian are given in Figures 10 and 11.

The free and combined amino acids of sheep bile are listed in Table 12 and are depicted in Figures 12 and 13. The four ninhydrin-positive spots, X_1, X_2, X_3, and X_4, in the hydrolysate were not identified. They may possibly be peptides which were not hydrolyzed during the preparation of the solution for chromatography.

Fig. 10

Dugesia sp. non-protein amino acids
160µl extract
Ninhydrin color reaction

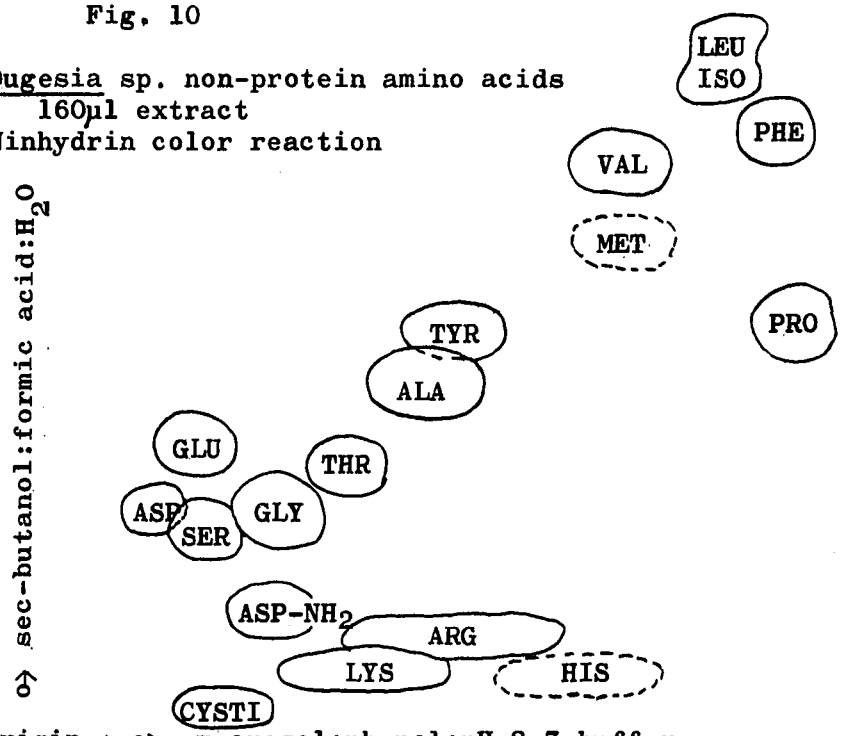


Fig. 11

Dugesia sp. protein amino acids
80µl extract
Ninhydrin color reaction

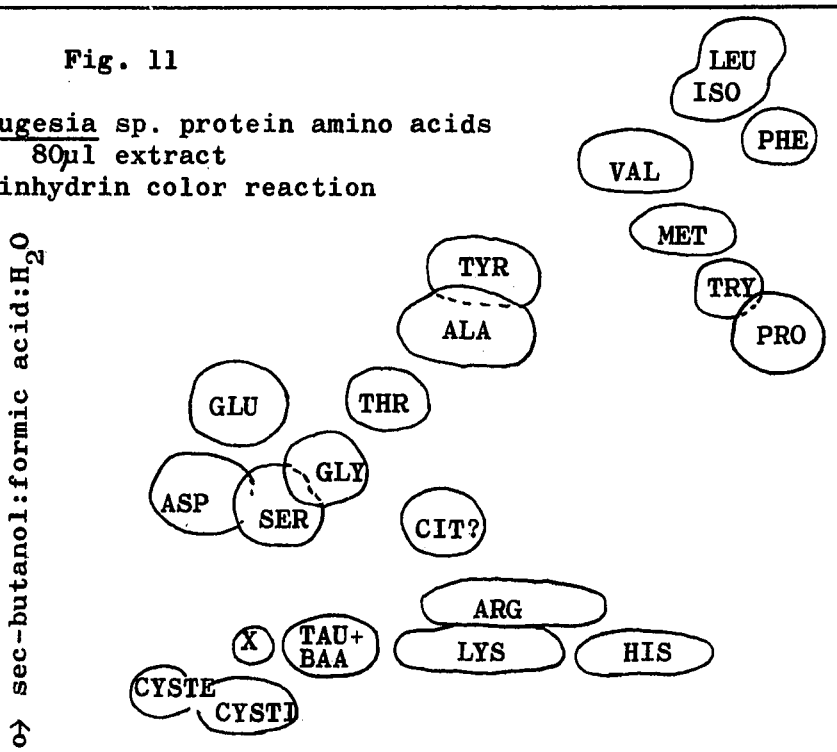


TABLE 12

AMINO ACIDS IN SHEEP BILE

Amino Acid	Free Plus Combined Amino Acids	Free Amino Acids
alpha-alanine	+	+
beta-alanine	+	+
arginine	+	+
aspartic acid	+	+
citrulline	+?	+?
creatine	-	-
cystine/cysteine	+	+
glutamic acid	+	+
glycine	+	+
histidine	+	-
leucine/isoleucine	+	+
lysine	+	+
methionine	+	+
ornithine	d	-
phenylalanine	+	+
proline	+	+
serine	+	+
taurine	+	-
threonine	+	+
tryptophan	+	-
tyrosine	+	+
valine	+	+
unidentified	4	1

+ presence of the amino acid
- absence of the amino acid
d see text for discussion
? identity not confirmed

Fig. 12

Bile free amino acids
120µl extract
Ninhydrin color reaction

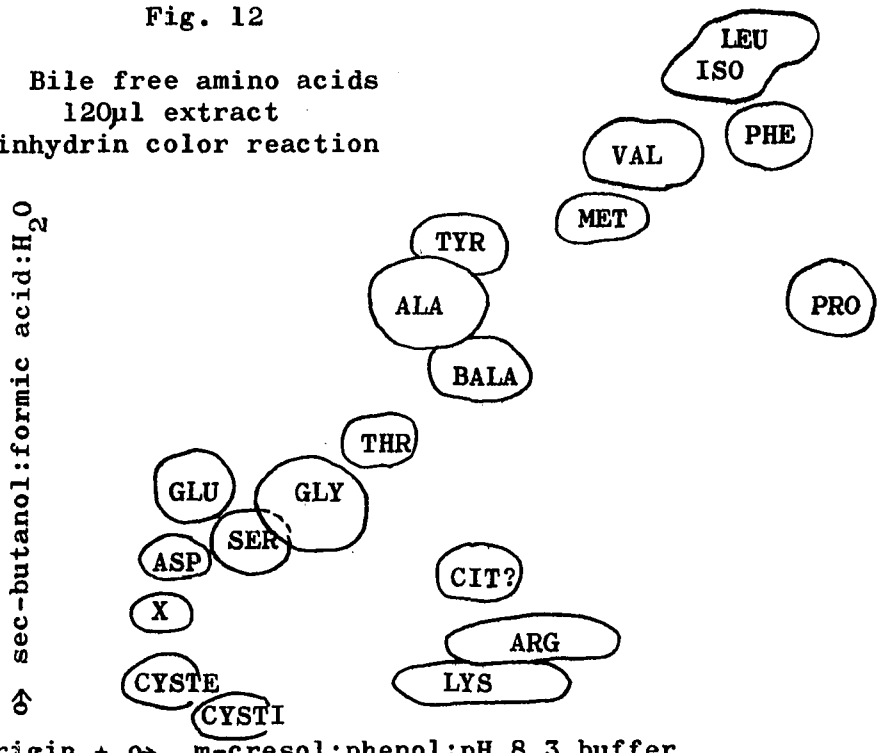
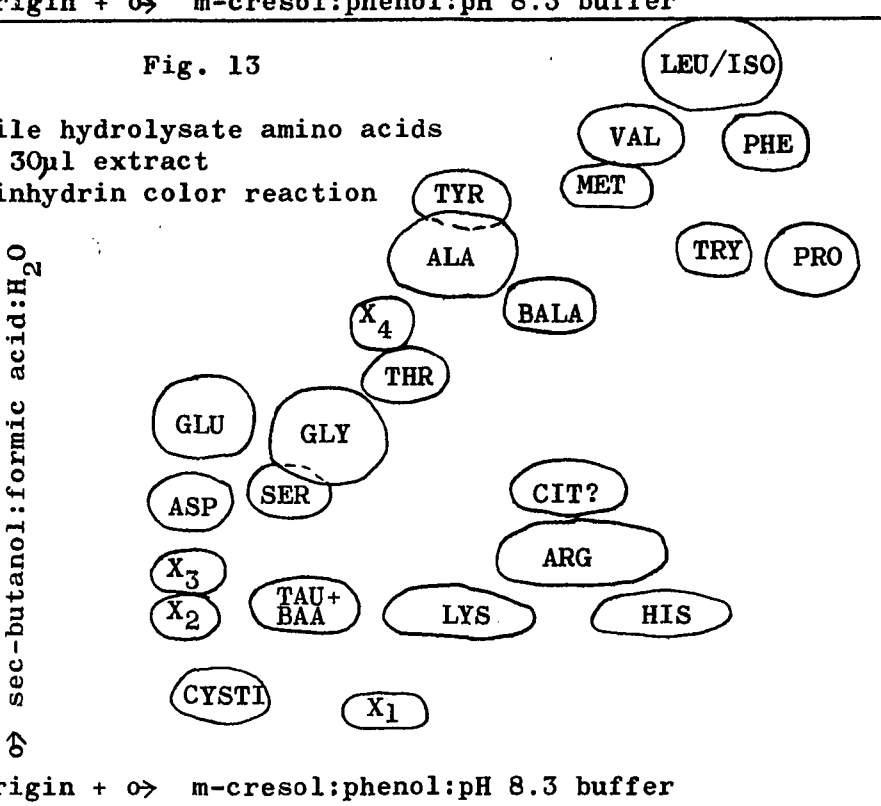


Fig. 13

Bile hydrolysate amino acids
30µl extract
Ninhydrin color reaction



CHAPTER IV

DISCUSSION

For comparative purposes, the percentages of protein which have been reported for cestodes, together with the present findings, are presented in Table 13. The protein content of cestodes, as well as other biological materials, is usually assumed to be 6.25 times the total nitrogen. The use of the factor 6.25 is based upon the observation that proteins contain on the average 16% nitrogen. On the basis of Salisbury and Anderson's (31) data, Reid (2) has pointed out that this assumption is questionable for cestodes. Salisbury and Anderson found 20% of the total nitrogen of lipid-extracted Cycticercus fasciolaris to be extractable with water. Of this 20%, only 7% was precipitable with trichloroacetic acid and could be considered protein. This leaves a large percentage of the total nitrogen of these larvae as non-protein nitrogen. In this investigation, the non-protein nitrogen averaged 16.2% for M. expansa, 12.7% for T. actinioides, and 9.6% for C. perplexa. Even the assumption that the ethanol precipitable nitrogen fraction represents the tissue proteins is in considerable error, due to the large amount of non-amino acid nitrogen in this fraction. Probably the most accurate estimate of the protein nitrogen of these helminths is the protein carboxyl nitrogen. The only error introduced in

TABLE 13

PERCENTAGE PROTEIN OF VARIOUS CESTODES

Specimen	Reference	% Water	% Nitrogen	% Protein[a] Wet Wt.	% Protein[a] Dry Wt
Cittotaenia perplexa	Present	72.85 (71.34-74.54)[b]	1.29 (1.22-1.39)	8.06 (7.63-8.69)	30.00
Cysticercus fasciolaris	(31)	---	5.19[c]	---	32.44
Diphyllobothrium latum	(29)	90.50 (80.99-96.61)	0.91 (0.63-1.52)	5.68 (3.94-9.50)	59.88
Echinococcus granulosus hydatid scolices	(33)	85.20 (±1.17)	1.47 (±0.08)	9.19 (±0.48)	62.06
Moniezia expansa	(14)	88.96 (86.79-91.51)	0.67 (0.56-0.82)	4.19 (3.50-5.13)	37.94
Moniezia expansa	(16, 17)	86.6	---	4.86	36.27
Moniezia expansa	Present	90.8 (89.18-92.58)	0.54 (0.43-0.77)	3.38 (2.69-4.81)	36.69
Raillietina cesticillus	(2)	79.46 (75.50-83.10)	1.14 (1.04-1.41)	7.13 (6.50-8.82)	34.69
Schistocephalus solidus plerocercoids	(32)	68.22 (±0.293)	a) 1.81 b) 1.84	a) 11.31 b) 11.50	36.00

TABLE 13 - Continued

Specimen	Reference	% Water	% Nitrogen	% Protein[a] Wet Wt.	% Protein[a] Dry Wt.
Taenia saginata	(26)	87.82 (86.90-88.50)	0.64 (0.56-0.73)	4.00 (3.50-4.56)	32.88
Taenia solium	(28)	91.29 (87.27-94.08)	0.65 (0.47-9.93)	4.06 (2.94-5.81)	46.63
Thysanosoma actinioides	Present	83.64 (82.07-84.84)	1.30 (1.16-1.50)	8.13 (7.25-9.38)	49.69

[a]6.25 X total nitrogen.

[b]Extreme values or standard deviation given in parentheses.

[c]Percentage of dry weight.

this estimate is the destruction of the amino acids by acid hydrolysis. On this basis, the average percentage protein of the dry weight of these species is, 21.84 for M. expansa, 29.03 for T. actinioides, and 20.60 for C. perplexa.

Data on the distribution of nitrogen in other species of cestodes are available only in the work of Salisbury and Anderson referred to above. They determined the nitrogen content of several fractions of the larval cestode Cysticercus fasciolaris. Different techniques were used to separate these nitrogen fractions and it would be difficult to compare their data with the data for M. expansa, T. actinioides, and C. perplexa.

The percentages of the total nitrogen present in the non-protein, non-protein carboxyl, protein, and protein carboxyl fractions of the three anoplocephalid cestodes studied in this work and Dugesia sp. have been calculated and are presented in Table 14. The percentage nitrogen in the non-protein fraction appears to be distinct for each species. Pollak and Fairbairn (42) have studied the nitrogen distribution in ovarian tissue of Ascaris lumbricoides, from swine. Their findings are also included in Table 14. In this tissue a much lower percentage of the total nitrogen is in the non-protein fraction than is the case in the cestodes analyzed in this work. Only 1.2% of the total nitrogen of this tissue is present as alpha-amino nitrogen in the non-protein fraction. This is in contrast to the percentages of 4.7, 6.3, and 6.4 found in the cestodes studied here. The small percentage nitrogen in the non-protein fraction of Ascaris ovary may be the consequence of a high anabolic rate which prevents a piling up of nitrogen in the free

TABLE 14

COMPARATIVE NITROGEN DISTRIBUTION IN *MONIEZIA EXPANSA*, *THYSANOSOMA ACTINIOIDES*, *CITTOTAENIA PERPLEXA*, *ASCARIS LUMBRICOIDES* OVARY, AND *DUGESIA SP.* (MEAN VALUES IN MG NITROGEN PER 100 GRAMS FRESH WEIGHT OF TISSUE)

Species	TN	NPN	NPCN	PN	PCN
Moniezia expansa whole worm	544	88	35	414	321
% of total nitrogen in fraction		16.2	6.4	76.1	59.0
Thysanosoma actinioides whole worm	1296	164	81	896	756
% of total nitrogen in fraction		12.7	6.3	69.1	58.3
Cittotaenia perplexa whole worm	1291	124	61	978	830
% of total nitrogen in fraction		9.6	4.7	75.8	64.3
Ascaris lumbricoides ovarian tissue (42)	1700	85	20.1[a]	1508	981
% of total nitrogen in fraction		4.7	1.2	88.3	57.8
Dugesia sp.	1687	230	125	1304	1023
% of total nitrogen in fraction		13.6	7.4	77.0	60.3

[a]Determined by conversion of alpha-amino nitrogen to ammonia by means of ninhydrin reaction.

nitrogen pool.

In comparing these cestodes with the planarian, Dugesia sp., certain differences are noted (Table 14). Dugesia sp. has a higher average nitrogen content generally, as well as in each fraction. On the basis of carboxyl nitrogen, protein accounts for an average of 38.10% of its dry weight. This is higher than any value found for the cestodes. On the other hand, the percentage of the total nitrogen present in each fraction of Dugesia sp. does not differ significantly from that of the cestodes. Asparagine was present in the non-protein fraction of Dugesia sp. in fairly high concentration, as estimated by the intensity of its reaction with ninhydrin on the chromatograms. This amide was not detected in the cestodes. The amino acids, beta-alanine, beta-aminoisobutyric acid, and gamma-aminobutyric acid, which are present in the non-protein fraction of the cestodes, could not be detected in this fraction of Dugesia sp. According to Hyman (64), planaria are aerobic organisms which do not contain glycogen as an energy reserve. This is in direct contrast to cestodes which are generally considered anaerobic and have a high rate of carbohydrate metabolism. The differences observed between the biochemical make-up of these two classes of flatworms undoubtedly represent divergent adaptations which have been made by cestodes as a result of their endoparasitic mode of life, on the one hand, and by planarian as a result of their free-living existence on the other.

Except for the failure to detect hydroxyproline, the present finding of the alpha-amino acid composition of M. expansa, T. actinioides, and C. perplexa do not differ significantly with what has been reported for H. diminuta. A high concentration of taurine was reported by Aldrich

et al. in the free amino acid extracts of H. diminuta (9). This sulfonic amino acid was in low concentration, when present, in the non-protein fractions of M. expansa and T. actinioides. Sobotka (65) reports taurocholic acid to be the major bile acid of sheep. However, in the present work, taurine could not be detected in the free amino acid extracts of sheep bile. It was present in the combined amino acid fraction but, in much lower concentration than glycine. Glycine was so abundant in both fractions that it made chromatography of these difficult. The concentrational distribution of taurine in H. diminuta and the two ovine cestodes studied here probably reflects differences in its availability in their respective hosts.

Pollak and Fairbairn (42) found glutamic acid and alanine to comprise 50% of the total free amino acids of ovarian tissue of Ascaris lumbricoides. In a later study of the intermediary amino acid metabolism of this tissue, these authors (66) showed the alanine-glutamate transaminase system to be the most active. If the relative concentrations of the amino acids are indicative of the rate of transaminase activity, it is postulated that the alanine-glutamate sytem is also the most active in the three species of anoplocephalids investigated. Aspartic acid gave only a faint ninhydrin reaction in the non-protein fractions of these worms while glutamic acid and alanine gave very strong reactions, resulting in large, dense spots on the chromatograms. The suggestion that the alanine-glutamate system is the most active in these cestodes is not consistent with previously reported results on H. diminuta. Aldrich et al. (9) report the aspartate-glutamate system to be the most active one in this tapeworm.

The present work is the first report of beta-alanine, beta-aminoisobutyric acid, and gamma-aminobutyric acid in cestodes. Beta-alanine and beta-aminoisobutyric acid occur in the non-protein fractions of all three species of worms. Gamma-aminobutyric acid occurs only in the non-protein fractions of the two intestinal species. From the present knowledge of the metabolism of these amino acids in other tissues, several postulates can be made concerning their presence in these cestodes.

Beta-alanine was first reported as a decarboxylation product of aspartic acid in bacteria by Virtanen and co-workers (67, 68). It is a constituent of carnosine, anserine, coenzyme A, and pantothenic acid. It occurs in the non-protein fraction of these cestodes in roughly the same concentration as alanine. This estimate is based upon the intensity of its reaction with ninhydrin on the chromatograms. Beta-alanine has previously been reported as a free or loosely combined amino acid in apples by Hulme and Arthington (69), in nerve tissue of the mouse and rabbit by Roberts et al. (70), in extracts of rye grass by Synge (71), in several species of insects by Clark and Ball (72), and Po Chedley (73), and in various tissues of the cat by Tallen et al. (74). Graff and Hoberman (75) showed it to be rapidly deaminated in vivo in the rat. The calculated rate of deamination was based upon the formation of urea from injected N^{15}-labeled beta-alanine. Roberts and Bregoff (76) found it to undergo transamination with alpha-ketoglutarate in brain and liver preparations of mice. Transamination of beta-alanine in rat and calf brain was reported by Bessman et al. (77). Presumably malonic semialdehyde is formed in this reaction, although this compound has not been isolated. Pihl and Fritzson (78) found 90% of the radioactivity of

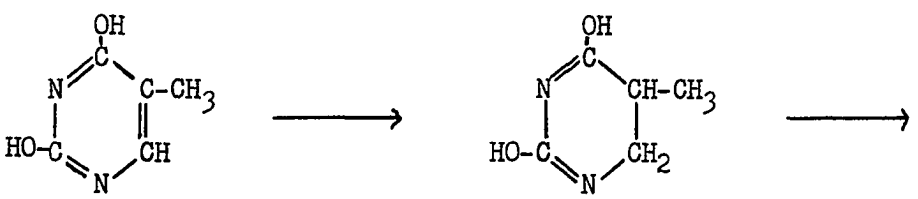
of injected 1-C^{14}-beta-alanine recoverable in the respiratory carbon dioxide of the rat within five hours after its administration. The oxidative pathway suggested by them involves deamination to formyl-acetic acid, with the subsequent decarboxylation of this compound to carbon dioxide and acetaldehyde. The acetaldehyde is further oxidized to acetate.

Three major synthetic pathways, other than transamination of malonic semialdehyde, are known for beta-alanine. First of all, it is formed from the decarboxylation of aspartic acid in bacteria (67, 68). Secondly, it is a degradation product of pyrimidines. Fink and co-workers (79, 80) showed beta-alanine to be formed when either dihydrouracil or beta-ureidopropionic acid was incubated with rat liver slices. Dihydrouracil was first isolated from a natural source by Funk and co-workers (81). Fink et al. (80) suggest that the degradation proceeds from dihydrouracil to beta-ureidopropionic acid to beta-alanine. Dihydrothymine is readily formed from thymine and an analogous reduction of uracil is assumed to occur. Since cestodes are in more or less constant contact with nucleoproteins or their digestion products, whether they occur in the intestine or in the biliary passages, the degradation of pyrimidines may be an important metabolic pathway for them. A third, and perhaps less direct method of beta-alanine formation, is suggested by the findings of Stadtman (82). Extracts of Clostridium propionicum catalyze the formation of beta-alanyl-coenzyme A and beta-alanyl-pantotheine from acrylyl-coenzyme A and acrylyl-pantotheine, respectively. Also, dried cell suspensions of these bacteria accumulate beta-alanine when oxidizing propionate. The oxidation of propionate has not been investigated in cestodes, although, Coutelen (83) reports this fatty acid in

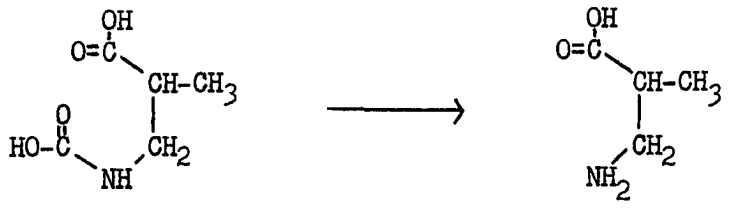
Echinococcus granulosus.

The presence of large amounts of lipids in cestodes has been reported by von Brand (14) and Warren and Daugherty (84). This suggests a high rate of lipid metabolism in these worms and, the presence of relatively high concentrations of beta-alanine may be associated with this metabolism. In the present work it was shown that the beta-alanine in the non-protein fractions could be a product of the hydrolysis of pantothenate. The same treatment of a calcium pantothenate solution as was used for the preparation of the worm extracts showed that it was extensively hydrolyzed. Hydrolysis also occurred during chromatography. (See Figure 9.)

Beta-aminoisobutyric acid was first reported from human urine by Crumpler et al. (85) and has since been reported to occur as a free amino acid in several tissues of the cat by Tallen et al. (74). Following the discovery of beta-aminoisobutyric acid in human urine, Fink and co-workers (86) found that the administration of thymine or thymine-containing nucleic acids to rats resulted in the appearance of this amino acid in the rats' urine in higher than normal concentrations. In vitro studies with rat liver slices have led to the elucidation of the following pathway for the degradation of this pyrimidine:



thymine dihydrothymine



beta-ureidoisobutyric acid

beta-aminoisobutyric acid

As was suggested in the case of beta-alanine, the occurrence of beta-aminoisobutyric acid in cestodes may be associated with the degradation of pyrimidines with which they are in contact.

Another aspect of the intermediary metabolism of beta-aminoisobutyric acid is its possible formation in the degradation of valine. Valine is glycogenic and occurs as one of the major amino acids in all the cestodes that have been studied. According to Meister (61), valine is metabolized through a series of steps, involving coenzyme A and diphosphopyridine nucleotide, to a three-carbon acid. This acid may be utilized for glycogen synthesis. In this oxidative sequence, two possible precursors of beta-aminoisobutyric acid are formed, methyl-acrylyl-coenzyme A and methylmalonic semialdehyde. On the basis of Stadtman's work (82) with acrylyl-coenzyme A, the amination of the methyl substituted compound could be postulated to take place. Methylmalonic acid semialdehyde could also be postulated to transaminate to beta-aminoisobutyric acid. These pathways have never been investigated, however, and their occurrence in cestodes, as well as other tissues, can only be speculation.

Gamma-aminobutyric acid has been determined as a free amino acid in nerve tissue by Awapara et al. (87), Roberts et al. (70), Tallen et al. (74), and Udenfriend (88). Tallen et al. (74) also report it in other

tissues of the cat. Its occurrence in plant tissues has been reported by Hulme and Arthington (69), Reed (89), Synge (71), and Westall (90). In animal tissues it occurs in highest concentration in brain and nerve tissue.

The enzyme systems in nerve tissue which catalyze the decarboxylation of glutamic acid to gamma-aminobutyric acid have been investigated by Roberts and Frankel (91, 92, 93) and by Awapara and co-workers (87, 94). The work on these enzyme systems in bacteria is reviewed by Gale (95).

The role of gamma-aminobutyric acid in intermediary metabolism has not been completely elucidated. Roberts and Bregoff (76) and Bessman and co-workers (77) found it to undergo transamination with alpha-ketoglutarate to form succinic semialdehyde and glutamic acid. The further oxidation of succinic semialdehyde to succinic acid results in the complete oxidation of the molecule. Glutamic acid occurs in fairly high concentrations in these cestodes and may possibly serve as a precursor for gamma-aminobutyric acid.

One significant difference between T. actinioides and the two intestinal cestodes, M. expansa and C. perplexa, is the absence of gamma-aminobutyric acid from the non-protein fraction of T. actinioides. Gamma-aminobutyric acid could not be detected in the non-protein fraction of this species even though attempts to do so were made by doubling the amount of solution normally analyzed by two-dimensional chromatography.

The contribution of the hepatic secretion to the intestinal environment is reviewed by Read (96). The biliary passages and gall bladder have never been considered as an environment for parasitic helminths.

In the present study, the amino acids available to T. actinioides in this environment are of interest. The general composition of bile is reviewed by Sobotka (65) and Popper and Schaffner (97). Sobotka reports several amino acids in bile and, according to Popper and Schaffner, these amino acids are present in the same concentration as they are in blood. All of the alpha-amino acids which occur consistently in the non-protein fraction of T. actinioides, with the possible exception of histidine, were detectable as free amino acids in sheep bile. Beta-alanine was also detectable in the free amino acid extract of bile. The absence of beta-aminoisobutyric acid in bile and its presence in the non-protein fraction of T. actinioides is good evidence that it is a product of the intermediary metabolism of this cestode. This raises the question as to whether the gamma-aminobutyric acid present in M. expansa and C. perplexa is a product of their intermediary metabolism or is taken up by them from their environment. On the basis of these findings, it is felt that the occurrence of T. actinioides outside the intestine of its host is not a valid basis for assuming that this species obtains its amino acids directly from the tissues of the host. Sufficient amino acids occur in sheep bile to satisfy its amino acid requirements. The ability to absorb these amino acids from the surrounding medium, as was demonstrated for H. diminuta and R. cesticillus by Daugherty (5) and Daugherty and Foster (7, 8), is the only prerequisite necessary for these worms to obtain amino acids. The presence of amino acids in bile may contribute to an explanation of the results that have been obtained by Chandler (1) and others, who have approached the problem of cestode nutrition through alteration of the host diet. Bile, being secreted continuously

by the liver, could supply amino acids to these intestinal cestodes and, the effects of elimination of protein from the host diet would be negligible. The work of Goodchild and Wells (4) lends support to this idea. In Thiry-Vella fistulas the contribution of bile to the environment of the helminth is eliminated. According to these authors, tapeworms maintained in these fistulas showed a quantitative decrease in their amino acid content. However, it is felt that there are differences in the intestinal and liver environments, especially in the availability of carbohydrate.

A comparison of T. actinioides with the intestinal species, on the basis of percentage protein of dry weights, reveals a difference not evident in the data based upon fresh weights. T. actinioides has a higher percentage protein than either of the intestinal forms. On the basis of carboxyl nitrogen, this difference amounts to approximately 10%. This is presumably a reflection of the availability of carbohydrate in the two environments. Sobotka (65) reports glucose to occur in bile, but its occurrence may be variable and in small amounts. A lowered availability of glucose would result in a lower glycogen content of the worms which would account for an increased percentage protein of the solid material. Reid (2) found this process responsible for the increase in the percentage nitrogen of R. cesticillus maintained in starved chickens.

In an overall comparison of the three species of anoplocephalid cestodes studied here, certain differences and similarities are noted. The nitrogen content of the fresh weight of M. expansa is approximately one-half or less that of C. perplexa and T. actinioides. This is also

true of the nitrogen content of the different fractions of these worms. C. perplexa and T. actinioides show significant similarities in the amount of nitrogen in the total nitrogen, non-protein carboxyl nitrogen, protein nitrogen, and protein carboxyl nitrogen fractions. This establishes a closer biochemical relationship between C. perplexa and T. actinioides than exists between either of these species and M. expansa. In the non-protein nitrogen fractions, each species forms a more or less distinct group. This presumably represents specific nitrogen turnover rates in these helminths. Each species is also distinct in the percentage of solid matter in its fresh weight. M. expansa contains only approximately 10% solid matter, C. perplexa about 25%, and T. actinioides is intermediate between the two with 16%. As was pointed out in the preceding paragraph, T. actinioides contains a higher percentage of this solid matter as protein than the other two species. Protein makes up 29% of this solid material for T. actinioides and only approximately 20% in both C. perplexa and M. expansa.

The amino acids present in the protein fractions of each species are qualitatively identical. A significant difference between the free amino acid pools of these cestodes is the presence of gamma-aminobutyric acid in M. expansa and C. perplexa and its absence from T. actinioides. Taurine and ornithine were detected occasionally in M. expansa and T. actinioides but never in C. perplexa. However, their occurrence in the two ovine species is so variable and in such small quantities that valid conclusions concerning species differences cannot be drawn. This is also true of the spot designated as citrulline. This spot occurred in the non-protein and protein fractions of a few individuals of M. expansa and in

the protein fraction of a few C. perplexa. Differences were also noted in the occurrence of the aromatic amino acids, phenylalanine and tyrosine, in the non-protein fractions. Phenylalanine occurred in M. expansa but not in the other two species. Tyrosine was detectable in T. actinioides and M. expansa but not in C. perplexa. The basic amino acid, arginine, was present in the non-protein fraction of M. expansa but not in T. actinioides and C. perplexa. Phenylalanine, tyrosine, and arginine, occur, however, in the protein fractions of all three species and must be taken up by them and utilized at some time. Due to this, it is felt that the failure to detect them in the non-protein fraction does not indicate a significant difference in the metabolism of one species from another.

CHAPTER V

SUMMARY

The nitrogen and amino acid composition of three species of anoplocephalid cestodes has been determined. The three species analyzed were, Moniezia expansa from the small intestine of sheep, Thysanosoma actinioides from the biliary passages and gall bladder of sheep, and Cittotaenia perplexa from the small intestine of cottontail rabbits. Total nitrogen, non-protein nitrogen, and protein nitrogen were determined by the Kjeldahl method. Non-protein carboxyl nitrogen and protein carboxyl nitrogen were determined according to the method of Van Slyke, MacFadyen, and Hamilton. The non-protein and protein fractions were separated with 80% ethanol. Amino acids in the non-protein and protein fractions were determined qualitatively by means of paper partition chromatography on buffered papers. The reaction of amino acids with ninhydrin was utilized as the general color reaction. Specific color reactions were also carried out on the papers. For comparative purposes, the nitrogen and amino acid composition of Dugesia sp., a free-living planarian, was determined. To test the postulate that cestodes satisfy their nutritional amino acid requirements by direct absorption from the host's tissues, the amino acids in sheep bile, which are available to T. actinioides, were determined.

On the basis of fresh weight, M. expansa is distinct from T. actinioides and C. perplexa in its nitrogen content. T. actinioides and C. perplexa show significant similarities in the amount of nitrogen in the total nitrogen, non-protein carboxyl nitrogen, protein nitrogen, and protein carboxyl nitrogen fractions. This establishes a closer biochemical relationship between C. perplexa and T. actinioides than exists between either of these species and M. expansa. Each species forms a more or less distinct group in the amount of nitrogen in the non-protein fraction. This is suggested as representing a specific nitrogen turnover rate for each species. Each species is also distinct in its percentage of solid matter. The percentage of this solid matter which is protein (protein carboxyl nitrogen X 6.25) is higher for T. actinioides than for the intestinal forms. This is attributed to differences in the availability of carbohydrate in the bile and intestinal environments. The protein amino acids are qualitatively identical for all three species. Differences were found in the amino acids of the non-protein fractions. The most significant of these differences is the presence of gamma-aminobutyric acid in the non-protein fractions of the intestinal forms and its absence from the hepatic form. The present findings obviate postulating a direct absorption of amino acids by cestodes from the host's tissues, at least in the case of T. actinioides. Sheep bile was found to contain sufficient amino acids to satisfy the requirements of this species.

This is the first report of the non-alpha amino acids, beta-alanine, beta-aminoisobutyric acid, and gamma-aminobutyric acid, in cestodes. Possible roles of these amino acids in the intermediary metabolism of cestodes are discussed.

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