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A COMPARATIVE STUDY OF THE FREE AMINO ACID AND PROTEIN AMINO ACID COMPOSITION OF THREE SPECIES OF THE CESTODE GENUS CORALLOBOTHRIUM

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APPROVED BY

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CHAPTER I

INTRODUCTION

Considerable use has been made of paper partition chromatography in the investigation of problems in systematics. In some cases it has seemed to show a close parallelism between levels of taxonomic affinity as revealed by morphological and other criteria and of biochemical similarity as revealed by amino acid chromatographic patterns.

Micks and Ellis (1951) reported the same eighteen free amino acids from each of seven species of mosquitoes investigated. They did however find quantitative differences in free amino acids of these various species as judged from relative densities on one-dimensional chromatograms prepared from measured amounts of extracts. Intergeneric distinctions were much more pronounced than intrageneric ones, and the patterns of two subspecies were strikingly similar. Buzzati-Traverso (1953) found that fluorescent and ninhydrin-positive patterns produced by identical tissues taken from various specimens of the same species of fish were remarkably constant, that patterns obtained from muscle tissue of

different species showed constant and easily recognizable differences, and that the closer the taxonomic position of the species the greater the similarity of their chromatographic patterns. He suggested that this technique could be used to distinguish stocks of the same species belonging to populations geographically separated.

Since the publication of the references cited above, many papers concerned with the use of paper chromatographic techniques in taxonomic studies of diverse organisms have appeared. These techniques have proved to be of value, not only in showing close relationships between groups, but also in showing biochemical differences between morphologically very similar groups. Paper chromatographic techniques have been used in taxonomic studies of bacteria, protozoa, echinoderms, snails, insects, fishes, and mammals; a comprehensive review of the use of these techniques in systematics has been made by Buzzati-Traverso (1960).

Three techniques have generally been used. One involves the separation of free amino acids, protein amino acids, or both. The amino acids are then identified or the ninhydrin-positive patterns noted without specific identification of the acids; the latter technique may be quantitative. A second purely qualitative technique involves chromatographing material mashed from the tissue or whole organism directly upon the filter paper or chromatographing a crude extract from the tissue or the whole organism. A third technique involves the separation of the amino acids from a given quantity of tissue extract and determination of the relative amounts of amino acids by densitometric readings of the spots.

Taxonomic problems of cestodes have not been directly investigated with paper chromatographic techniques. Several amino acids have been

identified in cestode tissue as a result of the interest in protein metabolism of the organisms. Kent (1947) reported the presence of ten or more amino acids in one of the protein-bile complexes of Moniezia expansa. Of these ten, eight were identified by paper chromatography to be aspartic acid, glutamic acid, valine, serine, alanine, leucine, histidine, and arginine. Later Kent (1948) confirmed the presence of glycine. Campbell (1960) reported the following amino acids from protein hydrolysates of M. expansa: alpha-alanine, arginine, aspartic acid, cysteine/cystine, glutamic acid, glycine, histidine, leucine/isoleucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurine, threonine, tryptophan, tyrosine, valine and the probable presence of citrulline. In addition to the amino acids listed, Campbell found beta-alanine, gamma-aminobutyric acid and beta-aminoisobutyric acid in the free amino acid extracts of the parasite; free tryptophan was not found. Several investigators have examined the amino acid composition of Hymenolepis diminuta. Aldrich et al. (1953) 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, phenlyalanine, proline, serine, taurine, tyrosine, and valine. Goodchild and Wells (1957) reported these same 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 the amino acids reported by Aldrich et al., they reported cystine, cysteine, histidine, hydroxyproline, methionine, threonine, and tryptophan. Kent (1957) identified

alanine, arginine, glutamic acid, glycine, histidine, leucine, methionine, serine, tyrosine, and valine in four of the protein complexes of H. diminuta. Except for the absence of citrulline, Campbell (1960) found identical amino acids present in the protein hydrolysates of Thysanosoma actinioides and M. expansa. Gamma-aminobutyric acid, arginine, citrulline, and phenylalanine were not identified from the free amino extract of T. actinioides. Campbell also found identical amino acids present in the protein hydrolysates of T. actinioides and Cittotaenia perplexa. He found that the free amino acid extract of C. perplexa differed from that of M. expansa in that it did not contain arginine, ornithine, phenylalanine, taurine, or tyrosine. Foster and Daugherty (1959) reported the following free amino acids and closely related compounds from Rallietina cesticillus: alpha-alanine, aspartic acid, citrulline and/or glutamine, glutamic acid, glutathione, glycine, methionine-leucine complex, proline, serine, taurine, threonine, tyrosine, and valine. Hydrolysates of this organism contained all of the amino acids found in the free amino acid extract except for taurine, citrulline and/or glutamine, glutathione, and revealed in addition arginine, lysine, and several unidentified compounds.

The present study was undertaken to determine if qualitative paper chromatographic determinations of free amino acids and protein amino acids could serve as a basis for differentiation between closely related species of a genus of tapeworms. The organisms selected for the study belong to the genus Corallobothrium Fritsch 1886 and are parasites in the small intestine of Pylodictus olivaris, the flathead catfish; Ictalurus lacustris punctatus, the southern channel catfish; and Ictalurus furcatus,

the blue catfish. The subgenus C. Megathylacoides was established by Jones et al. (1956) for those members of the genus distinguished by sphinctered suckers, "The sphincter consisting of a robust muscle partly encircling the orifice of each sucker, the sphincter being completed by a tendon or filament connecting the ends of the muscle." The three species investigated were: Corallobothrium (Megathylacoides) giganteum Essex 1927, C. (M.) procerum Jones, Kerley and Sneed 1956, and C. fimbriatum Essex 1927. This complex has afforded me excellent opportunity to test the usefulness of qualitative amino acid analyses in validating complex taxonomic affinities.

CHAPTER II

MATERIALS AND METHODS

Worms were removed from the intestine, all motile specimens rinsed free of debris, and placed in Tyrode's solution; all nonmotile worms were discarded. In order that the species could be morphologically determined, the scolex and two or three sexually mature proglottids were removed from each worm and prepared for microscopic study. Identification as to species was based on taxonomic characters used by Essex (1927) and Jones et al. (1956). The remainder of each worm was rinsed in three changes of distilled water, blotted, and wrapped in aluminum foil or placed in a vial. The samples were either quick-frozen and stored at -20° C or placed directly in a -20° C freezer.

Free amino acid extracts were prepared from pooled worms of a species according to the method of Awapara (1948), a method that permits recovery of ninety per cent of the amino acids present. The extracts were stored at -20° C.

Protein amino acids were prepared from the proteinaceous residue from the free amino acid preparations. The residue was dried in vacuo over sulfuric acid and refluxed for 24 hours at 120° C in 100 volumes of peroxide-free 6 N hydrochloric acid prepared according to Ingram and Salton (1957). 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. Alkaline hydrolyses for tryptophan were conducted by refluxing the protein residues for twenty hours at 120° C in 100 volumes of 0.38 N barium hydroxide. Barium ion was removed from the hydrolysates by neutralization with 2 N sulfuric acid. After filtering off the barium sulfate, the hydrolysates were evaporated to dryness and the residues taken up in 10% isopropyl alcohol to give approximately one mg. nitrogen per ml. of solution. The alcoholic solutions were chromatographed.

A modification of the ascending two-dimensional paper chromatography method of Williams and Kirby (1948) was employed for the analysis of the free amino acids and the protein amino acids. Eleven and one-fourth inch squares of Whatman No. 1 chromatography paper were used. The origin spot was always placed in the lower right hand corner of the paper so that the lower and right edges of its circumference were one and one-fourth inches from the lower and right edges of the paper. The first solvent was always run in the machine direction of the paper.

The first dimensional solvent was sec-butyl alcohol:formic acid: water in the ratio of 75:15:10 v/v (Hausmann 1952). Repeated development in the first solvent increased the degree of separation of most of the amino acids. Chromatograms developed in the first solvent were allowed to dry in air overnight, then placed in a forced-air drying oven at 60° C for fifteen minutes, followed by forced-air only for 10 minutes. Development and drying were then repeated.

Two different solvent systems were used for the development in the

second dimension. As shown in Figure 1 amino acids which moved to the upper half of the chromatogram in the first solvent were well separated in the second dimension by n-butyl alcohol:acetic acid:water in the ratio of 4:1:5 v/v (Partridge 1948). Two liquid phases form in this solvent: a bottom layer of butanol-saturated water, which was used to saturate the atmosphere of the chromatography chamber; a top layer of water-saturated butanol, which was used as the developing solvent. Phenol was used as the other second dimensional solvent for the separation of those amino acids which remained on the lower half of the chromatogram developed in the first solvent (Figure 2). It was prepared by mixing eight volumes of melted, redistilled phenol crystals (Baker Analyzed Reagent or Mallinckrodt, AR) with two volumes distilled water to give 80% (v/v) phenol. Removal of the second solvents was much like the removal of the first solvent, except that phenol-run papers were dried in the forced air for only fifteen minutes. Further removal of phenol was accomplished by washing the papers in a mixture of equal parts of acetone and petroleum ether (Block et al. 1958).

The analysis for the amino acids was accomplished by a series of three chromatograms, one spotted with the standard amino acid solution only, one with the standard plus the unknown solution, and one with the unknown solution only. The characterization of the migration of each amino acid in the three solvent systems employed was determined by comparing the corresponding spots after detection with ninhydrin.

A forty unit set of 0.01 M standard solutions (in 10% isopropyl alcohol) of amino acids and slightly modified derivatives (Shandon Scientific Co.) was used in determining the behavior of the amino acids in

the solvent systems used. R_f values of the amino acids proved to be quite variable; consequently special attention was given to the relative position of each spot, to the adjacent spots, and to the position of the spot on the chromatogram as a whole. Modified ninhydrin (Levy and Chung, 1953) was employed as the principal detection agent. In certain instances plain ninhydrin was used. Specific amino acid detection reagents recommended by Berry et al. (1951), Block et al. (1958), and Cramer (1955) were used to confirm identification of certain of the amino acids and modified derivatives. Methionine, methionine sulfoxide, and cystine-cysteine were confirmed by using the platinic iodide reagent. Isatin was used to confirm β-phenylalanine, methionine, tryptophan, tyrosine, γ-aminobutyric acid, proline, hydroxyproline, β-alanine, and ornithine. Tyrosine was further confirmed with α-nitroso-β-naphthol. Ehrlich's reagent was used to confirm tryptophan, citrulline, and methionine sulfone. Proline, hydroxyproline, taurine, ornithine, and sarcosine were confirmed with vanillin. Additional confirmation of several amino acids obtained from positional relationships on the chromatograms after use of a specific detection reagent by dipping the dried treated chromatogram in the ninhydrin-collidine, acetic acid reagent. This procedure is of no value when vanillin or the Sakaguchi reagent are used as initial detection reagents. Acetone was used as the solvent in most of the detection reagents because it does not cause streaking of the spots as do some of the other recommended solvents in which the amino acids are soluble. Ultraviolet was used to further identify hydroxyproline, proline, β-phenylalanine, tryptophan, and tyrosine.

Figures 1 and 2 are reproductions of chromatograms that show the

separation and the position of the two groups of amino acids in the different second dimension solvents. The chromatograms were prepared by spotting free amino acid extracts and aliquots of those standards that were known to be absent or present in very low concentration in the extract. Numerical subdivisions of the paper correspond to one inch on the chromatogram. Since the position of the origin spot is entirely within the first subdivision, the position of the amino acid spot does not indicate the true R_f value. Practically identical chromatograms can be made by spotting protein amino acids and adding aliquots of those standards that are known to be absent.

TABLE 1

LIST OF SHANDON AMINO ACID STANDARDS, ARRANGED NUMERICALLY TO SERVE AS A LEGEND FOR FIGURES 1 AND 2

Spot No.	Amino Acid	Spot No.	Amino Acid
1	α-aminooctanoic acid	21	Glycine
2	Diiodotyrosine	22	Serine
3	Leucine	23	Glutamic acid
4	Isoleucine	24	Aspartic acid
5	β-phenylalanine	25	Hydroxyproline
6	Tryptophan	26	Methionine sulfoxide
7	Norvaline	27	Methionine sulfone
8	Valine	28	Citrulline
9	Methionine	29	Glutamine
10	α-aminoisobutyric acid	30	Asparagine
11	α-aminobutyric acid	31	Histamine
12	β-aminoisobutyric acid	32	1-Methylhistidine
13	Tyrosine	33	Histidine
14	γ-aminobutyric acid	34	Arginine
15	Proline	35	Lysine
16	Alanine	36	Taurine
17	β-alanine	37	Ornithine
18	Ethanolamine	38	Cystine-Cysteine
19	Sarcosine	39	Homocystine
20	Threonine	40	Cysteic acid

Fig. 1 CHROMATOGRAM OF AMINO ACID STANDARDS PLUS FREE AMINO ACID EXTRACT

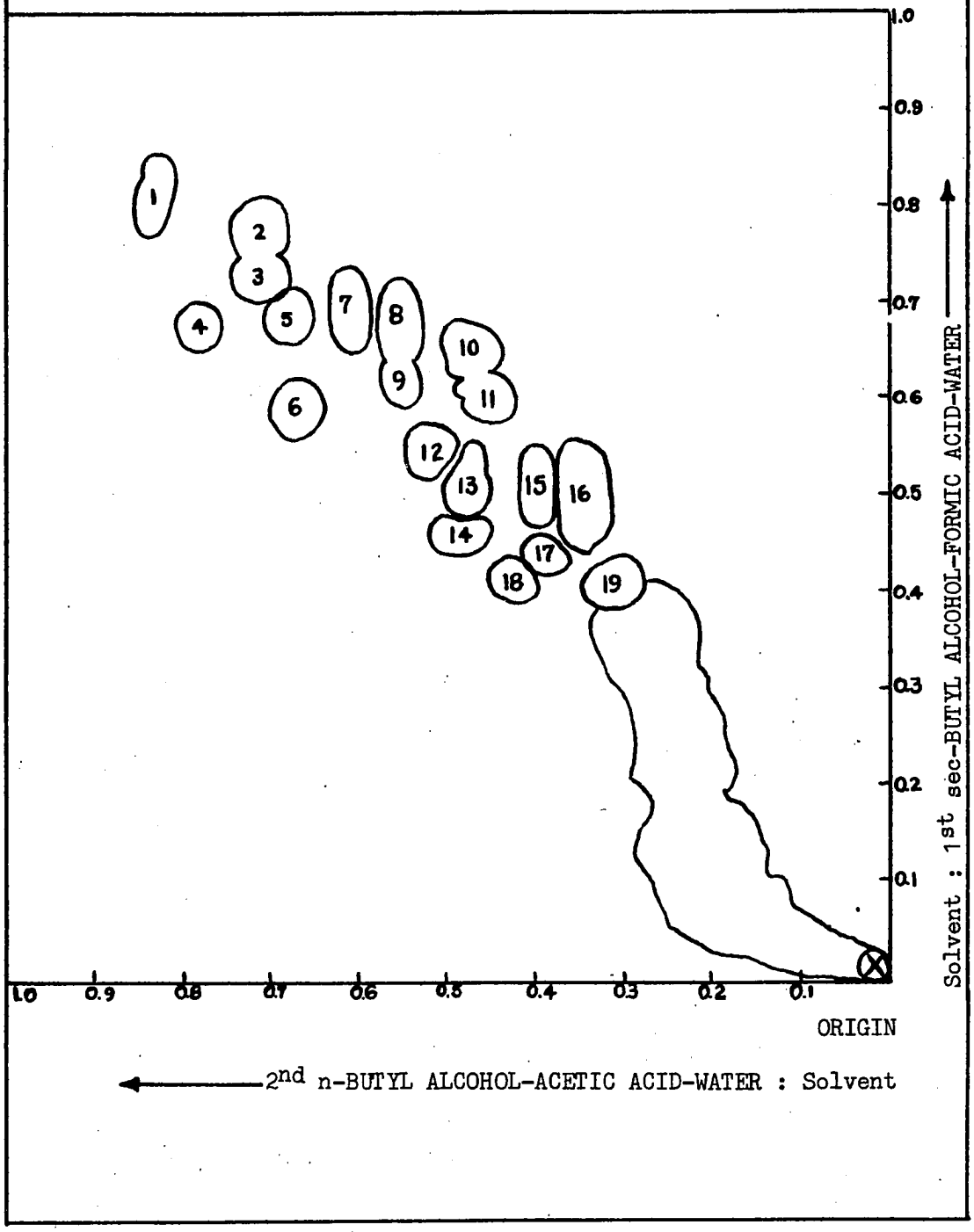
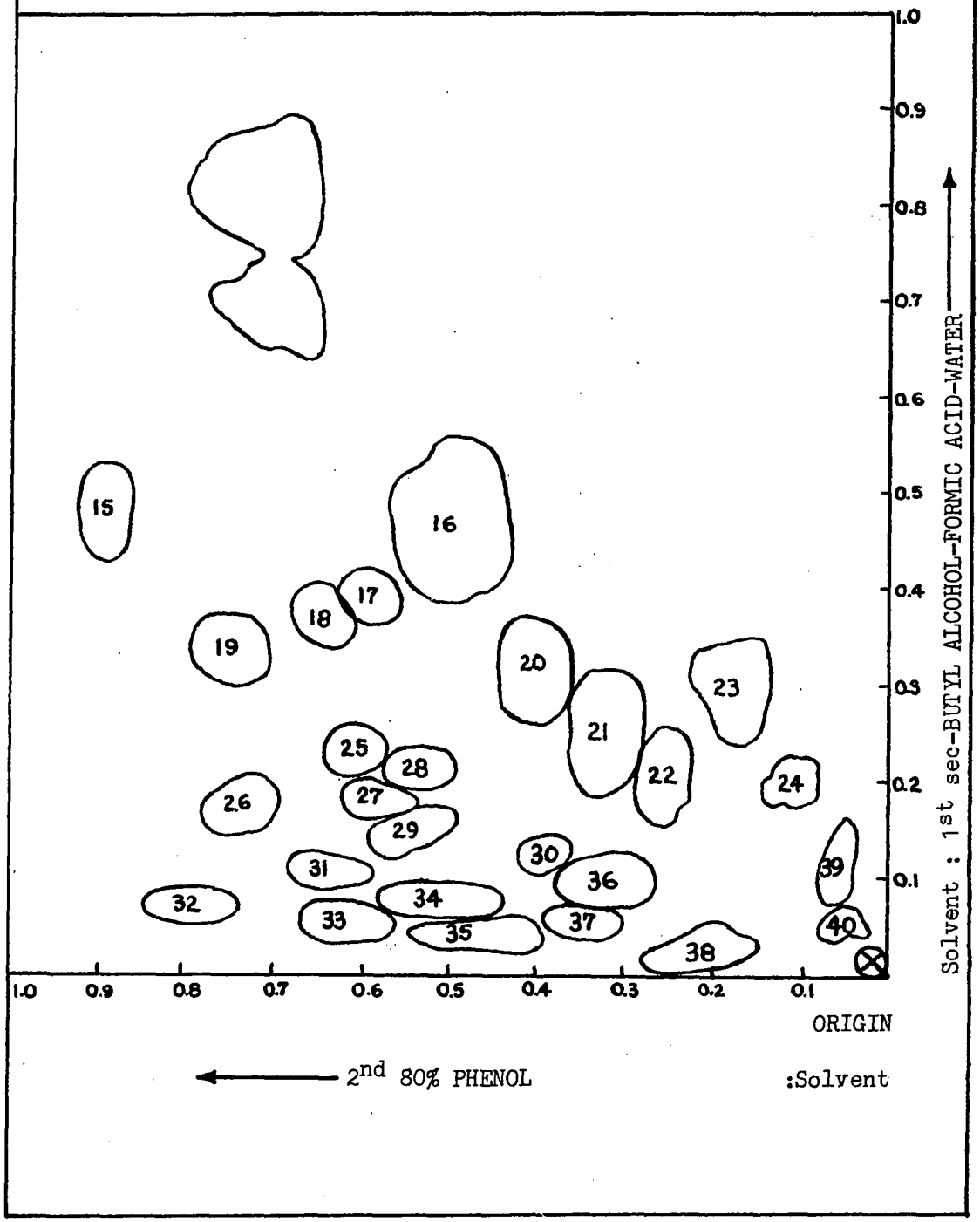


Fig. 2 CHROMATOGRAM OF AMINO ACID STANDARDS PLUS FREE AMINO ACID EXTRACT



CHAPTER III

RESULTS

The chromatographic analyses revealed that nineteen amino acids are common to the protein fractions of all three species of cestodes studied. From twenty-seven to twenty-three amino acids were identified in the free amino acid extracts; glutamic acid was tentatively identified from one extract. It was in the non-protein extracts that specific differences were noted. Table 2 lists the amino acids identified from each species. The term PAA designates the protein amino acids; the term FAA designates the free amino acids.

Alpha-aminoisobutyric acid and α-aminobutyric acid are not clearly separated by the butanol solvents (Figures 1 and 2). But since the chromatograms of the FAA extracts so closely duplicate those of the standards, they are both reported as being present.

As can be noted from Figure 2, methionine sulfone, citrulline, and glutamine are closely grouped on the chromatogram. In addition, each forms a poorly delineated spot on chromatograms developed in the second dimension in phenol; these characteristics make confirmatory identification of each of the compounds difficult. Glutamine was tentatively identified in free amino acid extract by co-chromatography with the standard solution. Citrulline is reported as being present because of

TABLE 2

COMPARATIVE AMINO ACID COMPOSITION OF *CORALLOBOTHRIUM* (*MEGATHYLACOIDES*) *GIGANTEUM*, *C.* (*M.*) *PROCERUM*, AND *C. FIMBRIATUM*

Spot No.	Amino Acid	*C. giganteum*		*C. procerum*		*C. fimbriatum*	
		PAA	FAA	PAA	FAA	PAA	FAA
3	Leucine	/	/	/	/	/	/
4	Isoleucine	/	/	/	/	/	/
5	β-phenylalanine	/	/	/	/	/	-
6	Tryptophan	/	/	/	/	/	/
8	Valine	/	/	/	/	/	/
9	Methionine	/	/	/	/	/	/
10	α-aminoisobutyric	-	/	-	/	-	/
11	α-aminobutyric	-	/	-	/	-	/
12	β-aminoisobutyric	-	/	-	/	-	/
13	Tyrosine	/	/	/	/	/	/
14	γ-aminobutyric	-	/	-	-	-	-
15	Proline	/	/	/	/	/	/
16	Alanine	/	/	/	/	/	/
17	β-alanine	-	/	-	/	-	/
18	Ethanolamine	-	/	-	/	-	/
20	Threonine	/	/	/	/	/	/
21	Glycine	/	/	/	/	/	/
22	Serine	/	/	/	/	/	/
23	Glutamic acid	/	/	/	/	/	/
24	Aspartic acid	/	/	/	/	/	/
28	Citrulline	-	/	-	/	-	/
29	Glutamine	-	/?	-	-	-	-
33	Histidine	/	/	/	/	/	/
34	Arginine	/	/	/	/	/	/
35	Lysine	/	/	/	/	/	/
36	Taurine	/	/	/	/	/	/?
37	Ornithine	-	/	-	/	-	-
38	Cysteine/cystine	/	/	/	/	/	/

/ amino acid present
- amino acid not present
? identity not confirmed

the color reaction with Ehrlich's reagent and because the behavior of the chromatographed worm extract so closely parallels the behavior of the chromatographed standard. Methionine sulfone could not be identified from the cestode material by any of the above procedures.

Taurine could not be identified in the FAA of C. fimbriatum with certainty, but a faint indeterminate spot was detectable.

Cystine and cysteine are reported together because it was found by Kent (1947) that cystine is converted to cysteine in certain procedures in chromatography.

An attempt was made to detect the presence in PAA and FAA the amino acids or amino acid derivatives only when standards for these were available. When large numbers of aliquots of PAA and FAA failed to produce chromatograms corresponding to the standards, they were assumed to be absent.

CHAPTER IV

DISCUSSION

Table 2 lists those amino acids found in the protein hydrolysates of C. (M.) giganteum, C. (M.) procerum, and C. fimbriatum. Kent (1947, 1948) and Campbell (1960) have reported the same amino acids from the hydrolysates of M. expansa; in addition, Campbell reported finding ornithine and tentatively identified citrulline. Campbell reported the same amino acids plus ornithine from T. actinioides and C. perplexa. Goodchild and Wells (1957) and Kent (1957) reported hydroxyproline, but not taurine, from hydrolysates of H. diminuta. Foster and Daugherty (1959) reported all those amino acids found in Corallobothrium sp. in hydrolysates of R. cesticillus except β-phenylalanine, cysteine/cystine, histidine, isoleucine, and tryptophan.

By examination of these data, it is obvious that qualitative differences in the protein amino acids have not been demonstrated in the species Corallobothrium that were investigated. Assuming the validity of the species, it must be concluded that this is not a fruitful taxonomic tool for demonstrating differences within this genus. I will also be noted that there is an intrageneric difference in the protein amino acid composition of each of the genera for which information is available.

TABLE 3
FREE AMINO ACID CONTENT OF CERTAIN CESTODES

Amino Acid	R. cesticillus	M. expansa	T. actinioides	C. perplexa	H. diminuta	C.(M.) giganteum	C.(M.) procerum	C. fimbriatum
Leucine.....	+	+	+	+	+	+	+	+
Isoleucine.....	-	+	+	+	+	+	+	+
β-phenylalanine...	-	+	-	-	+	+	+	-
Valine.....	+	+	+	+	+	+	+	+
Methionine.....	+	+	+	+	-	+	+	+
Tryptophan.....	-	-	-	-	-	+	+	+
α-aminobutyric....	-	-	-	-	-	+	+	+
α-aminoisobutyric.	-	-	-	-	-	+	+	+
β-aminoisobutyric.	-	+	+	+	-	+	+	+
Tyrosine.....	+	+	+	-	+	+	+	+
γ-aminobutyric....		+	-	+	-	+	-	-
Proline.....	+	+	+	+	+	+	+	+
Alanine.....	+	+	+	+	+	+	+	+
β-alanine.....	-	+	+	+	-	+	+	+
Ethanolamine.....	-	-	-	-	+	+	+	+
Sarcosine.....	-	-	-	-	-	-	-	-
Threonine.....	+	+	+	+	-	+	+	+
Glycine.....	+	+	+	+	+	+	+	+
Serine.....	+	+	+	+	+	+	+	+
Glutamic acid.....	+	+	+	+	+	+	+	+
Aspartic acid.....	+	+	+	+	+	+	+	+
Hydroxproline.....	-	-	-	-	+	-	-	-
Methionine sulfoxide....	-	-	-	-	-	-	-	-

TABLE 3 - Continued

Amino Acid	R. cesticillus	M. expansa	T. actinoides	C. perplexa	H. diminuta	C.(M.) giganteum	C.(M.) procerum	C. fimbriatum
Methionine sulfone.....	-	-	-	-	-	-	-	-
Citrulline.....	+	+?	-	+?	+	+	+	+
Glutamine.....	+	-	-	-	+	+?	-	-
Asparagine.....	-	-	-	-	-	-	-	-
Histamine.....	-	-	-	-	-	-	-	-
1-Methyl-histidine...	-	-	-	-	-	-	-	-
Histidine.....	-	+	+	+	-	+	+	+
Arginine.....	-	+	-	-	+	+	+	+
Lysine.....	-	+	+	+	+	+	+	+
Taurine.....	+	+	+	-	+	+	+	+?
Ornithine.....	-	+	+	+	-	+	+	-
Cystine/Cysteine.	-	+	+	+	-	+	+	+
Cysteic acid.....	-	-	-	-	-	-	-	-
TOTAL AMINO ACIDS	14	23	19	19	18	28	26	24

\+ presence of the amino acid
\- absence of the amino acid
? identity not confirmed

The amino acids found free in the cestode tissue investigated are listed in Table 2. Beta-phenylalanine and ornithine are absent from C. fimbriatum while they are present in the extract from C. (M.) giganteum and C. (M.) procerum. Taurine was not definitely identified in C. fimbriatum and, if present, occurs in very small quantities; it was definitely identified in the other two species. Gamma-aminobutyric acid is absent from C. fimbriatum and C. (M.) procerum, but it is present in C. (M.) giganteum. C. (M.) procerum, and C. (M.) giganteum differ further in that glutamine was not found in the former, but it was tentatively identified in the latter. The three species were found to have twenty-three amino acids in common.

The data show that there are qualitative interpecific differences in the free amino acid composition of species of Corallobothrium. They seem, also, to indicate a closer phylogenetic relationship between C. (M.) procerum and C. (M.) giganteum than between C. fimbriatum and either of the other two species, if it is assumed that smaller differences occur between more closely related organisms. The data help to justify the establishment of the Sub-genus Megathylacoides.

Table 3 summarizes the available information on the free amino acid composition of certain cestodes. The number of amino acids is the same for certain genera, but the amino acids are not identical. Of interest is the fact that M. expansa contains the same number of amino acids as does C. fimbriatum, but the amino acids are not identical. Thus, on the basis of the information presented it can be said that the complex of free amino acid composition is distinct for each of the organisms.

In the final evaluation of these data it should be noted that the

data from the present research represent a more thorough search for free amino acids than the data from at least some of the other works cited here. Further research might reveal more amino acids from those organisms already investigated.

Alpha-aminobutyric acid, tryptophan and α-aminoisobutyric acid are reported for the first time from cestode tissue.

CHAPTER V

SUMMARY

The free amino acid and protein amino acid composition of three species of Corallobothrium has been determined. The three species analyzed were: C. (M.) giganteum, C. (M.) procerum, and C. fimbriatum, parasitic in the intestine of Pylodictus olivaris, the flathead catfish, Ictalurus lacustris punctatus, the southern channel catfish, and Ictalurus furcatus, the blue catfish. The primary purpose of this research is to determine whether these analyses are of value in validating taxonomic affinities.

The free amino acids were separated from the protein fraction with 80% ethanol. The protein fraction was subjected to alkaline and acid hydrolysis. Qualitative determinations of the amino acids was made by two-dimensional paper chromatography. Leucine, isoleucine, tryptophan, valine, methionine, α-aminoisobutyric, tyrosine, proline, alanine, β-alanine, ethanolamine, threonine, glycine, serine, glutamic acid, aspartic acid, citrulline, histidine, arginine, lysine, taurine, and cysteine/cystine were found in the free amino acid fraction of all species investigated. In addition to these, C. (M.) giganteum and C. (M.) procerum were found to contain β-phenylalanine and ornithine. C. (M.) giganteum contained, in addition, γ-aminobutyric acid and what was

tentatively identified as glutamine. The protein amino acid composition of the three species was found to be qualitatively identical. The protein amino acids identified were leucine, isoleucine, β-phenylalanine, glycine, tryptophan, valine, methionine, tyrosine, proline, alanine, threonine, serine, glutamic acid, aspartic acid, histidine, arginine, lysine, taurine, and cystéine/cystine.

These data indicate that these species can be separated on the basis of free amino acid content.

Alpha-aminobutyric acid, α-aminoisobutyric acid, and tryptophan are reported for the first time from cestode tissue.

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