FREE AMINO ACID PATTERNS IN DEMOSPONGIAE; A BIOCHEMICAL APPROACH TO SPONGE CLASSIFICATION.

by

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Résumé

Les auteurs ont étudié les relations entre la répartition des acides aminés libres et la systématique des Démosponges ; les résultats sont discutés en fonction de la classification proposée par Lévi (1955, 1956).

L'étude comparée de la répartition des acides aminés libres peut fournir des données utiles à tous les niveaux de la classification.

INTRODUCTION

In recent years the great development of biochemical techniques has brought the field of biochemical taxonomy to the point where any experimentally inclined taxonomist can, in a relatively short time, conduct broadly based biochemical studies on large numbers of species or large population samples. Thus, quantities of data of a type inaccessible before, except in isolated instances, can now be utilized in classification.

There have been many such studies but none dealing with the great questions still remaining in the classification of the Demospongiae. Biochemical studies which have utilized Demospongiae have been concerned largely with identification and characterization of peculiar organic compounds, notably sterols and nucleosides. Bergmann and his co-workers made efforts to utilize sterol distribution in suggesting affinities within the Haplosclerida and Hadromerida, but the systematic aspect of their work was hampered by the fact that as chemists, they had no first hand knowledge of the biology and systematics of the organisms they worked with. Also, the conclusions

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reached by Bergmann et al. (1949, 1950) on relationships of sponges based upon sterol constitution must be accepted with reservation since it is now known that some substances which Bergmann considered as pure were mixtures and thus the actual sterol constitution of the sponges in question is still unknown.

Roche and his co-workers have contributed a great deal of information on the occurrence of guanidines and phosphagens in sponges and other organisms, but have never been concerned with the systematic implications of the occurrence of such compounds and have not sampled a great number species.

There have been two papers dealing directly with free amino acids in Demospongiae. Inskip and Cassidy (1955) determined that a considerable number of "unknown" amino acids were present in the thirteen species they sampled but they were unable to make any systematic sense out of the patterns. From the relatively small number of spots they observed on their chromatograms it is now apparent that the rugged pretreatment of their material in conjunction with the separation technique they used left much to be desired.

Kitteridge (1962) included two Demospongiae in his survey of invertebrate free amino acids. He did not publish full details of his chromatograms but commented on high glycine and taurine concentrations. Unfortunately, Kitteridge has misidentified one of his sponges. *Xestospongia vanilla* is not a calcareous sponge as he states, it belongs to the Haplosclerida (Demospongiae). This leaves some doubt as to whether Kitteridge was reporting on a misidentified calcareous sponge or actually on *Xestospongia.*

We are concerned primarily with the systematics of Demospongiae. They are animals for which new classificatory data is required urgently for many doubts still remain as to the correct ordinal classification of many genera and the families in general are poorly defined. Thus, here is a group with demonstrated biochemical peculiarities (diverse sterols and fatty acids, peculiar nucleosides) the systematics of which are difficult to resolve on the basis of morphological characters alone. For these reasons we have commenced a broadly based approach to biochemical taxonomy of the siliceous sponges utilizing in the first instance three techniques. These are DNA hybridization studies, acrylamide gel electrophoresis to compare soluble cellular protein patterns and thin-layer electrophoresis and chromatography to investigate free amino acid patterns. We wish, in this paper, to report on the methods and results of the amino acid investigation where we carried out an initial survey of twenty species of Demospongiae.

Before outlining the biochemical methodology, the systematic design of our species sample should be explained. Two aims were in mind when choosing the species to include in the study. First, we wished to determine at which taxonomic level free amino acid patterns were most useful as an aid to classification. Second, we wished to examine certain categories recognized by Lévi (1956) in his classification of the Demospongiae.

Lévi's (1956) classification of the Demospongiae provided a new viewpoint on the vexed question of relationships within the class. By

placing the major emphasis for the first time upon features of egg and larval development, he divided the Demospongiae into two subclasses, Ceractinomorpha with incubated parenchymella larvae and Tetractinomorpha with a diversity of reproductive patterns. Within the Tetractinomorpha, Lévi argued for the establishment of an order Clavaxinellida to include three groups which are more usually considered as separate orders, these are Epipolasida, Hadromerida, and

TABLE I
Systematic position of the species studied.
(High level classification after Lévi 1956 and Bergquist 1967)

Subclass	Order	Family	Genus and Species
CERACTINOMORPHA	DICTYOCERATIDA	SPONGIIDAE	*Spongia reticulata*
			Ircinia novae zealandiae
		DYSIDEIDAE	*Dysidea fragilis*
	DENDROCERATIDA	APLYSILLIDAE	*Aplysilla rosea*
	HAPLOSCLERIDA	HALICLONIDAE	*Haliclona heterofibrosa*
	POECILOSCLERIDA	MICROCIONIDAE	*Ophlitaspongia seriata*
		DESMACIDONIDAE	*Tetrapocillon novae zealandiae*
	HALICHONDRIDA	HALICHONDRIIDAE	*Halichondria moorei*
		HYMENIACIDONIDAE	*Hymeniacidon perleve*
	AXINELLIDA	RASPAILIIDAE	*Raspailia topsenti*
	HADROMERIDA	SUBERITIDAE	*Polymastia granulosa*
			Suberites cupuloides
			Aaptos aaptos
		CLIONIDAE	*Cliona celata*
TETRACTINOMORPHA	EPIPOLASIDA	TETHYIIDAE	*Tethya aurantium*
			Tethya ingalli
			Tethya sp.
	CHORISTIDA	STELLETTIDAE	*Stelletta arenaria*
		TETILLIDAE	*Cinachyra* sp.
	HOMOSCLEROPHORIDA	PLAKINIDAE	*Plakina monolopha*

Axinellida. The Clavaxinellida were characterized by oviparous reproduction. Other Tetractinomorpha, collectively the Tetractinellida, were in 1956, and still remain, poorly known as far as larval production is concerned. There are strong indications of oviparity in Choristida (Stellettidae: Liaci and Scisciola 1967) and of viviparity of an unusual type in the Tetillidae. The Homosclerophorida, insofar as their reproduction is known, (for single species of *Oscarella* and *Plakina* only) incubate curious amphiblastula larvae.

It is not possible to overestimate the important effect Lévi's ideas have had on sponge systematics. His new viewpoint revived interest

and debate among systematists on relationships within the Demospongiae. Further, the necessity, implicit in his classification, of knowing more details of gamete and larval production in a wide range of sponges has stimulated many workers, ourselves included, to gather and record such information. As a result, twelve years after the publication of the new classification, we have much more information than Lévi did on which to assess the validity of his arrangement of the Demospongiae. Most indications from morphological sources support Lévi's arrangement.

We aimed, in this investigation, to use biochemical systematics to provide additional information relevant to the following questions arising out of Lévi's work.

1. Can the division of the Demospongiae into two subclasses, Ceractinomorpha and Tetractinomorpha be supported?

2. What is the status of the Clavaxinellida. Can it embrace the Hadromerida, Epipolasida and Axinellida under one ordinal grouping as Lévi tentatively suggested. Is it a superorder? Need it be retained at all?

3. What relationships can be determined between the orders within each subclass?

The twenty species of Demospongiae selected for free amino acid studies are listed in Table 1. With the exception of one species, all sponges came from intertidal and shallow offshore waters near Auckland, New Zealand. The species of *Cinachyra,* included to enlarge the systematic coverage, is a West Indian sponge and was sampled as part of a later, continuing biochemical survey. It is included here since no tetillid sponges were readily available in New Zealand.

METHODS.

The system employed for separation of free amino acids from crude sponge extracts was two dimensional thin-layer electrophoresis followed by thin-layer chromatography. The method was modified only slightly from that detailed by Bieleski and Turner (1966).

a) Preparation of the sponge extract.

A known weight of sponge tissue, either fresh or killed by immersion in liquid air while fresh, is extracted by homogenizing in methanol/chloroform/water (MCW) 12/5/3 v/v. The volume of solvent used is 15-20 times the weight of tissue to be extracted. After centrifugation of the homogenate the supernatant is decanted and chloroform and distilled water, always in the ratio $CHCl_3/H_2O/1/1.5$ v/v, are added to it. (e.g. for 15 MCW, add 4 ml $CHCl_3$, 6 ml H_2O). The resulting two phase mixture is centrifuged after being well shaken, the aqueous (upper) layer is retained to be dried under vacuum or freeze dried, the chloroform layer is discarded.

When required for use the dried extract is resuspended in 10 p. 100 isopropanol and spotted directly onto the chromaplate.

b) Application of the sample.

The sample is applied along a linear origin located 2.5 cm in from one corner of a 20 × 20 cm plate spread with a mixed cellulose (MN 300)/silica gel (Kieselgel H) layer (12.5 gm/5.0 gm in 100 ml H_2O), 250 µ in thickness. The concentration of the extract applied is 10 µl of extract equivalent to 10 mg wet weight of tissue.

c) Electrophoresis.

Buffer (pH 2.0) is sprayed over the plate, the excess blotted off with chromatography paper, wicks of miracloth encased in dialysis tubing are wetted with buffer, laid across opposite ends of the plate and weighted by a glass frame. Electrophoresis is performed for 25 minutes at 1000 V/14 mA in a simple perspex box (1) using Standard Oil Varsol, saturated with buffer, as coolant to hold the plate temperature between 12°-16 °C.

d) Chromatography.

Before chromatography the electrophoretically produced bands are eluted to spots in 1 p. 100 acetic acid solution. The plates are dried and then chromatographed twice in the second dimension, first in methyl ethyl ketone/pyridine/water/acetic acid 70/15/15/2v/v and second *n*-propanol/water/*n*-propyl acetate/acetic acid/pyridine 120/60/20/4/1. Plates are dried between each step. Spots are visualized by spraying with ninhydrin (1 gm ninhydrin dissolved in a stock solution of 100 ml methanol, 1 ml acetic acid, 0.1 gm cadmium acetate). The plates, after spraying are developed at 60 °C for one hour before the spots are recorded and quantitative estimates made.

RESULTS.

In the following discussion, the designation "free amino acids" will be used to apply to those ninhydrin positive materials extractable by MCW and detectable on two dimensional chromatograms.

After visualization of the chromatogram, the pattern was traced, the spots numbered and, as far as possible, identified. Each spot was scored on a five point scale for size and intensity, both of which are rough quantitative indicators.

Verification of the identification of all amino acids named in

(1) For construction plan, see Bieleski (1965).

TABLE II

Distribution of Amino Acids and unidentified ninhydrin positive compounds in selected Demospongiae

	1 Tau	2 Hyptau	3 Taucy	4 i	5 ii	6 iii	7 iv	8 Sarc.	9 Pip.	10 β Ala	11 β amino isobut	12 γ amin isobut	13 Gly.	14 Ala.	15 Thr.	16 Ser.	17 Glu.	18 Gln.	19 Asn.	20 Arg.	21 Lys.	22 His.	23 Val.	24 Ileu.	25 Leu.	26 Phe.	27 Met.	28 Pro.	29 Cys.
Plakina monolopha	x							xx					x	x			xxx			x				x	x			x	
Stelletta arenaria	x												xxx	xx	x	xxx	xxx			x			x	x	x	x		x	
Cinachyra sp.	xxx	xx	xx					xxx		xx			xx	xx	xx	xx	xxx	xxx	x			x	x	x	x	x			xx
Tethya aurantium	xxxx	xxx	xx			xx			x		xx	x	xx	xx	x	x	xx	x	x	x	x	x	x	x	x	x	x	x	x
Tethya ingalli	xxx	x	x			x					x		xx	xx	x	x	x	x	x		x	x	x	x	x		x		
Tethya sp.	xxx	xxx	x			x			x		x		xx	x	x	x	x	x	x	x		x	x	x	x	x	x		x
Cliona celata	xxx	xxxx	x	x									xx	x	xx	x	x		x	x			x	x	x				
Aaptos aaptos	xxx	xx	x										xxx	xx	x	x	x		x				x	x	x				
Suberites cupuloides	xxx	xxxx	x	xx					x				xx	x	xx	x		x	x			x	x	x				x	
Polymastia granulosa	xxx	xx		x									xxxx	x	x	x	x		x	x	x		x	x	x	x		x	
Raspailia topsenti	xxx	xxxx							x				x	x	xx	x													
Hymeniacidon perleve	x	x											xxxx	x	x	x	xx	x	x	x	x	x	x	x	x	x	x		x
Halichondria moorei	xx	x											xx	x	x	x	x	x	x	x	x	x	x	x	x	x			x
Tetrapocillon novae zealandiae	xx				xxx								xxx	x	xx		x	x	x		x	x	x	x	x		x		x
Ophlitaspongia seriata	xxx				xxx								xxxx	xx	x	x	x	xx	x	x	x	xx	xx	x	x	x	x	x	x
Haliclona heterofibrosa	xx	x											x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Aplysilla rosea	xx												xxx	x	x	x			x		x	x	x	x	x	x			x
Dysidea fragilis	x			x					x				xxxx	xx	x	xx	xx	xx	x	xx	xx	xx	x	x	x	x	x	x	x
Ircinia	xx								xxx				xxxx	xx	x	xx	xxx	xx	xxx	x	xx	x	x	x	x	x	x		x
Spongia reticulata	x					xxx			x				xxx	xx	x	x	xxx	x	x	xx	x	xx	xx	x	x	x	x		xxx

Table II has been made using co-chromatography, comparison with the R_fGly. of known standards, alternate staining proceedures (isatin, Sakaguchi) and hydrolysis of extracts in 6N HCl before electrophoresis and chromatography. Several important spots appearing in our chromatograms are as yet unidentified, these are numbered i-iv (4-7) in Table II. Investigation of their identity is still proceeding. The major components of the free amino acid patterns from the twenty species studied and the quantity in which are present in each species is indicated in Table II.

From consideration of the table and the supporting figures, two interesting facts emerge. There are tremendous quantitative variations in certain components of the pattern, notably glycine, taurine, hypotaurine and glutamine; also there is considerable variation in the number of amino acids which run near taurine and hypotaurine. Further, these features are consistent within certain systematic groups. It is also obvious from the chromatograms figured, that the free amino acid content of sponge tissue is high relative to most other invertebrates and is quantitatively comparable with that found in *Renilla* (Kitteridge et al. 1962). This observation is diametrically opposed to that made recently by Hammen and Florkin (1968). Their observation was based on the results obtained by Inskip and Cassidy (1955) and, as already noted, the extraction proceedure and chromatographic system used by those workers was not particularly efficient.

CONTROLS.

Sponges harbour such a wealth of commensal and symbiotic organisms that it is necessary to build controls into biochemical studies to ensure that sponge amino acids are being sampled and not those of algae, bacteria or other organisms.

Care was always taken to ensure that the sample of sponge tissue extracted was free of organisms large enough to be visible under the binocular microscope. Portions of each sponge were also fixed and sectioned for microscopic examination which allowed estimation of the quantity of microbial symbionts. It is known that certain sponges harbour huge quantities of intracellular bacteria but our observations suggest that this is a feature only of sponges belonging to the Veronginae, none of which are included in this sample. In all species considered here the sponge tissue was sufficiently free of symbionts to ensure swamping of any contaminants.

We have studied samples of the same sponge from different locations (Open Coast/Harbour) to acertain whether interaction with a different planktonic microfauna and microflora would produce differences in amino acid patterns. No differences were found.

We also considered that variation in reproductive state of the sponge might lead to seasonal fluctuation in amino acid pattern, at least on a quantitative basis. This has been investigated in one species (*Halichondria moorei*) and no differences were found.

DISCUSSION.

Considering first the free amino acid patterns of Ceractinomorpha in contrast with those of Tetractinomorpha. The Ceractinomorpha appear as a relatively homogeneous group with free amino acid patterns dominated by large quantities of glycine associated with a consistent representation of most of the protein amino acids. The Tetractinomorpha, on the other hand, is not a homogeneous group, four types of amino acid patterns are recognizable within the subclass.

The Homosclerophorida (*Plakina*) has a low free amino acid content by comparison with all other groups and the simple pattern is dominated by an exceptionally high quantity of glutamic acid and by the presence of sarcosine (methyl glycine).

Within the Choristida, both families represented show distinct amino acid patterns. The Stellettidae (*Stelletta*) has a pattern which is most like that of the Poecilosclerida where protein amino acids are represented most strongly, but in smaller numbers than appear on poecilosclerid chromatograms. The Tetillidae (*Cinachyra*), on the other hand (Fig. 1, Pl. I, 1), has a complex of discreet compounds which occupy the neutral region of the chromatogram and run near taurine. These include hypotaurine and taurocyamine. In the central area of the chromatogram glutamine, glutamic acid and sarcosine are present in high amount. The presence of β alanine in *Cinachyra* is interesting, it occurs nowhere else in the sample.

The Epipolasida (*Tethya*), Hadromerida (*Suberites, Cliona, Polymastia*) and Axinellida (*Raspailia*), which together constitute the Clavaxinellida (Lévi, 1955, 1956), have broadly similar patterns. In general, all species in this group have quantities of free amino acids with the greatest concentration in the neutral region near taurine. Hypotaurine is present in all cases, taurocyamine is absent only from *Polymastia.* Other unidentified compounds are also present in this region (Fig. 2, Pl. I, 2). The centre of the chromatogram is dominated by glycine with other protein amino acids present in moderate amount. There is great similarity between the patterns shown by *Tethya* and

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FIG. 1

Map of free amino acid pattern of *Cinachyra* sp.

Numbers 1-29 correspond with numbers in Table II. Additional compounds not tabulated are 31 Cysteic acid, 32 Methionine sulphone, 33, 34, 35, 36 unidentified, 37 Tyrosine.

FIG. 2

Map of free amino acid pattern of *Tethya aurantium.*

Notation as detailed for Fig. 1.

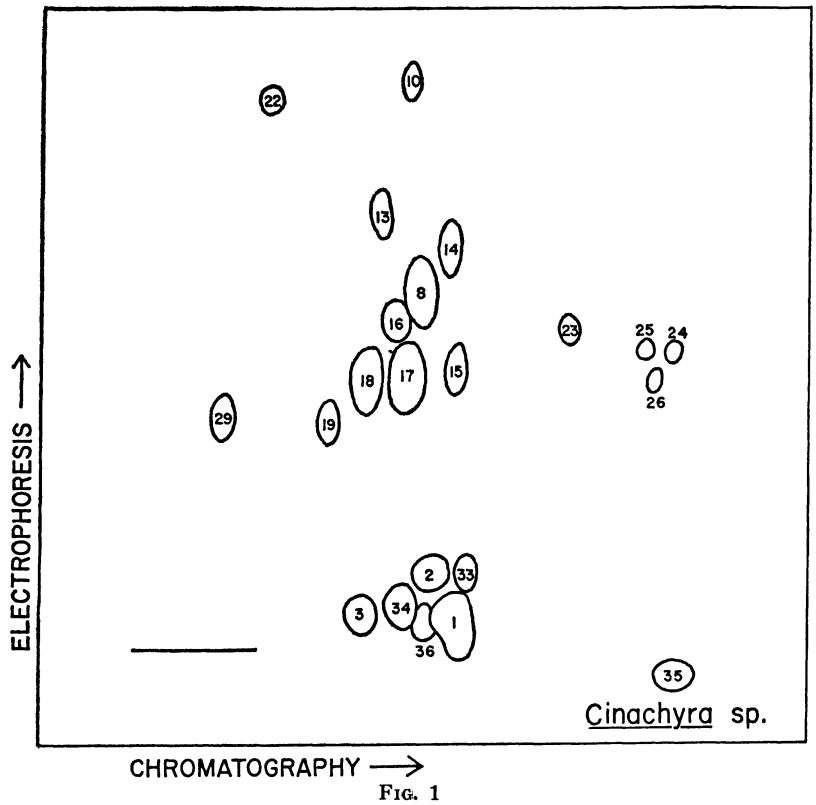
ELECTROPHORESIS
CHROMATOGRAPHY
Cinachyra sp.
22
10
13
14
8
16
23
25
24
18
17
15
26
29
19
2
33
3
34
1
36
35

FIG. 1

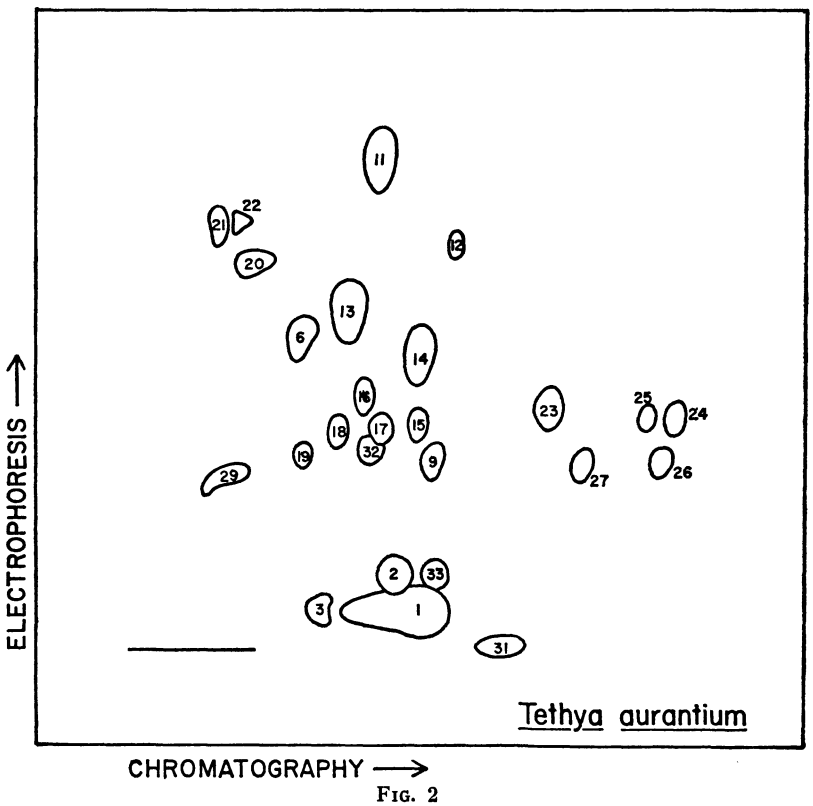
ELECTROPHORESIS
CHROMATOGRAPHY
Tethya aurantium
11
22
21
20
12
13
6
14
16
23
25
24
18
17
15
19
32
9
27
26
29
2
33
3
1
31

FIG. 2

all Hadromerida, but it should be mentioned here that the Axinellida emerges as quite a distinct group when detailed comparison are drawn.

If we examine the chromatographic patterns for any indication of relationship between recognized orders in each subclass, it is apparent that, within the Ceractinomorpha, differences between orders are not as great as in the Tetractinomorpha. However, two types of pattern are distinguishable. The Haplosclerida (*Haliclona*) and Halichondrida (as exemplified particularly by *Halichondria*) have a very characteristic pattern where most or all of the twenty protein amino acids occur in moderate and equal amount. In the neutral region of the chromatogram taurine is accompanied by hypotaurine (Fig. 3). The other pattern (Fig. 4, Pl. I, 3) which is characteristic of the Poecilosclerida (*Ophlitaspongia, Tetrapocillon*), Dictyoceratida (*Spongia, Ircinia, Dysidea*) and Dendroceratida (*Aplysilla*) is dominated by huge quantities of glycine with other components present in unequal amounts. Hypotaurine never occurs. The presence of great quantities of glycine in *Hymeniacidon* (Halichondrida) produces a pattern which is to some extent intermediate between the two types described. However, the presence of hypotaurine and the uniform representation of components other than glycine places *Hymeniacidon* closest to the haplosclerid/halichondrid group.

It is not possible on the basis of our present sample to point to any major feature of the amino acid pattern which separates the Haplosclerida and Halichondrida. However the Poecilosclerida are distinguished by high quantities of an unidentified compound (Compound ii in Table II) which has an R_FGly of 0.92 and R_EGly 0.3, while all genera of the Dictyoceratida are distinguished by the presence of pipecolic acid which is found nowhere else within the Ceractinomorpha. The Dendroceratida, as judged by one species show no remarkable features, they are very close to the Dictyoceratida but lack the pipecolic acid and have a relatively low free amino acid content.

As for relationships within the Tetractinomorpha, we have already noted the distinctness of the Homosclerophorida and both families of the Choristida from each other and from the three orders referred to collectively as the Clavaxinellida.

We should elaborate, however, on the relationships within the clavaxinellid group. On the basis of our present sample the Epipolasida, represented unfortunately only by one family, the Tethyiidae, is extremely close to the Hadromerida. The Axinellida are quite distinct. They share the emphasis on taurine, hypotaurine and associated compounds found in other clavaxinellids but they lack many of the common protein components and do not have large quantities

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FIG. 3
Map of free amino acid pattern of *Haliclona heterofibrosa*.
Notation as detailed for Fig. 1.

FIG. 4
Map of free amino acid pattern of *Dysidea fragilis*.
Notation as detailed for Fig. 1.

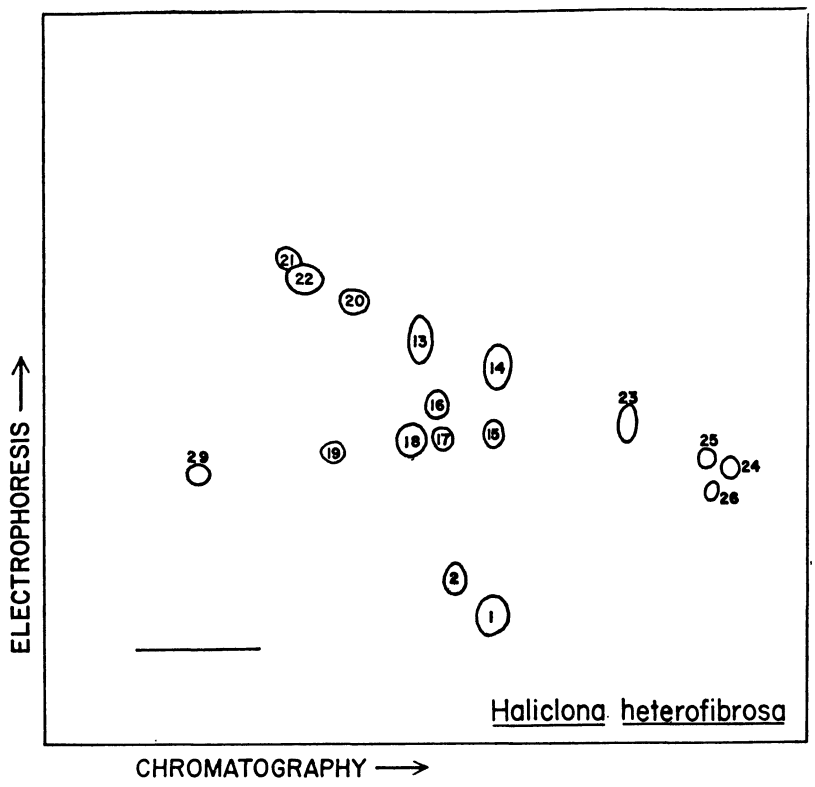
ELECTROPHORESIS
CHROMATOGRAPHY
Haliclona heterofibrosa

FIG. 3

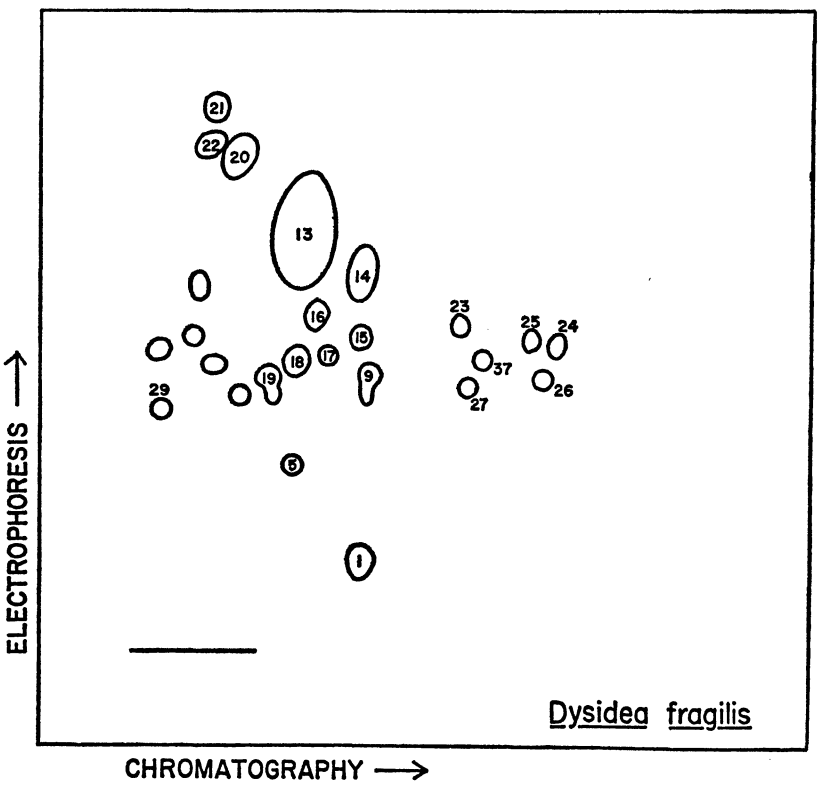
ELECTROPHORESIS
CHROMATOGRAPHY
Dysidea fragilis

FIG. 4

of glycine. The possible systematic implications of this difference will be discussed later.

The above are the salient points in which the free amino acid patterns differ at the ordinal level and above, more detailed analysis of the patterns can be utilized to differentiate genera within a family and species within a genus, at least in the sample tested thus far.

There is a problem at the family level since no two sponge systematists can yet agree on the number and composition of families within the Demospongiae. It is therefore futile to discuss family relationships except in those cases where there has been, either for

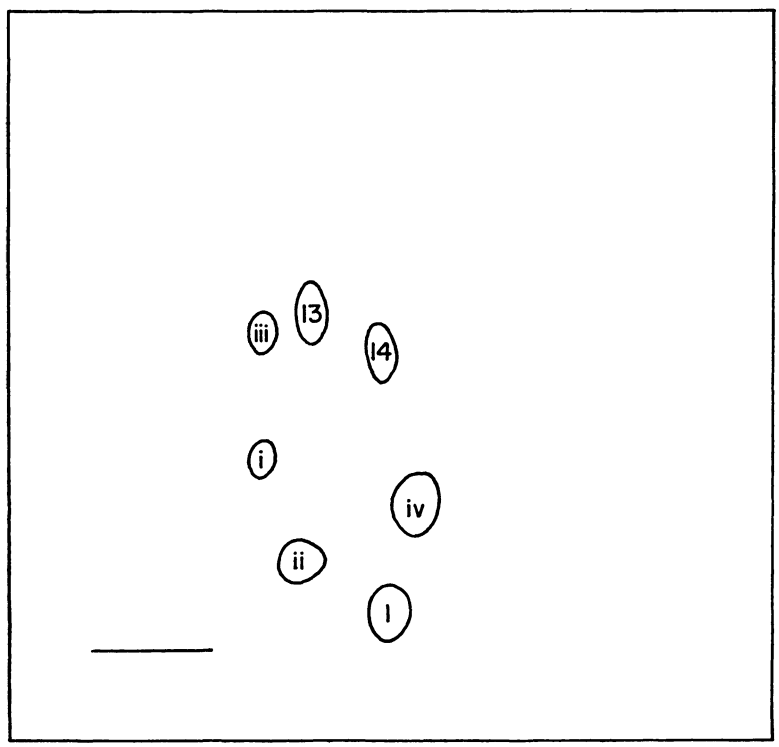

FIG. 5
Map of unidentified compounds i-iv.
Reference amino acids 13 Gly, 14 Ala, 1 Tau.

reasons of ignorance or knowledge, little change in family classification regardless of author. Most recent workers have considered the Tetillidae to be a family of the Choristida (Lévi 1956, De Laubenfels 1936). Certain systematists, notably Dendy (1922), maintained that in fact the tetillids were very distinct from the Choristida, and placed them in a different suborder (Note: in Dendy's classification the suborders were huge categories by comparison with more recent classifications).

While the basis for Dendy's separation of the Tetillidae from the asterose choristids is not here considered sound, the idea that the

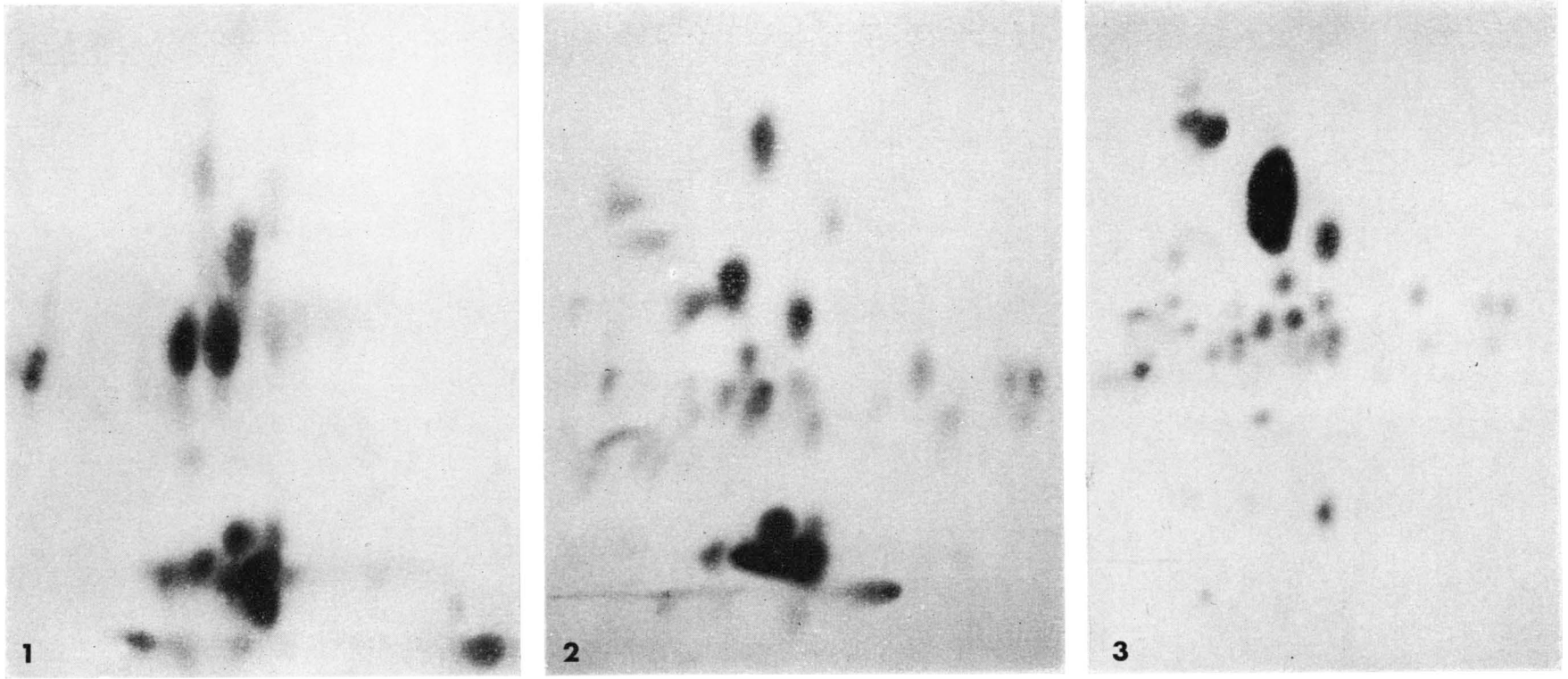

Plate I
Chromatograms.
1 - *Cinachyra* sp. 2 - *Tethya aurantium*. 3 - *Dysidea fragilis*.

two groups are quite separate is interesting in view of the very distinct amino acid patterns shown by representative species of both groups. The differences are certainly greater than those existing between any other of families in our sample. Also, a good case can be made on reproductive and morphological grounds for the recognition of the Tetillidae as a separate order.

When we consider the possibility of distinguishing between related genera on the basis of free amino acid patterns the indications are equivocal. At this point the need for a larger species sample is apparent and a more extensive investigation is now under way utilizing the Jamaican sponge fauna. At this point we can only say that on our results to date we can distinguish the three genera of Suberitidae and the two genera of Spongiidae. To take the latter example, *Spongia* and *Ircinia* differ markedly in the quantity of pipecolic acid present, *Ircinia* lacks Compound iv which is present in high quantity in *Spongia.* The sample of genera and species within the Spongiidae has been enlarged considerably in the Jamaican survey (twelve species belonging to five genera). The results of this work will be published elsewhere, but it is desirable to note here that preliminary indications that genera can be differentiated on the basis of amino acid content have proved valid.

At the species level the only example we have tested is *Tethya* and here the three species are separable by the details of amino acid pattern. All three species possess β-amino-iso-butyric acid and compound iii, *Tethya aurantium* also has γ-amino-iso-butyric acid. Pipecolic acid is present in *T. aurantium* and *T. sp.* but not in *T. ingalli.* The latter also has a much smaller quantity of hypotaurine than the other two species.

SYSTEMATIC IMPLICATIONS OF THE CHROMATOGRAPHIC RESULTS.

On the basis of the differences observed in the free amino acid patterns of the twenty species selected, the following suggestions can be made in response to the questions posed earlier.

The Ceractinomorpha as constituted by Lévi (1956) is a very homogeneous group of five orders in which the only substantial difference in free amino acid pattern lies between the Halichondrida and Haplosclerida on the one hand and the Poecilosclerida and Dictyoceratida on the other. The Tetractinomorpha, however, is more difficult to comprehend as a single group, in fact there appear on the basis of biochemical evidence to be four distinct categories, not all of which coincide with recognized ordinal groupings. The groups are the Homosclerophorida, the Spirophorida (2), the Choristida proper

(2) The name Spirophorida is derived from the family name, Spirophoridae used by Brien (1968) for sponges previously termed the Tetillidae. Brien attributes this name to Lévi (1956) but this is incorrect, Lévi has not to our knowledge published the name. The name could however, be in manuscript under Lévi's name. It is utilized here since it is now published and serves admirably as a name for the ordinal category we propose to recognize.

and the Clavaxinellida. These categories coincide to a great extent with the arrangement proposed by Topsent (1928) on the basis of morphological evidence. There is the added factor that four different types of reproductive pattern characterize these four tetractinomorph groups.

1) Clavaxinellida—are oviparous producing either parenchymella larvae or curious "amphiblastulae".
2) Choristida—are oviparous with larval development and morphology still unknown.
3) Spirophorida—are viviparous, incubating complete young sponges which are sexual in origin. Larvae are unknown.
4) Homosclerophorida—incubate curious amphiblastulae in the two examples for which details of reproduction are known (*Plakina, Oscarella*).

Our view is that these groups represent four divergent lines of equal systematic rank within the Tetractinomorpha. Many details of family and generic placement need to be decided between these groups and a great deal more information, particularly on reproductive biology, is required to enable affinities within this subclass to be debated in the context of Lévi's classification.

It will suffice here to draw attention to two groups which raise special problems, the Epipolasida and the Clavaxinellida.

The Epipolasida is represented in our sample only by species of *Tethya* and it is clear that the Tethyidae show strong affinities with the Hadromerida particularly with the genus *Aaptos*. We suggest that a logical systematic arrangement should include the Tethyiidae as a family within the Hadromerida as suggested by Topsent (1928) and at least implied by earlier workers such as Sollas (1888) and Lendenfeld (1889). This leaves us with no information, reproductive or biochemical, relevant to sponges which may belong to that ill-defined order, Epipolasida. Sponges of the genera *Epipolasis, Sollasella* and some of those classified as species of *Jaspis* must be investigated before any decision can be made on the precise status and affinities of the Epipolasida. Such sponges are not common, they are in general deep water forms, occurring in shallow water only in the tropics. This makes the necessary information more difficult to obtain.

If we adopt the view that the Tethyidae belong to the Hadromerida and accept that we have little information on sponges which might remain in an order Epipolasida were it to be retained as Lévi (1955, 1956) and De Laubenfels (1936) suggest, then the necessity for a group, Clavaxinellida becomes less obvious.

Although there are undeniable similarities in reproduction and biochemistry between the Hadromerida and the Axinellida, there are sufficient differences, when morphology is also considered, to treat these groups as separate orders (Bergquist 1967). At the same time on morphological grounds, it is likely that most "epipolasid" genera which are not either axinellid or hadromerid will fall eventually into the Choristida and Homosclerophorida. It is not absolutely clear from Lévi's work (1955, 1956) whether he favors retention or abolition

of the Epipolasida (cf. 1955, p. 78 and p. 83; 1956, p. 167 and 169) or, if it is to be retained, where he would place it.

We consider that it is desirable to discontinue use of the name Epipolasida and to assign the disputed genera to the appropriate position in other orders. Also, the term Clavaxinellida seems no longer useful except to imply that the relationship between the Hadromerida and Axinellida is closer than any other relationship existing between orders of the Tetractinomorpha.

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Summary

Free amino acid patterns of Demospongiae follow lines coincident with recognized systematic groupings and are thus useful in deciding ordinal affinities of species the position of which is still debatable. Details of the amino acid patterns can be utilized to elucidate classification at all levels at east in the sample examined thus far.

The division of the Demospongiae into two subclasses proposed by Levi (1955, 1956) can be supported on our evidence but the case for a category Clavaxinellida cannot. Morphological and biochemical evidence combine to suggest abandoning the order Epipolasida, placing the Tethyidae within the Hadromerida and recognizing the Choristida and Spirophorida as separate orders.

Zusammenfassung

Es wurde der Zusammenhang zwischen dem Verteilungsmodus freier Aminosäuren und der systematischen Stellung von Demospongien untersucht. Gewisse, von Lévi (1955/56) vorgeschlagene systematische Kategorien, werden im Lichte der biochemischen Evidenz diskutiert.

Die Resultate lassen erkennen, dass der Vergleich des Verteilungsmodus freier Aminosäuren nützliche Anhaltspunkte liefert für alle Stufen der Klassifikation.

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