A RE-EVALUATION OF THE RELEVANCE OF ACID MUCOPOLYSACCHARIDES IN SPONGE TAXONOMY

by

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Résumé

Des études antérieures **sur** les Spongiaires et d'autres Invertébrés ont amené **à** l'hypothèse de l'utilisation possible des mucopolysaccharides **(AMPs)** dans la taxonomie. Ainsi, Stempien **(1966) a** proposé que la composition et la localisation des **AMPs à** l'intérieur des tissus des Spongiaires soient utilisés **pour** résoudre quelques-uns des problèmes taxonomiques complexes de ce groupe. Plus récemment, Rahemtulla et Lovtrup **(1975 b)** ont suggéré que la répartition des **AMPs** particuliers **à** l'intérieur des différents phylums d'Invertébrés puisse servir de base **à** l'établissement de rapports phylogénétiques.

Tenant compte de ces études antérieures, le caractère et la localisation cytologiques des **AMPs** dans une vaste gamme de Demospongiae ont été examinés **en** détail en **vue** d'établir une base ferme pour un rôle possible des **AMPs** dans la taxonomie de cette classe de Spongiaires. L'analyse des **AMPs** par les procédés électrophorétiques et histochimiques employés dans cette étude plus approfondie **a** cependant prouvé l'inexactitude des hypothèses existantes. Le type et la localisation des **AMPs** ne peuvent **pas,** en effet, être considérés comme des paramètres taxonomiques valables dans la classe des Demospongiae.

INTRODUCTION

The acid mucopolysaccharides (AMPs) are considered to serve important functions in many biological processes since they are known to be integral components of the connective tissues of most organisms. Because of their wide distribution in nature, attention has been recently drawn again to their possible use in taxonomy (Rahemtulla and Lovtrup, 1974 a, b; 1975 a, b). A much earlier study had suggested that the composition of AMPs and their localization within sponge tissues could provide a useful basis for establishing taxonomic relationships within the Phylum Porifera (Stempien, 1966).

Prevailing problems in sponge classification are such that information obtained from cytochemical and biochemical studies which can be used in taxonomy is of immense benefit as a supplement to the traditional means of classification. The difficulties inherent in the classification of sponges also exist in any histological studies,

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since the numerous sponge cell types remain poorly defined. It is here suggested that five principal cell types may be differentiated with the Phylum Porifera (Class Demospongiae) :

1. The totipotent *archaeocytes,* which are large, nucleolate cells with the ability to change into most other cell types;

2. The flagellated *choanocytes,* which are responsible for generating the water currents essential for feeding and physiological processes;

3. The *pinacocytes,* which form the protective internal and external lining layers;

4. The *blast cells,* which produce either spicules or fibres for the sponge skeleton. This category incluses spongoblasts, scleroblasts and the ubiquitous collencytes;

5. The *amoebocytes,* which possess amoeboid activity and which are differentiated from the archaeocytes by their smaller size and lack of a nucleolus. Amoebocytes are frequently classified according to the presence or absence of granular inclusions, and many secretory cells fall into this category.

The multitude of names characterizing specialized sponge cell types which has arisen out of detailed studies on individual species has not thus far allowed any generalized approach to sponge cytology. Many specialized cell types have been established merely on the presence or absence of some recognizable inclusion, or some poorly defined cell function. The practice of classifying cell types according to function is rendered dubious by the existence of the totipotent archaeocytes. The presence of these cells implies that the ability to perform particular functions may be inherent within every cell and that changes in the cell environment, for example, may produce functional changes within the cell to meet the immediate demands of the sponge. This would be particularly evident during development or in aggregation studies where dissociation and subsequent aggregation produce new environments for the cell. For these reasons, the cytochemical observations reported here are confined largely to the principal cell types outlined above, and reference to specialized cell types will be made only when considered necessary.

The histological studies detailed here were designed to test the suggestion that the cytological localization of AMPs within the Class Demospongiae may prove of taxonomic value. A preliminary report on electrophoretic experiments aimed at characterizing these AMPs is included. The new species referred to in this paper are not named for reasons of priority but will be described in subsequent publications.

EXPERIMENTAL

In the cytochemical study, thirty-two specimens representing nine orders of the Class Demospongiae were collected from various localities, fixed immediately in cetyl pyridinium chloride-formalin (Williams and Jackson, 1956) and **paraffin** sections were prepared using

standard histological techniques. The cytological localization of AMPs was demonstrated by a variety of methods including alcian blue at pH 1.0 and 2.5 (Steedman, 1950), the critical electrolyte stain (Scott *et al.,* 1964) and the alcian blue—periodic acid Schiff technique (Mowry, 1963). Using a combination of these staining procedures, it is possible to differentiate between the sulphated and non-sulphated AMPs, neutral mucosubstances and sialomucins. The general morphology and fixation of each species was checked by examining sections stained with Mallory's triple stain (summarized in Humason, 1967).

AMPs were extracted for electrophoresis from a selection of sponges by a modification of the micro-method of Breen *et al.* (1970). Small samples of each sponge blotted dry and weighing 5 g were mechanically dissociated through 60μ gauge bolting cloth into a solution containing 8ml of 0.5M sodium acetate buffer pH 7.5, 0.5ml of 10mM $CaCl_2$, and 2ml of Pronase B (Calbiochem) at 1mg/ml. Proteolytic digestion was carried out for 24 h at 50°C and the digest was then cooled to 4°C before the protein was precipitated over 20 min with 5 per cent trichloroacetic acid. The protein was removed by centrifugation (8000rpm, 20min, 0°C) and the AMPs were then precipitated over two days at 0"C by the addition of 3 volumes of absolute ethanol and 1ml of supersaturated LiCl. The AMPs were pelleted by centrifugation (8000 rpm, 20min ,0°C), washed in esthanol, ethanol and ether (1:1), and then ether before being air dried and resuspended in 0.1ml of distilled water. Electrophoresis was performed on cellulose acetate membranes in a Beckman microzone electrophoresis apparatus using 0.2M $ZnSO_4$ and 0.05M LiCl as the electrolyte systems. After electrophoresis, the membranes were stained with 1 per cent alcian blue (pH 2.5), washed and cleaned before being air dried. The following standards were employed at lmg/ml : Heparin (Sigma Grade I), chondroitin sulphate mixed isomers (Sigma Grade III), hyaluronic acid (Sigma Grade I) and dermatan sulphate (Sigma). Heparan sulphate standard could not be obtained but its electrophoretic mobility was determined from previous studies (Breen *et al,* 1970). The AMPs were identified over a number of runs employing both electrolyte systems by calculating Rx values relative to chondroitin sulphate and relating these to similar values for the standard chemicals.

RESULTS

The results of the cytological localization study have been summarized in Tables 1 and 2.

In *Plakina monolopha,* the sole representative of the Order Homosclerophorida, acidic sulphated mucosubstances were most prevalent and were localized predominantly in the choanocytes and the pinacocytes (Plate I, 1). The critical electrolyte stain was most intense at 0.1 $MgCl_2$, although the pinacocytes retained their reactivity at 1.0M $MgCl_2$ suggesting the presence of highly sulphated AMPs such as heparin, as well as the chondroitin sulphates. Although only

one species was examined from this order, the results are largely in agreement with that of Stempien **(1966).**

Within **the** Order Choristida, slight staining of the amoebocytes and the collencytes of *Stelletta maori* was apparent. Using the critical electrolyte stain, no reaction was noticeable within the amoebocytes below **0.1M** $MgCl_2$ suggesting the presence of an unsulphated AMP and alcianophilia was lost from the collencytes at 0.2M $MgCl_2$ suggesting the presence of the chondroitin sulphates in this cell type. In *Geodia* n.sp., some alcianophilia was confined to the pinacocytes but, generally speaking, the AMPs seemed to be dispersed throughout the mesohyl. The localization of AMPs within this order was thus seen to differ between the two genera considered. No previous study of the localization of AMPs within the Order Choristida has been made.

Within the Order Axinellida, the exopinacocytes of *Raspailia topsenti* were found to contain sulphated AMPs. The specimen examined contained eggs and many cellular structures had been resorbed or redisposed. Thus various cell types, such as the archaeocytes, were not evident. The eggs were of granular composition, staining positively for mucosubstances, sialomucins **and sulphated AMPs.** Staining reactions indicative of the chondroitin sulphates were found in the collencytes and pinacocytes of *Trachycladus stylifer* and the mesohyl of this species was found to contain unusually large amounts of sialomucins. The staining reactions of the pinacocytes, amoebocytes and choanocytes of *Axinella australiensis* suggested the presence of hyaluronic acid or weakly acidic sulphated mucopolysaccharides in these cells. The choanocytes were also found to contain some sialomucins. The specimen of *Desmoxya* n.sp. examined was in the early stages of egg production but still retained most of its normal cellular characteristics. Sulphated mucosubstances were found in the amoebocytes, pinacocytes and choanocytes but not within the collencytes. It is apparent that the results are variable within the Order Axinellida, and no reference can be made to other studies since this group has not been considered previously.

Four species of the genus *Tethya* represented one family within the Order Hadromerida and provided an opportunity to examine interspecific differences in the localization of AMPs. The results were found to vary between each species. The genus *Tethya* has often been classified in the Order Epipolasida (Topsent) but, even if recent taxonomic changes had not been made (Bergquist and Hogg, 1969), there is no evidence to support the conclusion of Stempien (1966) that AMPs are always found as inclusions within the amoebocytes and choanocytes. As can be seen from Table 1, the choanocytes and pinacocytes of *Suberites cupuloides* contained a mixture of sulphated AMPs and neutral mucosubstances or sialomucins. These two particular cell types also displayed alcianophilia in both species of *Polymastia,* but traces of similar materials were also found in the collencytes and amoebocytes of *Polymastia fusca.* In the specimens of *Aaptos aaptos* examined, many spermatozoa occupied the flagellated chambers. The spermatozoa were seen to be associated with sialomucins, neutral mucosubstances and weakly acidic mucosubstances by their characteristic staining reactions. Localization of alcian blue positive material in this species was restricted to the collencytes and

TABLE 1
Summary of cytochemical results Sub-class Tetractinomorpha

Species	Cell Type					Notes
	Ar	Ch	Co	Pl	Am	
Plakina monolopha		A.		A.		
Stelletta maori	P.		A.P.		A.P.	Dispersed within the mesohyl
Geodia n.sp.				A.		Dispersed
Axinella australiensis		A.P.	A.	A.	A.	
Raspailia topsenti				A.		Reproducing:eggs
Trachycladus stylifer			A.	A.P.		
Desmoxya n.sp.	A.	A.		A.P.		Reproducing:eggs
Tethya aurantium			A.	A.	P.	
Tethya ingalli		A.P.	A.	A.	A.	
Tethya n.sp.A		A.P.	P.		A.	
Tethya n.sp.B					A.	Poor reaction
Latrunculia braevis		A.P.			A.P.	
Aaptos aaptos			A.	A.	P.	Reproducing:sperm
Polymastia granulosa		A.		A.		Poor reaction
Polymastia fusca		A.	A.	A.	A.	Poor reaction
Suberites cupuloides		A.P.		A.P.	P.	Dispersed
Cliona celata		A.P.		A.	P.	

A: alcian blue positive cells; P: periodic acid Scbiff positive cells; Ar: archaeocytes; Ch: choanocytes; Co: collencytes; Pi: pinacocytes; Am: amoebocytes.

TABLE 2
Summary of cytochemical results Sub-class Ceractinomorpha

Species	Cell Type					Notes
	Ar	Ch	Co	Pl	Am	
Aplysilla rosea		A.P.		A.	A.	
Aplysilla sulphurea		A.P.			A.	Ectosome intense: Reproducing:larvae
Aplysilla violacea				A		Dispersed
Dysidea fragilis					A.	Dispersed
Spongia n.sp.			P.	A.	A.P.	Dispersed
Ircinia novaezelandiae		P.	P.	A.	P.	Dispersed
Haliclona heterofibrosa		A.	A.		A.	Reproducing:larvae
Microciona coccinea	A.	A.			A.	
Anchinoe incrustans		A.	A.		A.P.	
Adocia venustina		A.		A.P.	A.P.	
Tedania ignis			A.P.		A.P.	Intense reaction
Mycale macilenta		A.	A.P.	A.P.	A.P.	
Halichondria moorei	P.	A.P.		A.	A.	Reproducing:gemmules
Hymeniacidon perleve					A.	Poor reaction

For abbreviations, see Table **1.**

pinacocytes. *Cliona celata* and *Latrunculia braevis* displayed alcianophilia in the choanocytes, pinacocytes and amoebocytes indicating the possible presence of the chondroitin sulphates.

The Order Dendroceratida was represented by three species of the genus *Aplysilla,* each of which differed in their staining reactions with alcian blue. In *Aplysilla rosea,* alcianophilia was confined to the

amoebocytes, choanocytes **and** pinacocytes **(Plate I, 2).** The amoebocytes of this species reacted strongly with alcian blue at **pH 1.0,** but at 0.2 **M** $MgCl_2$, this reaction was absent. These reactions suggested that the granules within the amoebocytes of *Aplysilla rosea* were composed predominantly of the chondroitin sulphates. In *Aplysilla sulphured,* alcian blue staining was confined to the choanocytes and the amoebocytes and, in the latter cell type, the reaction was strongest at 0.2M $MgCl_2$. Staining of granules within the amoebocytes of this species did not disappear until 0.8M $MgCl_2$ suggesting the possible presence of the chondroitin sulphates and heparin. The amoebocytes of *Aplysilla violacea* failed to display any significant alcianophilia and localization was only evident in the pinacocytes. The results of specific staining for AMPs in this genus, particularly the failure of the amoebocytes of *Aplysilla violacea* to display any alcian blue positive material, suggested a large amount of inter-specific variation within this order.

The **AMPs** in species belonging to the Order Dictyoceratida were generally dispersed throughout the mesohyl although some localization was apparent in the pinacocytes of *Ircinia novae-zelandiae,* the amoebocytes of *Dysidea fragilis* and both the pinacocytes and amoebocytes of *Spongia* **n.sp,**

Stempien (1966) did not divide the former Order Keratosa into the Orders Dictyoceratida and Dendroceratida as used here and thus a direct comparison of results is difficult. Nevertheless, there is no evidence to suggest consistent localization of AMPs within the choanocytes and the pinacocytes as previously suggested.

The Order Haplosclerida was represented by *Haliclona heterofibrosa* and sulphated AMPs were seen to be localized in the collencytes of this species. Non-sulphated AMPs or weakly acidic sulphated mucosubstances were found in the choanocytes and in the granular amoebocytes. Although only one species was considered from within this order, the results were again found to be at variance with the earlier study.

The staining reactions of the five species representing the Order Poecilosclerida were found to differ markedly and the AMPs were not confined to the amoebocytes as previously suggested (Stempien, 1966) but were found in a number of cell types. The presence of sulphated AMPs possibly including heparin was suggested in the archaeocytes, amoebocytes, choanocytes and rhabdiferous cells of *Microciona coccinea.* The staining reactions of *Anchinoe incrustans* indicated the presence of sulphated AMPs in the choanocytes, collencytes, and amoebocytes. At 1.0M $MgCl_2$, the alcianophilic material was confined to granules of the amoebocytes, suggesting the presence of sulphated AMPs similar to heparin. The collencytes and amoebocytes of *Tedania ignis* contained some alcian blue material but further localization was obscured by the intense staining reaction of the mesohyl of this species. This reaction was presumably due to the heavy mucous discharge which occurred during collecting. *Adocia venustina* and *Mycale macilenta* displayed similar staining reactions although they were representative of two separate families. Thus the choanocytes, amoebocytes and pinacocytes of each species reacted positively with alcian blue.

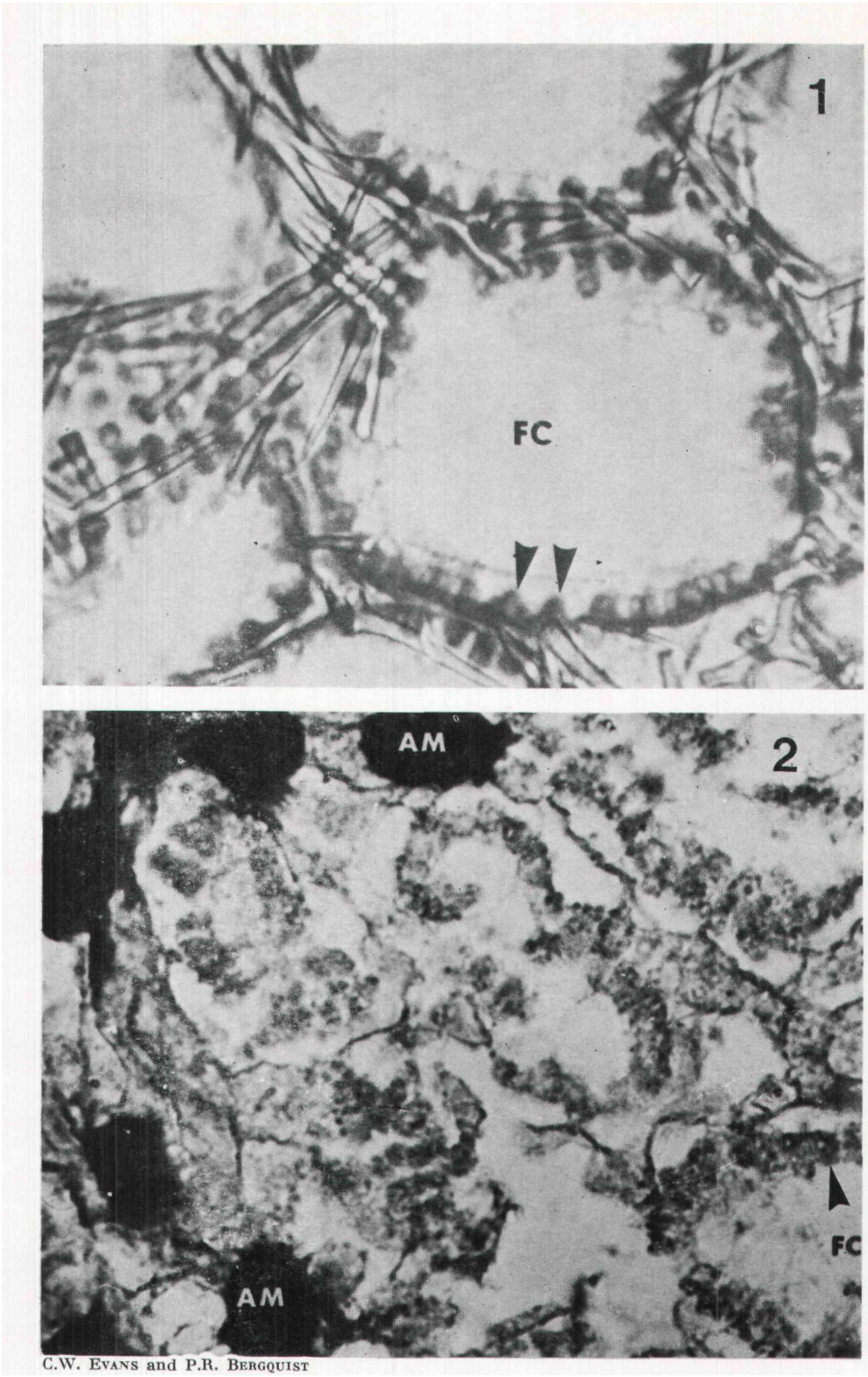

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PLATE I

1 : At pH 2.5 alcian blue positive choanocytes (arrowed) are visible lining the flagellated chambers (FC) of *Plakina monolopha* 790x.

2: Using the alcian blue/periodic acid Schiff technique at pH 1.0 intense-blue staining amoebocytes (AM) are seen in the vicinity of the flagellated chambers of *Aplysilla rosea*. The choanocytes (arrowed) lining the chambers are periodic acid Schiff positive and have stained red. Some alcianophilic material was apparent within the choanocytes when the staining sequence was used at pH 2.5. 630x.

Halichondria moorei and *Hymeniacidon perleve* represented two families of the Order Halichondrida and displayed different staining reactions. *Hymeniacidon perleve* reacted poorly to the stains employed but some sulphated mucosubstances were confined to the amoebocytes. The reactions of *Halichondria moorei,* on the other hand, suggested that various AMPs were present in the choanocytes, pinacocytes and the amoebocytes. The Order Halichondrida has not previously been considered in a cytochemical survey of AMP localization.

The results of the electrophoretic analysis of AMPs from 16 species of sponges are presented in Table 3. $ZnSO_4$ buffer separated hyaluronic acid from dermatan sulphate and LiCl distinguished hyaluronic acid from heparin and the other AMPs which ran together.

TABLE it

Results of electrophoretic analysis of AMPs from 16 species of sponges.

Species	Acid Mucopolysaccharides				
	unknown	HA	DS	CS	H
Plakina monolopha	*	*	*	*	*
Ancorina alata	*	*		*	
Raspailia agminata	*			*	
Tethya aurantium		*	*	*	
Tethya ingalli (1)		*?	*?		
Tethya n.sp.B		*		*	
Polymastia granulosa	*			*	
Cliona celata		*	*		
Aplysilla rosea		*	*	*	
Spongia reticulata		*			
Haliclona heterofibrosa	*	*		*	
Microciona n.sp.		*		*	*
Halichondria moorei	*	*	*		
Halichondria n.sp.	*	*			
Hymeniacidon perleve	*	*	*		
Ciocalypta polymastia	*	*			
Average Rx (0.2M $ZnSO_4$)	0.2	0.6	0.75	1.0	—
Rx (0.05M LiCl)	0.1	0.6	—	1.0	1.2

1. — The bands produced on the electrophoresis of the AMPs extracted from *Tethya ingalli* were broad and indistinct.
The asterisk denotes the presence of an AMP.
HA: hyaluronic acid; DS: dermatan sulphate; CS: chondroitin -4 and -6 sulphate; H: heparin.

Heparan sulphate is known to band between hyaluronic acid and dermatan sulphate (Breen *et ah,* 1970). The chondroitin sulphate isomers could not be separated by electrophoretic means.

Hyaluronic acid was found to be almost universally present and an unknown AMP displaying poor electrophoretic mobility (Rx = 0.2 in $ZnSO_4$) was also detected in several species. The biochemical nature of this AMP remains to be established but its electrophoretic mobility suggests a high molecular weight coupled with a relatively low charge.

It is apparent from Table 3 that variation in **AMP** content is obvious both within a single order, and between two species of the

same genus. Thus the two species of *Halichondria* considered, for example, have different AMP contents and contain both sulphated and non-sulphated AMPs.

DISCUSSION

The persistent difficulties in sponge classification which are largely the product of traditional taxonomic techniques, emphasise the need for all available criteria to be considered when taxonomic relationships within this phylum are examined. The hypothesis presented by Stempien (1966), which was based on the structure and localization of AMPs, raised the possibility of an additional aid to sponge classification. Stempien's study was performed on a relatively small sample of sponges and his suggestion required confirmation by investigation of a larger and more detailed sample before it could be evaluated as a taxonomic parameter. Recent taxonomic changes also demanded closer examination of his hypothesis and it was predominantly for these reasons that the cytological investigations were performed.

Based on the results presented here the suggestion of Stempien (1966) cannot be substantiated, since our investigation has shown that the cellular localization of AMPs can vary from species to species within a single genus. The type of AMP found within particular cell types as detected by cytochemical methods or within the whole sponge as demonstrated by electrophoresis provided no further information of taxonomic significance since, again, interspecific variation exists.

Stempien (1966) isolated both sulphated and non-sulphated AMPs from *Microciona prolifera,* but he could only extract non-sulphated AMPs from *Haliclona viridis.* He suggested that differences of this nature together with the cytochemical localization of AMPs were of potential use in sponge taxonomy. The identification in the present study, however, of both sulphated and non-sulphated AMPs in other species of the same genera used by Stempien *(Haliclona heterofibrosa; Microciona* n.sp.) does not support this hypothesis.

Rahemtulla and Lovtrup (1975 b) have recently attempted to use the distribution of AMPs in invertebrates as a basis for establishing phylogenetic relationships at the supra-ordinal level. Studies are still in progress on the AMP composition of other sponge classes but the present results do not support the use of AMP distribution as a taxonomic parameter at or below the ordinal level of classification. The use of AMP distribution at higher taxonomic levels is also questioned, since the present results suggest that whether a particular AMP is found to be present or absent within a particular taxon would be dependent on the species selected for analysis.

Although the data reported here on the localization and type of AMP found in the Class Demospongiae do not support the idea that they have potential as taxonomic characters within this group, the analysis of other attributes of AMPs or of the enzymes involved in their synthesis (Hunt, 1970) may ultimately be of use in supplementing present taxonomic techniques.

Summary

Previous studies on sponges and other invertebrates have suggested that acid mucopolysaccharides (AMPs) may be of possible use in taxonomy. Thus Stempien (1966) suggested that the composition and localization of AMPs within sponge tissues may be of use in resolving some of the complex taxonomic problems within this group. More recently, Rahemtulla and Lovtrup (1975 b) have suggested that the distribution of particular AMPs within various invertebrate phyla may be used as a basis for establishing phylogenetic relationships. With these earlier studies in mind, the nature and cytological localization of AMPs within a wide range of Demospongiae were examined in detail in an attempt to establish a firm basis for any possible role of AMPs in taxonomy within this class of sponges. Analysis of AMPs by the electrophoretic and histochemical procedures employed in this more extensive study, however, failed to yield evidence supporting the existing hypotheses. The type and localization of AMPs are thus not considered to be valid taxonomic parameters within the Class Demospongiae.

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