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A STUDY OF THE COMPARATIVE BIOCHEMISTRY

OF SOME MARINE INVERTEBRATES

APPROVED BY

Leon S Cieslo

Bernard C. Patten

J. C. Colbert

D. H. Wenkam

Harriet Harvey

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

The Comparative Aspects of the Distribution of Phosphagens in the Animal Kingdom

In 1927 Eggleton and Eggleton (1, 2) postulated the presence of a labile organic phosphate compound in frog muscle. They proposed the name 'phosphagen' for this compound which interfered with their determination of inorganic phosphate. The same year Meyerhof and Lohmann (3) demonstrated that it was creatine phosphate. Subsequently Meyerhof and Lohmann (4) isolated another organic phosphate, arginine phosphate, from the muscle of the lobster, an invertebrate.

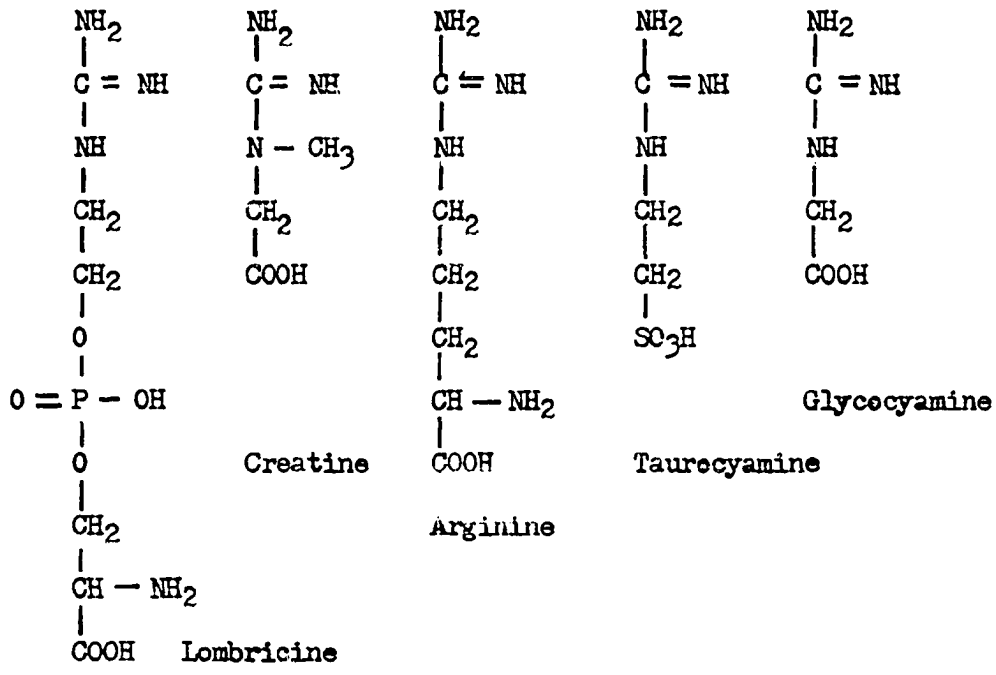
The role of guanidine phosphate compounds in muscle metabolism was not clarified until the work of Lohmann (5) in 1934 showed that creatine phosphate took part in the reaction:

$$\text{Creatine phosphate} + \text{Adenosine diphosphate} \rightleftharpoons \text{Creatine} + \text{Adenosine triphosphate}$$

The reversibility of this reaction was shown by Lohmann (6) in 1935. Currently a phosphorylated guanidine base which takes part in the following generalized Lohmann reaction (Hobson and Rees (7)) is termed a phosphagen.

FIGURE 1

THE STRUCTURE OF SOME GUANIDINE BASES AND PHOSPHAGENS



Phosphagen + Adenosine diphosphate $\rightleftharpoons$

Guanidine base + Adenosine triphosphate

The formulas of the guanidine bases and corresponding phosphagens are shown in figure 1. In contracting muscle adenosine triphosphate (ATP) is the immediate energy source. In the contraction process ATP loses one of its high energy phosphate groups, and adenosine diphosphate (ADP) is formed. Glycolysis will reconvert ADP to ATP, but regeneration is slow. The role of the phosphagen can be seen clearly from the Lohmann reaction. Phosphagen can quickly regenerate ATP in the contracting muscle, and even in the absence of glycolysis the muscle will be able to continue working until all the phosphagen is gone.

No sooner had the chemical nature of the phosphagens been determined than apparent regularities in their distribution were noted. Meyerhof (8), Kutscher (9), and Eggleton and Eggleton (10) all agreed that creatine phosphate appeared to be the characteristic phosphagen of vertebrates and arginine phosphate the common invertebrate phosphagen. Needham and Needham in 1932 summarized the data available and came to the following conclusions:

1. Phosphagens appear to be universally distributed.
2. Creatine phosphate is present in the vertebrates.
3. With the single exception of the jaw muscles of the echinoid, Strongylocentrotus lividus, a sea urchin, arginine phosphate is present in the invertebrates.
4. Among the protochordates, forerunners of the vertebrates, there is a transition from arginine phosphate to creatine phosphate.

The subphylum Tunicata, the sea squirts, represented by Ascidia

mentula, contained arginine phosphate.

The subphylum Enteropneusta, represented by Balanoglossus salmoneus, contained both arginine phosphate and creatine phosphate.

The subphylum Cephalochorda, represented by Amphioxus lanceolatus, contained only creatine phosphate.

These results provided neat biochemical evidence for the theory of Bateson (12, 13, 14) who believed that the enteropneusts were related to the echinoderms. The results also indicated that there is a gradual transition from one phosphagen to the other, rather than a sudden complete change.

The theory of Needham and Needham remained unchallenged for many years. However a few reports appeared in the literature which did not fit into the picture. Verjbinskaya, Borsuk and Kreps (15) reported finding arginine and creatine phosphates in the holothurian, Cucumaria frondosa (a sea cucumber). Verjbinskaya (16) also found a creatine phosphate like phosphagen in a chaetognath, Sagitta the arrow worm, and in some brachiopods, the lamp shells. In 1933 Arnold and Luck (17) were unable to find any evidence of arginine in annelids or gephyreans and again in 1937 Kurtz and Luck (18) found no trace of arginine phosphate in annelids. Using the reproductive organs of various invertebrates for his studies Greenwald (19) found evidence for creatine phosphate among some of them and, in 1947, Wajzer and Brochart (20) isolated arginine phosphate from the sperm of guinea pigs and swine.

At this point it may be well to comment on some of the weaknesses of the earlier work. Some of the data tabulated by Needham and Needham in their review were obtained by indirect means of analysis. It has

been found that the rate of hydrolysis of creatine phosphate in acid is greater than the hydrolysis rate of arginine phosphate in acid, especially in the presence of molybdate ion. Thus the phosphagen, extracted from the tissue by trichloroacetic acid was not isolated but was hydrolysed in solution. The rate of hydrolysis of the phosphagen was measured by the colorimetric determination of the inorganic phosphate liberated by the hydrolysis. The phosphagen was then characterized as arginine or creatine phosphate, depending upon whether the hydrolysis rate had been slow or rapid respectively. Another difficulty, though not an error, resides in the fact that some workers used the whole animal in their analyses. In some species this can hardly be avoided if enough material is to be obtained for the analysis. However, as Greenwald's work demonstrates, it is preferable to deal with only one tissue at a time since the phosphagen present in the reproductive organ may differ from that of the muscle tissue and one phosphagen may obscure the other in analysis, especially in the indirect analysis.

Although the above results were casting some doubts on the views of Needham and Needham, nevertheless Baldwin (21) in 1948 maintained that the situation was not seriously altered and that the transition from arginine phosphate to creatine phosphate in the protochordates was especially interesting from an evolutionary viewpoint. Of interest in connection with the present work is his statement that the coelenterates have not been studied but possess muscle cells and 'further investigation in this direction would be amply repaid.'

In 1950 Baldwin and Yudkin (22) began the most serious undermining of the supports of the theory of Needham and Needham. They

studied twenty-nine species of annelid and gephyrean worms. Using trichloroacetic acid they extracted the phosphagens and characterized them by their rates of hydrolysis. In the one oligochaete they examined, Lumbricus terrestris the earthworm, they found no phosphagen. Among the polychaete worms they found thirteen species containing a phosphagen hydrolysing at a rate similar to that of arginine phosphate, five species with a phosphagen behaving like creatine phosphate and six species apparently containing both compounds by virtue of their complex hydrolysis curves. Baldwin and Yudkin do not claim that these compounds are arginine phosphate and creatine phosphate. In fact they did obtain the picrate of the arginine phosphate-like compound which they refer to as 'AP'. The 'AP' picrate obtained from the polychaetes, Amphitrite johnstoni and Phascolosoma elongatum, melted at 277°C while an authentic sample of arginine picrate melted at 217°C. Thus the authors themselves pointed out the necessity for isolating the phosphagens or their free bases before positive identification can be made.

Baldwin, always ready to classify, noted at first that there appeared to be a distinct grouping of polychaetes into errant polychaetes containing creatine phosphate-like 'CP' and sedentary polychaetes containing 'AP'. This apparent grouping was based on observations made on the species at Woods Hole. When the authors continued their comparisons of polychaetes at Plymouth they found that this classification could not be retained. They state:

> At present therefore the occurrence of the two phosphagens cannot be correlated with the physiological factor of overall muscle activity, with the ecological habit or with any other environmental factor: indeed, their distribution appears to be entirely erratic.

The authors conclude that the annelids and gephyreans contain a new

unknown phosphagen 'AP' sometimes present together with 'CP' (yet to be positively identified as creatine phosphate) and that in some cases 'CP' is present alone.

In the same paper Baldwin and Yudkin again consider the case of the echinoderms and protochordates. In four species of asteroids (starfish), five species of holothurians (sea cucumbers) and two species of crinoids (sea lilies) they and other workers found only arginine or arginine phosphate present. They discredit the work of Verjbinskaya, Borsuk and Kreps (15) who reported arginine and creatine phosphates in Cucumaria frondosa. In three species of ophiuroids (brittle stars) examined, Baldwin and Needham (23) and Baldwin and Yudkin (22) found only creatine phosphate. Finally both arginine phosphate and creatine phosphate were found in four of the six species of echinoids studied while the remaining two species, which were both members of the genus Arbacia, were shown to contain only arginine or arginine phosphate. To Baldwin and Yudkin this was an indication that the replacement of arginine by creatine was not a sudden mutation but a gradual process. The same gradual change was mentioned earlier in the case of the protochordates and these authors chose to believe that this is evidence supporting the theory that the vertebrates evolved from the echinoderms via the hemichordates. This theory failed to achieve further confirmation when two more species of enteropneusts were examined. In both cases the animals, Saccoglossus kowalevskyi and Saccoglossus horsti, were shown to contain only creatine phosphate.

Inasmuch as Baldwin and Yudkin had found at least one new phosphagen and possibly creatine phosphate among the annelids and gephyreans

it was necessary that they fit this in with their biochemical theory of evolution. This they account for by saying that the annelids and gephyreans replaced arginine phosphate in a process of convergent chemical evolution and not because they are close relatives of the chordates.

In 1953 Baldwin (24) published his latest work on biochemical evolution. In it he again draws attention to the apparent non-conformity of the genus Arbacia with the others of the echinoid class and also to the variation in the enteropneust class between the three species studied, Balanoglossus salmoneus, Saccoglossus kowalevskyi and Saccoglossus horsti. It is well worthwhile to quote his comments on this:

> . . . .it must be emphasized that, in view of the unexpected variability already discovered within both these groups, it is not advisable to base theories of chemical evolution upon observations of too small a number of species. If comparative biochemistry is to be fruitful and give birth to new ideas on evolution it must, above all things, be comparative enough.

Baldwin then makes an admission which is most damaging to the theory of Needham and Needham. In trying to confirm the original work of 1932 on Balanoglossus salmoneus Baldwin collected a species known as Balanoglossus clavigerus and found no arginine phosphate present. Baldwin states that it now seems likely that the specimen examined in 1932 was not Balanoglossus salmoneus but was Balanoglossus clavigerus. This means that all the enteropneusts studied now seem to contain only creatine phosphate and thus this class no longer appears biochemically as an evolutionary stepping-stone from the echinoderms. Baldwin, always ready with an explanation, does not let this fact pass without a challenge. He asks:

> Is it, perhaps, just conceivable that at some time between 1931 and 1951 the local race of Balanoglossus has undergone an odd mutation and lost its arginine phosphate once and for all? Arginine phosphate has been lost before in the course of evolution, as it evidently has been in the ophiuroids, for example.

These results bring us to the most recent work on phosphagens, most of which has been done by three groups of workers led by Jean Roche in France, A. H. Ennor in Australia, and Hobson and Rees in England.

It would seem that Roche and his group were originally interested in the metabolism of guanidine compounds, and these studies almost naturally led to the comparative study of the distribution of the phosphagens. In 1948 (25) and in 1950 (26) Roche and his group had studied the bacterial degradation of glycocyamine and the distribution of bacterial deguanidases. Then in 1951, Thoai and Robin (27) related the methylation of guanidoacetic acid (glycocyamine) to the distribution of creatine among marine invertebrates. Their original suggestion was that the creatine precursor, glycocyamine, and the transmethylating enzyme converting glycocyamine to creatine must both be present if the animal contains creatine. Lack of one or the other of these factors would lead to the absence of creatine in the animal. To test their theory they prepared aqueous extracts from four marine invertebrates, the ophiuroid Ophiothrix fragilis, the echinoid Paracentrotus lividus Lmk., the mollusk Pecten maximus and the arthropod Palinurus vulgaris L., a lobster. These extracts were incubated with glycocyamine and a methyl donor, either methionine or choline. After incubation, the creatine formed by each extract was quantitatively determined. In all extracts but the lobster extract considerable quantities of creatine were produced. This indicated that the lobster, which does not contain creatine, also does not possess the transmethylating enzyme. The mollusk likewise does not contain creatine, yet its extract produced creatine. This indicated that it possesses the transmethylating enzyme but lacks the creatine precursor

glycocyamine in its tissues.

It was in this period that better identification techniques were being developed. In 1948 Ennor and Stocken (28) studied in detail the Barritt (29) modification of the Vosges and Proskauer (30) diacetyl-∝-naphthol color reaction for the estimation of creatine. In the same year these authors published a method for the preparation of sodium phosphocreatine (31). This method has recently been adopted as a standard biochemical preparation (32). Then in 1951 Roche, Felix, Robin and Thoai published their first paper on the chromatography of monosubstituted guanidine derivatives (33). Prior to this there had been no satisfactory way of distinguishing between the monosubstituted guanidines present in a mixture.

In 1952, Ennor and Rosenberg (34) turned their attention to the distribution of phosphocreatine in animal tissues. Studying the rat, rabbit and cat they found phosphocreatine not merely in nerve or muscle, but in all the tissues of these animals. They suggest that phosphocreatine may play a role in the metabolism not only of fast acting tissues as nerve and muscle, but in other tissues as well. Also in this year Roche, Thoai, Robin, Garcia and Hatt (35) were able to study the metabolic derivatives of arginine in the mollusks, crustaceans and echinoderms. Using their newly developed chromatographic technique they were able to show that arginine, γ-guanidobutyric acid and δ-guanido-∝keto-valeric acid exist among all the invertebrates studied, while agmatine and octopine occurred in some species of mollusks and crustaceans. Glycocyamine was found in the echinoderms, Ophiothrix fragilis and Paracentrotus lividus Lmk. These two species contain creatine and the discovery

of glycocyamine in these animals is consistent with the theory that glycocyamine is a precursor of creatine and must be present if creatine is present. Roche, Thoai, Garcia and Robin (36) then extended these studies to the annelids and here found some interesting results as had been forecast by Baldwin and Yudkin. They found by chromatographic methods that three species of errant polychaete worms, Eulalia viridis Muller, Halosydna gelatinosa Misars and Nereis diversicolor Muller, and one nemertean worm, Lineus gesserensis Muller, all contained arginine and a new monosubstituted guanidine. A second new monosubstituted guanidine was found present in four species of sedentary polychaetes, Arenicola marina L., Lanice conchilega Pallas, Myxicola infundibulum Renier, and Sabellaria alveolata L. Of these four sedentary polychaetes only Lanice conchilega contained arginine. Two gephyrean worms, Phascolosoma elongatum Keferstein and Sipunculus nudus L. contained both the new guanidine compounds plus arginine and still a third new monosubstituted guanidine. The single oligochaete worm examined, Lumbricus terrestris, contained none of the known guanidine compounds. The authors tentatively concluded that the annelids do contain new monosubstituted guanidines, one of which seems common to the errant polychaetes and one to the sedentary polychaetes. Since arginine appeared only in traces, and since L-amino acid oxidase and oxidation products or arginine were also absent it appears that arginine is not oxidatively metabolised in these worms. Finally, since arginine was present only in traces while the new guanidine compounds were abundantly present it seemed more than likely that the new compounds acted as phosphagens. In a note added to this paper the authors announced that the new guanidine derivative from Arenicola marina had

been crystallised. The discovery of the new guanidines was also mentioned in a paper by Thoai, Roche and Robin (37).

The next step forward was taken shortly afterwards. In 1953, Thoai, Roche, Robin and Thiem (38) published an article in which they identified two of the new guanidines as glycocyamine and taurocyamine. From Nereis diversicolor, an errant polychaete, they isolated glycocyamine as the free base. From Arenicola marina they obtained taurocyamine as the picrate and as the free base. Then by chromatographic procedures they identified the monosubstituted guanidine in other errant polychaetes, Nephtys humbergii, Halosydna gelatinosa, and in a nemertean, Lineus gesserensis, as glycocyamine. Similarly they found taurocyamine in another sedentary polychaete, Sebellaria alveolata, and in a gephyrean, Phascolosoma elongatum. In all these animals arginine was found only in the digestive tract. In the same paper the authors report the isolation of the phosphagens of these worms. Using the method of Fawaz and Seraidarian (39) they synthesized glycocyamine phosphate and taurocyamine phosphate and found that the hydrolysis products of these compounds, when chromatographed, yielded spots with the same R_f value as the hydrolysis products of the phosphagens of Nereis diversicolor and Arenicola marina respectively.

Also in 1953 Thoai, Roche, Robin and Thiem (40) published an additional note on the new phosphagens, taurocyamine phosphate and glycocyamine phosphate. They draw attention to the fact that this is the first time taurocyamine has been found in living material. Glycocyamine was known to be present in living tissues before this time, but it now adds to its role as creatine precursor the role of phosphagen in some annelids.

Again in 1953, which might be described as a banner year for the identification of new phosphagens, Thoai, Roche, Robin and Thiem (41) reported finding a new phosphagen in the earthworm, Lumbricus terrestris, an oligochaete. The compound was extracted by the usual trichloroacetic acid extraction and after removal of inorganic phosphates and calcium the phosphagen was chromatographed and its R_f value was found not to correspond to that of arginine or creatine phosphates. The phosphagen was hydrolysed with sulfuric acid and the compound guanidoethylserylphosphate was identified in the hydrolysate. On re-extracting some phosphagen and then hydrolysing it, inorganic phosphate was liberated and this was quantitatively determined. For each mole of the guanidine base (guanidoethylserylphosphate) present in the hydrolysate there was one mole of liberated inorganic phosphate present. There are, therefore, two phosphate groups in the phosphagen molecule.

In 1954, Thoai and Robin (42) reported on the identificiation of glycocyamine and taurocyamine in more detail. They mention that these two compounds were always found localized in the muscle or alimentary tract of the annelids studied, while any arginine present was found only in the alimentary tract.

Ennor and Rosenberg (43) studied the turnover rates of the organic phosphates in muscle and published a study of the properties of creatine phosphokinase which they isolated from sheep skeletal muscle (44). Creatine phosphokinase is the enzyme required to bring about the previously described Lohmann reaction and is a transphosphorylating enzyme. Ennor and Rosenberg found that when the pH is 7.2 the reaction proceeds towards the formation of ATP, and at pH 10.5 the reverse reaction, the phosphorylation of creatine, is favored. Calcium and magnesium ions are required

for enzyme activity.

Roche, Thoai and Hatt (45) further improved their chromatographic technique for the analysis of guanidine derivatives. They identified a variety of guanidine compounds present in the ray, Torpedo mormorato Risso, the actinian, Actinia equina L., the alcyonarian Alcyonium palmatum Pallas and the sponge, Hymeniacidon carencula Bowerbank. Arginine was present in all of them. The ray and the alcyonarian contained creatine and methylguanidine. The actinian and the alcyonarian contained guanidine and finally, dimethylguanidine was present in the alcyonarian. The authors observe that one cannot state definitely which compound acts as the phosphagen. In the same journal Thoai and Robin (46) gave some more details about the isolation of lombricine and lombricine phosphate.

Other workers in Roche's laboratory studied the guanidine derivatives in sponges, and Garcia and Miranda (47) reported finding arginine, agmatine and glycocyamine in three sponges, Halichondria panicea Johnstone, Hymeniacidon caruncula and Thetia lyncurium L. It would appear that Roche, Thoai and Hatt (45) missed agmatine and glycocyamine when they studied Hymeniacidon caruncula. The sponge, Thetia lyncurium, also contained creatine and a new monosubstituted guandine. The 'new' compound was identified by Roche and Robin (48) as taurocyamine. At first the assumption was made that glycocyamine and taurocyamine phosphates were the phosphagens in these sponges. However, when the phosphagen of Hymeniacidon caruncula was extracted by the usual trichloroacetic acid procedure it was found to be arginine phosphate and not glycocyamine phosphate, despite the high concentration of glycocyamine in this species. Similarly the true phosphagen of Thetia lyncurium was found to

be creatine phosphate even though this sponge contained large amounts of taurocyamine. Isolation of the guanidine base does not always reveal the nature of the phosphagen.

Robin and Roche (49) studied some coelenterates and sponges and found taurocyamine in the actinian, Actinia equina and in the sponge already mentioned, Thetia lyncurium. Other species of coelenterates studied, namely Anemonia sulcata Penn, Bunodes gemmaceus Ellis and Sagarlin parasitica Couch contained no trace of taurocyamine. Yet all contained taurine and hypotaurine.

In 1955 the two English workers, Hobson and Rees published their first work in this field (50). In the case of the annelids they found enzymes in the body walls of these worms capable of transferring phosphate to the guanidine base present. In a later publication (51) they described the results of their analysis by the method of Baldwin and Yudkin (22) of the phosphagens of seventeen species of annelids. Since the hydrolysis tests were not conclusive they described a method for the direct chromatography of the phosphagens. Of interest from the comparative viewpoint is their finding of differences within the single genus Glycera. The species Glycera gigantea contained both creatine and arginine phosphates while the species Glycera convoluta contained only creatine phosphate. Thus the authors conclude again, as did Baldwin and Yudkin, that the distribution of phosphagens among the annelids is entirely erratic, and they add that Roche, in a private communication, has also agreed that the phosphagen present is not related to the sedentary or errant nature of the worms. In their discussion they also indicate the belief that the presence of creatine phosphate in the annelids does not support the close relationship between the annelids and the chordates,

postulated by Dohrn (52) and by Semper (53). Instead the presence of creatine phosphate appears to be an example of convergent chemical evolution.

In 1955, Rosenberg and Ennor extended their studies on creatine phosphokinase (54). In the same year Ennor, Morrison and Rosenberg (55) published a note on the isolation of barium phosphoarginine. The full details were published in 1956 (56). From the crayfish, Jasus lalandii, they obtained 9.8 grams of arginine phosphate per 1600 grams of muscle. Their procedure was the first modification of Meyerhoff's original work of 1928 (4). Also in 1956, Morrison, Griffiths and Ennor (57) reinvestigated the nature of the phosphagen present in the tunicates. Using the atrial muscles of two tunicates, Pyura sp. and Pyura stolonifera, they extracted the phosphagen by the classical trichloroacetic acid procedure and identified the phosphagen by chromatographic methods as creatine phosphate. They also isolated the base as the picrate and identified it as creatine picrate. An aqueous extract of the muscle showed creatine phosphokinase activity and in none of the work was there any evidence found for the presence of arginine, arginine phosphate or arginine phosphokinase. These findings are in direct opposition to the findings of Needham et al. (58) who found only arginine phosphate in one species of tunicate. The authors conclude that 'the present work is consistent with and provides biochemical support for the accepted phylogenetic classification of the tunicates..' Nevertheless, it would seem that from the experience with the annelids it would be worthwhile to reinvestigate by modern direct means the phosphagen present in the tunicate studied by Needham, Ascidia mentula. Also, as cautioned by Baldwin, many more species

should be examined before it be concluded that tunicates as a class contain only creatine phosphate.

Rosenberg, Ennor and Morrison (59) studied the Sakaguchi reaction and found that the diacetyl, ∝-naphthol reaction is preferable for the estimation of monosubstituted guanidines or arginine when only one guanidine is present.

In 1957 Morrison, Griffiths and Ennor (60) described the purification and properties of arginine phosphokinase, obtained from the sea crayfish Jasus verreauxi. The enzyme phosphorylated arginine very quickly but it phosphorylated homoarginine and canavanine very slowly. Other guanidines were unaffected. In a second paper, the kinetics of the reaction were reported (61). Thoai (62) studied taurocyamine phosphokinase and glycocyamine phosphokinase present in Arenicola marina and in Nereis diversicolor respectively and found a strict specificity of these enzymes towards the characteristic guanidine base in the animal from which the enzyme was extracted. The reactions:

ATP + taurocyamine ⇌ ADP + taurocyamine phosphate

and, ATP + glycocyamine ⇌ ADP + glycocyamine phosphate

both show a pH optimum for the forward direction of 8.9 and a pH optimum for the reverse reaction of 7.1. Thoai had difficulty in purifying the enzyme because in 16 hours of dialysis its activity was reduced by a half.

Hobson and Rees (63) also reported their work on the annelid phosphokinases. They prepared enzyme extracts from the body walls of several annelids, and these they incubated with a series of substrates, including arginine, creatine, glycocyamine and taurocyamine. After

incubation the phosphagen formed was isolated by the usual trichloroacetic acid extraction. The phosphagens were then chromatographed directly or hydrolysed with N hydrochloric acid at 100°C for five minutes and the hydrolysate chromatographed against known bases. Seven annelids were studied in this way and extracts of four of them were able to phosphorylate two of the guanidine bases tested as substrate while three contained only one phosphokinase. The authors did not attempt to separate the phosphokinases in the cases where two bases were phosphorylated but concluded that the enzyme systems for the synthesis and utilization of phosphagens completely duplicated each other. Arginine was the only substrate not phosphorylated. Thus the authors state:

> The experimental results again emphasize that creatine phosphate, acting as a phosphagen, is not confined to the echinoderms, protochordates and vertebrates, but is also present, often in considerable quantity, in the annelids. At the present time the distribution of creatine phosphate appears quite arbitrary. This fact lessens the value of the identification of the phosphagens in evolutionary studies in the animal kingdom.

Griffiths, Morrison and Ennor (4) reinvestigated the phosphagens, guanidines and phosphokinases in the echinoids. They found that of two Australian echinoids, Gentrostephanus rodgersii and Heliocidaris erythrogramma the former contained arginine, arginine phosphate and arginine phosphokinase while the latter contained arginine, arginine phosphate and arginine phosphokinase together with creatine, creatine phosphate and creatine phosphokinase. The authors, perhaps somewhat unfairly, state that the assumption of Baldwin and Needham that both arginine phosphate and creatine phosphate are present in echinoids is unfounded. Baldwin himself had found that in Arbacia punctulata only arginine phosphate was present. It is also interesting to note that the authors just after

stating, ". . .and results emphasize the necessity for examining a number of species within a class before concluding that a particular phosphagen (s) is characteristic of that class," then theorize that so far arginine phosphate and creatine phosphate have been found together only in the Camarodonta order of echinoids[1], while in all other orders studied[2] (two species!!) only phosphoarginine was found. These workers made another contribution in demonstrating that in Heliocidaris erythrogramma two phosphokinases, one for arginine and one for creatine, were both present in the same muscle. As yet there is no explanation of the functions of the two phosphagens in the same muscle.

The year 1957 also marked the discovery of a new phosphagen. Robin, Thoai and Pradel (65) isolated from 1 kilo of leeches, (Hirudo medicinalis L.), 300 mg. of a new monosubstituted granidine. This compound differed from all previously studied guanidines and was obtained as a crystalline picrate. Its formula is $C_7H_{17}N_5$ and it was given the name hirudonine. Since it is the only guanidine base present its probable role is that of a phosphagen. In a later report, Robin, Thoai and Roche (66) note that arcaine is also present in the leech but not in the muscle and therefore the arcaine could not serve as the phosphagen.

Roche, Thoai and Robin (67) also published a review article in which they summarized the results of their work on thirty-seven animal species ranging from protozoa to arthropods and echinoderms. They analyzed all of them for the presence of creatine or arginine. They

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[1]Species of Camarodonta order studied were Paracentrotus lividus, Sphaerechinus granularis, Strongylocentrotus lividus, Echinus esculentus, and Heliocidaris erythrogramma.

[2]Order Aulodonta, species Centrostephanus rodgersii, Order Sterodonta, species Arbacia punctulata.

noted two aspects to the distribution of creatine. One is a permanent phenomenon, the presence or absence of creatine in the muscle. The other is a temporary phenomenon, the presence or absence of creatine in the spermatozooids. They observed that creatine was encountered uniquely in those species in which the spermatozooids were flagellated, while the ameboid spermatozooids contained no creatine. Possibly the more labile creatine phosphate contributes to the motility of the spermatozooids. With respect to muscle, the authors report that creatine occurs widely but irregularly throughout the various phyla studied. The view of Roche and his group on the significance of the occurrence of creatine in the invertebrates may be quoted:

> Notons enfin que la créatine est plus repandue chez les Invertébrés qu'on ne l'a longtemps supposé; on la rencontre en effet chez des Echinodermes, des Annélides, des Géphyriens, des Coelentérés et des Spongiaires, ou elle ne saurait cependant constituer un caractère de classe, puisque certaines espèces en sont dépourvues. Etant donnée la diversité de cette répartition, la signification biologique de la créatine dans la degré d'evolution des organismes étudiés n'apparaît pas clairement. Toutefois les anciennes théories, bases sur la présence de créatine chez les Echinodermes et faisant de ces animaux une classe de transition entre les Vertébrés et les Invertebres ne sauraient désormais être retenues.

Finally, Thoai and Thiem (68) have isolated and synthesized the ammonium salts of taurocyamine phosphate and glycocyamine phosphate. These products were obtained by treating phosphorus oxychloride with the appropriate monosubstituted guanidine. The melting points of the synthesized products corresponded with those of the natural substances. Shortly afterwards, Morrison, Ennor and Griffiths (69) described what they consider an easier preparation of barium monophosphotaurocyamine.

This brings the literature survey of the distribution of phosphagens up to the present. It would be well to summarize what has been learned. So far there is no serious challenge to the fact that creatine

phosphate is the characteristic phosphagen of the vertebrates. Among the invertebrates studied the picture is less straightforward. No less than six phosphagens are now known to be present in the invertebrates and their distribution follows no definite pattern. One fact is clear however, and that is that many theories have been proposed regarding phosphagen distribution according to habitat or phylogenetic classification and these theories have been proposed from the study of as few as two members of a class or even of a phylum of the animal kingdom. Although these theories may be stimulating, they cannot be considered seriously until the comparisons are, as Baldwin says, comparative enough.

Pigments in Marine Invertebrates

Many books and articles have been written about animal and plant pigments. This is not surprising for the phenomenon of color fascinates everyone. Fox (70) author of one monograph on animal pigments, states:

> Anyone who undertakes the preparation of a treatise on colour in the living world is necessarily confronted with the task of keeping separate the deeply aesthetic and otherwise subjective aspects from the purely physical, chemical and metabolic sides of the topic.

Other authors of books about natural pigments include, Goodwin (71), Karrer and Jucker (72), Perkin and Everest (73) and Mayer and Cook (74).

Specific information about marine invertebrate pigments is limited, and detailed knowledge about the pigments of gorgonians is almost entirely lacking. Fox and Pantin (75) have reviewed the pigments found in coelenterates and they note that as early as 1880 MacMunn (76) had found 'lipochromes' widely distributed in this phylum. Merejkowski (77) in 1881 found 'zooerythrin' and 'tetroerythrin' in the sea anemone

Actinia equina. For several years no work was reported in this field. Then in 1914, Studer (78) observed orange-red droplets of carotenoid in the coenenchyme (but not in the polyps) of the gorgonian Eunicella verrucosa. Lonnberg (79, 80, 81, 82) and Lonnberg and Hellstrom (83) found evidence of carotenoids in the alcyonarians, actinarians, madreporians and ceriantharians. However in 'clean' diatom-free Antennularia antennina no carotenoids were found.

Actual identification of the carotenoids started when Lederer (84) and Fabre and Lederer (85) found α- and β-carotene and a red carotenoid ester, actinoerythrin, in Actinia equina. Heilbron, Jackson and Jones (86) isolated the acid of this ester, called it violerythrin, and found that it had an absorption spectrum very different from that of the original ester. Other pigments found include sulcatoxanthin found first in Anemonia sulcata by Heilbron, Jackson and Jones, then in Cribrina xanthogrammica by Strain, Manning and Hardin (87) and in Metridium senile by Fox and Pantin (88), who also found metridin and taraxanthin in Metridium senile. In Tealia felina Heilbron et al. (86) found actinioerythrin and astacin, which were also found in Epiactis prolifera by Fox and Moe (89).

Two questions arise concerning the carotenoids present in the coelenterates. The first is whether or not the species examined was contaminated by algal symbionts which could be responsible for the presence of the pigment. Fox and Pantin (88) report that the sulcatoxanthin they extracted was obtained from clean algal-free specimens of Metridium senile. However, Lonnberg (80) found no carotenoids when clean Antennularia antennina were studied. It is therefore important to use clean specimens when possible.

The other question which arises is concerned with the formation of the carotenoids by the coelenterates. They may be formed 'de novo' by the animal, or they may be formed by altering ingested carotenoids. Goodwin (71) flatly states his belief that the marine invertebrates are incapable of synthesizing carotenoids 'de novo'. Schultze (90) starved specimens of Hydra circumcinta and found that they lost their color but regained it when fed colored copepods. Similarly, Abeloos-Parize (91) found that specimens of Actinia equina raised on a carotenoid-free diet contained no carotenoids, but that when they were fed carotenoids, their original species color, red, green or brown, returned. This strongly suggests that the animals can alter ingested carotenoids.

The color of alcyonarians is also derived from pigments in the spicules themselves. In this, they differ from other coelenterates. The only spicule pigment from an alcyonarian which has been examined is that of Alcyonium palmatum. Durivault (92) found that the spicules of this species contained a yellow to violet-red pigment which was destroyed by acids. He also detected iron in the spicules by the Prussian blue and the thiocyanate reactions while they were dissolving in dulute acid. He was unable to extract the pigment with any organic solvents.

Finally, mention should be made of a pigment found in the tunicate Styelopsis grossularia by Abeloos and Teissier (93). This compound is red in the natural state, and is soluble in water and alcohol. In acid solutions it is green, and in basic solution it is red. This is the only report of a natural pH indicator pigment found in the tunicates, and it is of interest because a similar pH indicating pigment has been found in the siphon muscles of Polycarpa obtecta by the author.

Waxes and Oils in Marine Invertebrates

Very little work has been done in this field. Bergmann has studied the sterols of many marine invertebrates and as a sideline it would appear he has reported some of his findings on waxes and oils in these animals. In 1942, Kind and Bergmann (94) reported finding octadecyl alcohol and batyl alcohol in Plexaura flexuosa. An acetone extract of Xiphigorgia sp. yielded cetyl palmitate. In an earlier paper (95) Bergmann discussed the relationship between coral reefs and petroleum formation. He noted that in a 'brown Florida gorgonian' one kilogram dry weight yielded 33 grams of non-saponifiable material which was 20% sterol, 50% cetyl alcohol and 30% semisolid hydrocarbon. Similarly a 'yellow gorgonian' contained large amounts of sterols, alcohols and hydrocarbons. Finally, in two more papers (96) (97) he notes the high organic and fat content of coelenterates. Apart from the alcohols mentioned above, Bergmann's interest has been restricted to the sterols and little is known about other organic compounds present.

CHAPTER II

EXPERIMENTAL

Collection of Animals and Initial Preparation of Samples

Animals were collected from the shallow waters around Bimini and Bermuda. The Bermuda species were:

1. The holothurian or sea cucumber, Stichopus badionotus Selenka, found on the sandy bottom of Castle Harbour.

2. The holothurian, Synaptula hydriformis Lesueur, found on rocks near the south entrance to Castle Harbour.

3. The echinoid or sea urchin, Tripneustes esculentus Leske, found under rocks and in caves near the entrance to Castle Harbour.

4. The ascidian or sea squirt, Polycarpa obtecta, found in the quiet waters of The Reach.

Several species of gorgonians or horny corals were obtained on the coral reefs near Somerset, Bermuda. These were:

1. Eunicea grandis Verrill.
2. Eunicea tourneforti Milne-Edwards and Harrison.
3. Plexaura crassa Ellis and Solander.
4. Plexaura dichotoma Esper.
5. Rhipidogorgia flabellum (L).
6. Xiphigorgia citrina (Esper).

Four more species of gorgonian were found in Castle Harbour.

7. Muricea muricata (Pallas).
8. Plexaura esperi Verrill.
9. Plexaura homomalla Esper.
10. Pterogorgia acerosa (Pallas).

Two species were collected from North Rock.

11. Plexaurella nutans Duchassaing and Michelotti.
12. Pterogorgia americana (Gmelin).

One species was taken from Burchall's Cove.

13. Plexaura flexuosa Lamouroux.

Some of the Bermuda species of gorgonians also occurred at Bimini but four species found at Bimini did not occur at Bermuda. These were:

1. Briareum asbestinum (Pallas).
2. Eunicea mammosa Lamouroux.
3. Plexaura flavida Stiasny.
4. Xiphigorgia anceps (Pallas).

The first three of these Bimini species were collected off the Rabbit Keys while the Xiphigorgia anceps were obtained near Turtle Rock. The gorgonians were identified by Dr. Elisabeth Deichmann of the Museum of Comparative Zoology, Harvard College.

The gorgonians were hung up to dry in the sun as soon as possible after they were collected. Drying was completed in an oven at about 60°C. Some species, possessing a thick inner skeleton of horny protein, were stripped of their cortex i.e., the calcareous exoskeleton containing the dried polyps. The cortex was then crushed or ground to a powder to be ready for extraction. In others the inner skeleton was very fine and the entire animal was crushed and ground.

The other animals were placed in aquaria with running sea water

as soon as they were landed. Whenever they were to be used they were placed in ice-cold sea water and left there until they became torpid. They were then variously treated.

The holothurian, Synaptula hydriformis, was treated by the method of Hobson and Rees (51). The entire animal was blended in a Waring blendor with two volumes of ice cold distilled water and the resultant extract, after standing for ten minutes, was treated with five volumes of ice-cold acetone. The extract was filtered and the acetone powder was dried under vacuum and stored at 2°C in a desiccator over calcium chloride.

Over 100 specimens of Tripneustes esculentus were cut open after cooling and the Aristotle's lanterns were removed. The jaws were dropped into ice-cold acetone to be dehydrated and were then dried under vacuum and stored as above.

The Polycarpa obtecta were cut completely in half and then the siphon muscles were removed and placed in ice-cold acetone. They were then treated in the same manner as the lantern jaws of Tripneustes esculentus.

The holothurian, Stichopus badionotus, was cut open lengthwise and the five longitudinal body wall muscles were removed. Some were placed in two volumes of ice-cold distilled water and blended. The extract was then treated with five volumes of ice-cold acetone and filtered. The resultant acetone powder was dried under vacuum and stored in a desiccator. A second lot of the longitudinal muscles amounting to over 400 grams were treated in a different manner as described by Ennor, Morrison and Rosenberg (56) in their isolation of arginine phosphate from the crayfish, Jasus lalandii.

The longitudinal muscles were dropped into a 9% (w/v) trichloroacetic acid solution as soon as they were removed from the animal. One hundred grams of muscle were added to each 450 ml. of ice-cold trichloroacetic acid solution. This mixture was then blended in a Waring blendor for two minutes and filtered by suction into a flask containing enough 10N NaOH to raise the pH above 9.0. The residue was re-extracted with 5% trichloroacetic acid solution, filtered and combined with the first filtrate, and the pH of the combined filtrates was adjusted to 9.0 with 5N HCl. The water-insoluble barium salts were precipitated out by adding 1.0 M barium acetate until no further precipitation occurred. The pH was again adjusted to 9.0, this time with 5N NaOH. The supernatant was removed by decantation or centrifugation and the soluble barium salts were precipitated by the addition of three volumes of 95% ethanol, while maintaining the pH at 9.0 with N NaOH. After settling overnight, the crude "water soluble, ethanol insoluble" barium salts were washed with ethanol and ether, dried under vacuum and stored in a desiccator with calcium chloride. All the above steps were carried out at 2°C.

Study of the Guanidine Bases Present

All animals collected were examined to determine the nature of the guanidine base or bases present. The method used is described by Roche, Thoai and Robin (67). Ten grams of the dried, ground gorgonian cortex of each species was used for the analyses (with the exception of Briareum asbestinum of which there was only two grams of material available). Other samples used were: one gram of the dried acetone powder of Stichopus badionotus muscle, dried muscle from five lantern jaws of Tripneustes esculentus, ten dried atrial muscles of Polycarpa obtecta

and one gram of dried, powdered Synaptula hydriformis.

Five volumes of 2% acetic acid were added to each sample, and the mixture was heated for five minutes on a boiling water bath. The calcium carbonate spicules in the gorgonians caused these samples to foam considerably. The samples were then filtered, and the cooled filtrates were passed through Amberlite IRC50 cation exchange columns in the hydrogen form. Each of the columns was washed with 200 ml. of water and then the adsorbed guanidine bases or basic amino acids present were eluted with 2N ammonium hydroxide. The ammonium hydroxide eluates were concentrated to near dryness by vacuum distillation and each was made up to a volume of 2 ml. with water. The samples were chromatographed on Whatman #1 paper, 0.05 ml. of each sample being used for a run. Five micrograms of arginine and of creatine were run as standards. The following six solvent systems were employed:

System #1. n-butyl alcohol, acetic acid, water (73 : 10 : 17)

System #2. pyridine, isoamyl alcohol, acetic acid, water (80 : 40 : 10 : 40)

System #3. pyridine, isoamyl alcohol, water (80 : 40 : 70)

System #4. pyridine, isoamyl alcohol, ammonium hydroxide, water (80 : 40 : 10 : 40)

System #5. n-propyl alcohol, acetic acid, water (73 : 10 : 17)

System #6. n-propyl alcohol, ammonium hydroxide, water (73 : 20 : 7)

It took from ten to fourteen hours at 25°C to run a chromatogram. The papers were then removed from the solvent and dried. Each sample was run three times in each solvent system in order that the papers could be developed by three different sprays. The sprays used were:

1. The Sakaguchi spray which revealed the presence of arginine or any

other monosubstituted guanidine. The paper was sprayed with a fresh mixture of 0.2 ml. each of 40% NaOH solution, 40% aqueous urea solution and 1% alcoholic α-naphthol solution all diluted to 10 ml. with water. The paper was dried at room temperature and sprayed with a solution prepared by diluting 1 part of a stock solution of 0.9 ml. bromine in 100 ml. 10% NaOH with 3 parts water. The monosubstituted guanidines appeared as pink spots on a white background. The test was sensitive to one microgram of arginine.

2. The second spray revealed creatine, other disubstituted guanidines and monosubstituted guanidines. The paper was sprayed with a fresh solution of 16 ml. water, 2 ml. 40% NaOH, 2 ml. 1% alcoholic α-naphthol solution plus one drop diacetyl. The guanidines appeared as violet spots on a cream background. As described by Roche, Thoai and Robin, the test was sensitive to 2 micrograms of creatine or other disubstituted guanidine and was sensitive to 20 micrograms of monosubstituted guanidine. It was found that the sensitivity of the test could be increased by examination of the paper under ultraviolet light. The background fluoresced a bright blue, while the violet spots, some too faint to see in daylight, appeared as dark non-fluorescing spots under ultraviolet light. Examined this way the presence of as little as 0.1 microgram of creatine could be detected. However caution had to be exercised not to overload the paper with spray, lest the background fluorescence obscure the weaker non-fluorescing spots.

3. The third spray was a 0.3% solution of ninhydrin in 95% ethanol. It was used to confirm the presence of arginine and to reveal the presence of any other amino acids. After spraying, the paper was dried at room

temperature, then placed in an oven at 100°C for ten minutes. Amino acids appeared as blue spots on a white background.

The results are listed in table I. The Sakaguchi test was termed positive if any spot with a pink color appeared after spraying. In all but three cases, the R_f of the spot which appeared corresponded to that of arginine. The α-naphthol, diacetyl test was termed positive whenever a violet spot appeared and in all cases the R_f of the spot corresponded to that of creatine. The ninhydrin test was called positive only if a blue spot developed corresponding to either the Sakaguchi or α-naphthol, diacetyl test spots. It should be noted that several other blue or yellow spots appeared when ninhydrin was used, none of these spots corresponding to any revealed by the guanidine sprays. Pterogorgia acerosa, Pterogorgia americana and Rhipidogorgia flabellum gave faintly positive Sakaguchi spots at the same R_f value as the creatine spot and also gave strongly positive α-naphthol, diacetyl tests at these spots. From the literature it appears that glycocyamine has R_f values close to creatine so that the positive Sakaguchi test plus the negative ninhydrin test suggest the possibility that there is a small amount of glycocyamine present with the creatine.

Attempt to Extract Arginine Phosphate from Stichopus Badionotus

The crude barium salts extracted from Stichopus badionotus were brought back to Norman for purification. The method of Ennor and Morrison (56) was followed and 4 grams of the crude barium salts were dissolved in 150 ml. of ice-cold water. Sufficient 5N Hydrochloric acid was added to dissolve the salts and the pH was brought to 9.0 with 5N sodium hydroxide.

TABLE I

RESULTS OF GUANIDINE BASE DETERMINATIONS

Species or compound tested	R_f value in different solvents of spot found						*Color Test		
	1	2	3	4	5	6	1	2	3
Arginine	0.05	0.13	0.11	0.13	0.08	0.12	+++	+++	-
Creatine	0.13	0.17	0.24	0.21	0.13	0.15	-	-	+++
Eunicea grandis	0	0	0	0	0	0	-	-	-
Eunicea tourneforti	0	0	0	0	0	0	-	-	-
Muricea muricata	0	0	0	0	0	0	-	-	-
Plexaura crassa	0	0	0	0	0	0	-	-	-
Plexaura esperi	0	0	0	0	0	0	-	-	-
Plexaura flexuosa	0.13	0.17	0.24	0.21	0.13	0.15	-	-	+
Plexaura homomalla	0.13	0.17	0.24	0.21	0.13	0.15	-	-	+
Plexaurella nutans	0.13	0.17	0.24	0.21	0.13	0.15	-	-	++
Plexaurella dichotoma	0.13	0.17	0.24	0.21	0.13	0.15	-	-	++
Pterogorgia acerosa	0.13	0.17	0.24	0.21	0.13	0.15	+	-	++
Pterogorgia americana	0.13	0.17	0.24	0.21	0.13	0.15	+	-	+++
Rhipidogorgia flabellum	0.13	0.17	0.24	0.21	0.13	0.15	+	-	+++
Xiphigorgia citrina	0	0	0	0	0	0	-	-	-
Briareum asbestinum	0	0	0	0	0	0	-	-	-
Eunicea mammosa	0.13	0.17	0.24	0.21	0.13	0.15	-	-	++
Plexaura flavida	0.13	0.17	0.24	0.21	0.13	0.15	-	-	++
Xiphigorgia anceps	0	0	0	0	0	0	-	-	-
Tripneustes esculentus	0.05	0.13	0.11	0.13	0.08	0.12	+++	+++	-
Stichopus badionotus	0.05	0.13	0.11	0.13	0.08	0.12	+++	+++	-
Synaptula hydriformis	0.05	0.13	0.11	0.13	0.08	0.12	+++	+++	-
Polycarpa obtecta	0.05	0.13	0.11	0.13	0.08	0.12	+++	+++	-

*Color Test: 1. Sakaguchi; 2. Ninhydrin; 3. α-naphthol, diacetyl.

The heavy precipitate of barium phosphate which formed was centrifuged down, and the supernatant was removed. The precipitate was re-extracted with water, centrifuged again, and the supernatant was combined with the first supernatant. The combined supernatants were filtered and 6 ml. of cupric chloride solution (50 g. per 50 ml. water) was added to the filtrate. The pH was adjusted to 6.5 with 5N sodium hydroxide, and the resultant precipitate was centrifuged down. It was at this point that the very small amount of precipitate obtained made it clear that most of the arginine phosphate originally present must have decomposed, probably while in transit from Bermuda to Oklahoma in hot weather. This would account for the heavy barium phosphate precipitate, since hydrolysis of the arginine phosphate would liberate inorganic phosphate. When a drop of the final supernatant was placed on paper and sprayed with the Sakaguchi spray a strongly positive arginine or monosubstituted guanidine reaction was obtained.

Pigment from Polycarpa obtecta

When the siphon muscles of Polycarpa obtecta were extracted with 2% acetic acid in the determination of the guanidine base present, a red solution was obtained. The pigment extracted by the acid was not retained by the Amberlite IRC50 cation exchange resin used in the hydrogen form. This behavior would indicate that the pigment was not basic. It was found that hot water alone could extract the pigment. On standing from two to three hours, the pigment separated out as a red flocculent mass, leaving a clear yellow solution. Heating brought the pigment back into solution, but did not restore the full intensity of the color. In aqueous solution the pigment also passed through an Amberlite IRC50 cation exchange resin

in the hydrogen form but was strongly absorbed on both Dowex 1 - X_1 and Dowex 1 - X_{12} anion exchange resins which were in the chloride form. None of the eluants tried including sodium sulfate and sodium hydroxide was successful in moving the pigment off the column. However, the sodium hydroxide changed the color of the pigment on the column from red to green.

The most unusual feature of the pigment was that it acted as a pH indicator. It was green in alkaline solutions, red in neutral or slightly acid solutions and yellow in strongly acid solutions. A buffer series ranging in pH from 2.36 to 10.9 was prepared using veronal, sodium hydroxide, sodium acetate and hydrochloric acid as described by Gortner (98). It was found that the color change from red to green was quite reversible but the change from red to yellow was irreversible. The apparent pK of the pigment was 7.4. The solutions were clear except above pH 10 or in the range from pH 5.2 to 6.3.

The absorption spectral curves of the pigment at pH 8.5, pH 6.3 and pH 2.4 are shown in figure 2. The ultraviolet absorption spectra provide further evidence that there is a protein present. The maxima in this region remain near 280 - 285 millimicrons, where proteins absorb in the ultraviolet. The absorbance in the ultraviolet was greater than in the visible, and it should be noted that the solutions were diluted to obtain the ultraviolet curves. The green solution showed maximum absorbance in the visible at 430 and 580 millimicrons, and the ultraviolet at 235 and 285. One of the absorption peaks shifted to the shorter wavelengths in the case of the red solution. The maxima were at 430 and 520 in the visible and at 235 and 285 in the ultraviolet. However, for the

FIGURE 2

ABSORPTION SPECTRUM OF THE PIGMENT EXTRACTED FROM POLYCARPA OBTECTA

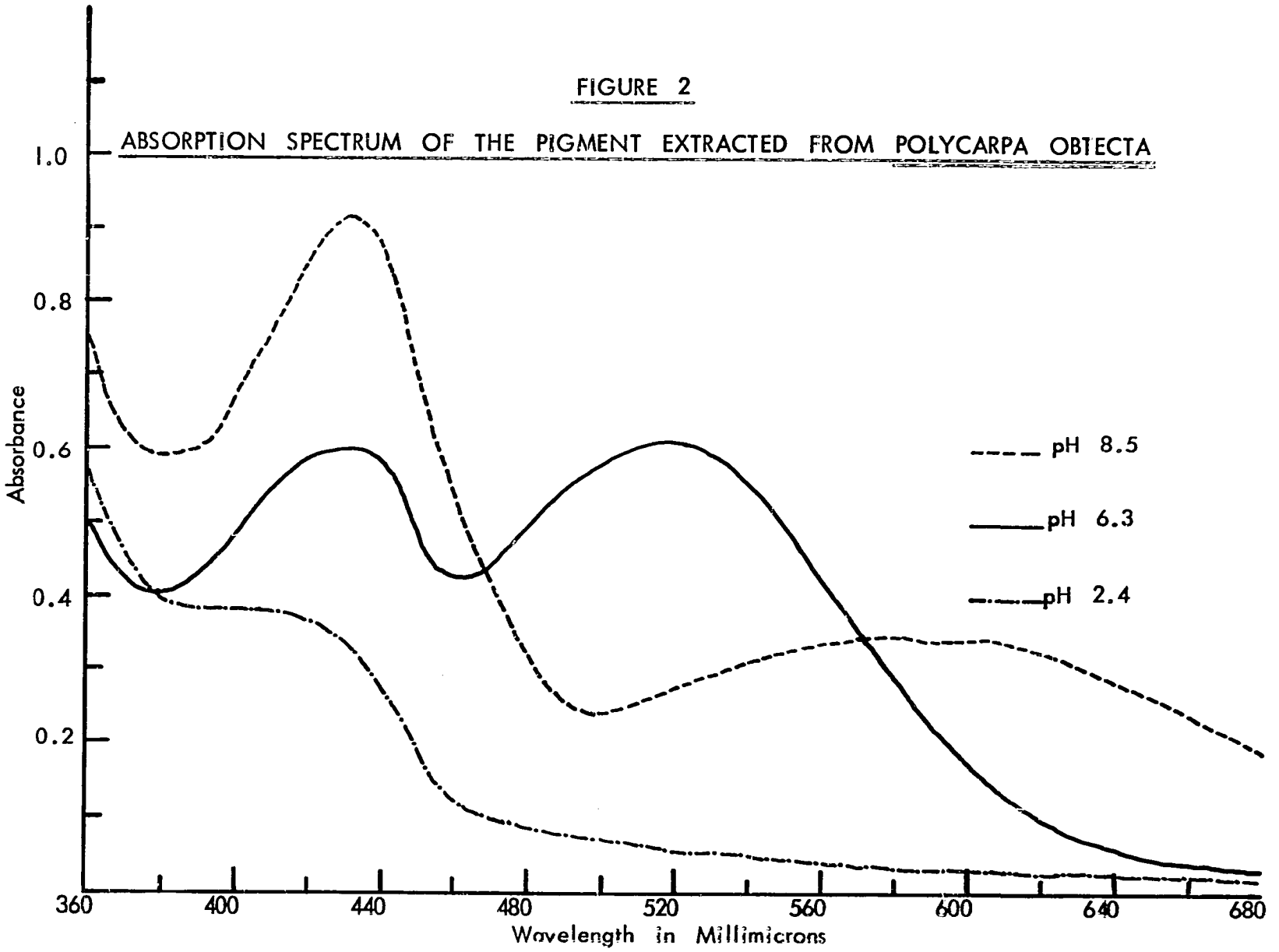
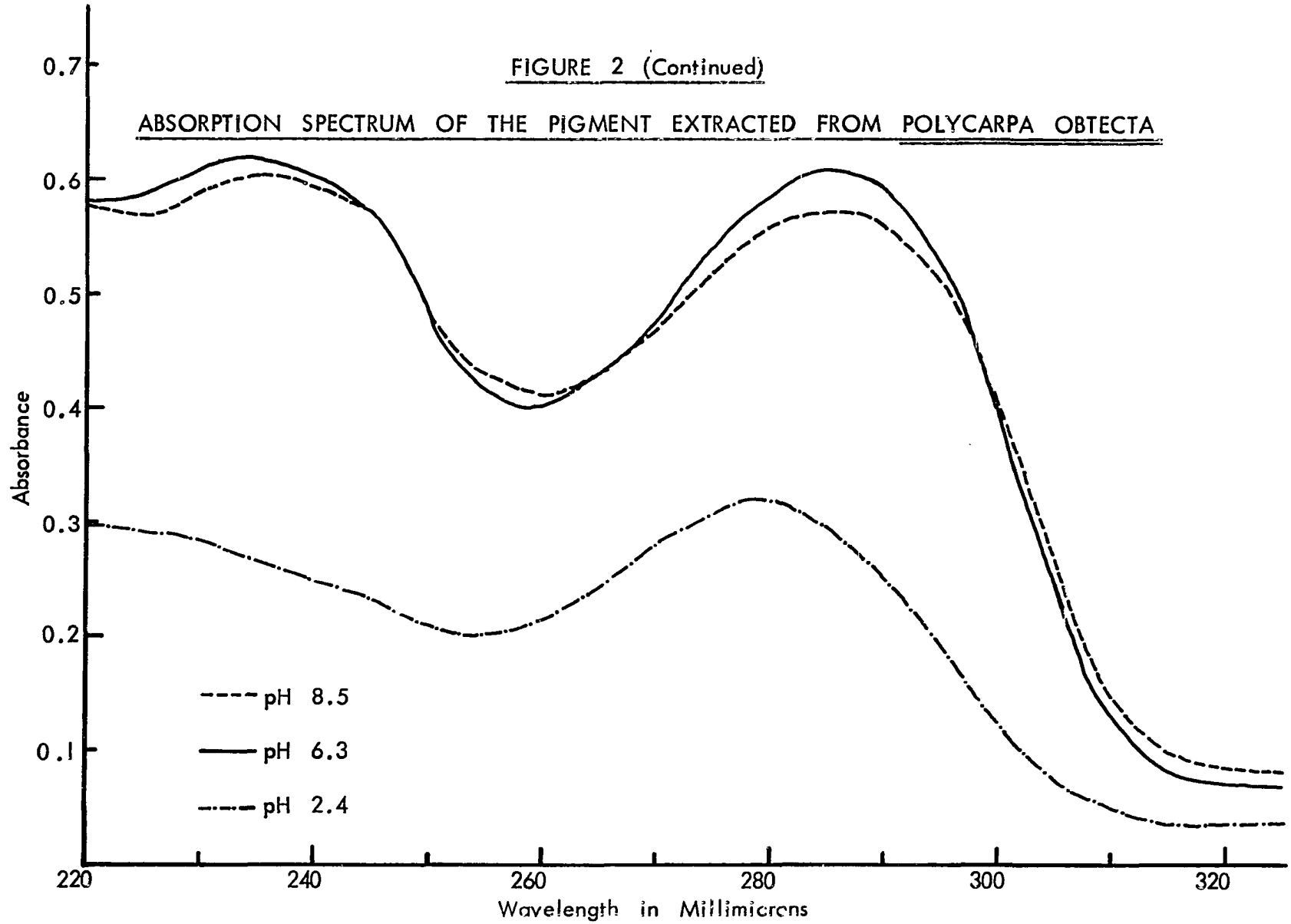


FIGURE 2 (Continued)

ABSORPTION SPECTRUM OF THE PIGMENT EXTRACTED FROM POLYCARPA OBTECTA



yellow solution, the visible absorption peaks practically disappeared, and only a shoulder remained in the curve at about 420 millimicrons. In the ultraviolet the absorption maximum was at 279 millimicrons. A slight peak was visible in the curve at about 230 millimicrons. If this is a chromoprotein, possibly the chromophoric group is destroyed in the more acid medium, or the protein may be split from the prosthetic group. It was also noted that the pigment could not be dissolved nor extracted into any organic solvent tested (ether, methanol, benzene and acetone). This coincides with the expected behavior of a protein.

The compound in solution was subjected to a number of other tests. Treatment of the neutral extract with hydrogen peroxide produced no effect. Sodium hydrosulfite changed both neutral and alkaline solutions to a pale yellow color which was only very slightly restored to the original colors by the addition of hydrogen peroxide. Dilute potassium cyanide solution changed the red solution to green, a pH effect, but had no visible effect on an alkaline solution. On standing exposed to air at room temperature, the color of the solutions gradually faded to a pale yellow. The solution at pH 6.8 retained its color for the longest period before fading.

Isolation of Pigments and Organic Compounds from Gorgonians

Preliminary extractions of some gorgonians with organic solvents had shown that these animals contained a variety of pigments and waxes. Dr. Cieraszko had also found some non-waxy colorless organic compounds in some of the extracts, particularly in pentane extracts of Eunicea mammosa and Plexaura crassa and in a methanol extract of Briareum asbestinum.

It was therefore decided to make a systematic study of the species available to try to isolate some more compounds and to identify them and the pigments present.

The starting material for these extractions was the gorgonian cortex described previously. A 100 gram sample of cortex from each animal was placed in a Soxhlet extraction apparatus and was extracted 24 hours with pentane. The pentane residue was briefly air-dried, then extracted 24 hours with diethyl ether. The process was repeated with acetone and finally with methanol. The order of solvents used went from non-polar to polar. Thus four extracts were obtained from each of the seventeen species of gorgonians available. Each of these extracts was concentrated to a volume of 100 ml. and a description of the extracts is given in Table II.

The pentane extracts were divided into three portions. Fifty ml. was used for the determination of the carotenoid pigment present, forty-nine ml. was used to determine the chlorophyll or porphyrin compounds present and one ml. was used to determine the amount of organic matter extracted. The formulas of the compounds discussed are shown in figure 3.

Isolation and Identification of Carotenoids in Gorgonians

Pentane extracts of the gorgonians had previously been chromatographed on Magnesol[3] columns. In most cases the order of elution from a column was:

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[3] Trade name for magnesium silicate sold by the Westvaco Chlor-Alkali Division of Food Machinery and Chemical Corporation.

TABLE II

DESCRIPTION OF GORGONIAN EXTRACTS

Species	Property*	Pentane solution	Diethyl ether solution
E. grandis	Vis.	Deep yellow brown	light orange brown
	U.V.	Gray orange	olive
	Res.	Slightly gray (spicules	slight dark residue
E. tourneforti	Vis.	Medium yellow green	Pale yellow
	U.V.	Buff	Blue green
	Res.	Gray residue	Dark oily residue
M.muricata	Vis.	Very dark olive green	Medium orange brown
	U.V.	Orange red	grayish pink
	Res.	White and waxlike	slight, whitish
P. crassa	Vis.	Deep yellow brown	Dark orange brown
	U.V.	Buff	Brick-red
	Res.	Large, dark crystals	White, slight
P. esperi	Vis.	Very deep yellow brown	Medium brown
	U.V.	orange red	orange brown
	Res.	Dark, oily	slight, dark
P. flexuosa	Vis.	Light yellow green	Medium orange brown
	U.V.	pale orange	gray green
	Res.	slight	slight, gray
P. homomalla	Vis.	clear olive green	dark orange brown
	U.V.	reddish brown	yellow brown
	Res.	slight, dark	none
P. nutans	Vis.	deep orange brown	dark orange
	U.V.	buff	dark orange brown
	Res.	slight	dark, slight
P. dichotoma	Vis.	deep yellow brown	medium orange
	U.V.	buff	greenish yellow
	Res.	dark, slight	dark, slight
P. acerosa	Vis.	orange	medium orange
	U.V.	green	green
	Res.	none	none
P.americana	Vis.	Rich deep orange brown	dark orange brown
	U.V.	greenish	orange brown
	Res.	slight, (spicules)	slight

*Property: Vis.= appearance in daylight
U.V.= appearance under ultraviolet light
Res.= appearance of insoluble portion of residue

Acetone solution	Methanol solution
Medium orange	very dark red brown
gray orange	gray orange
none	oil and white crystals
Colorless	light yellow
colorless	yellow green
slight, gray	dark oil and crystals
green yellow	orange
orange red	green
none	white crystals
deep greenish brown	orange brown
buff	green
heavy, white	oily
medium orange brown	yellow
orange red	yellow green
slight, dark, oily	dark, oily
light yellow	orange
pale orange	green
slight, brown	white crystals
medium orange brown	red
brown	green
none	dark oil
medium orange brown	red
pale orange	greenish gray
slight, dark	none
olive	very dark orange red
gray yellow	gray green
dark	crystals and oil
orange	orange
green	yellow green
none	none
deep orange brown	orange
gray yellow	gray green
none	dark oil

TABLE II (Cont.)

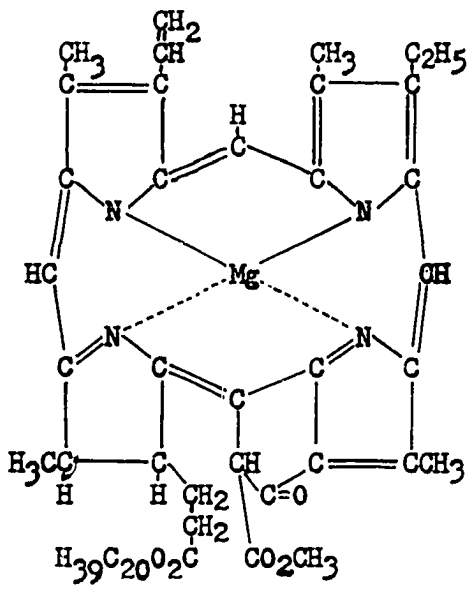
Species	Property*	Pentant solution	Diethyl ether solution
R.flabellum	Vis.	light yellow	light orange
	U.V.	green	yellow green
	Res.	none	none
X. citrina	Vis.	medium olive	light olive
	U.V.	buff	gray red
	Res.	slight grayish	slight, white (spicules)
B. asbestinum	Vis.	very light olive green	medium olive
	U.V.	gray pink	brick red
	Res.	slight whitish crystals	slight gray
E. Mammosa	Vis.	very dark olive green	medium brown
	U.V.	bright orange red	orange brown
	Res.	orange, crystalline	none
P. flavida	Vis.	olive green	medium brown
	U.V.	orange red	brick red
	Res.	none	none
X. anceps	Vis.	olive green	greenish brown
	U.V.	orange red	brick red
	Res.	white wax-like	white-slight

*Property: Vis. = appearance in daylight
U.V. = appearance under ultraviolet light
Res. = appearance of insoluble portion of residue

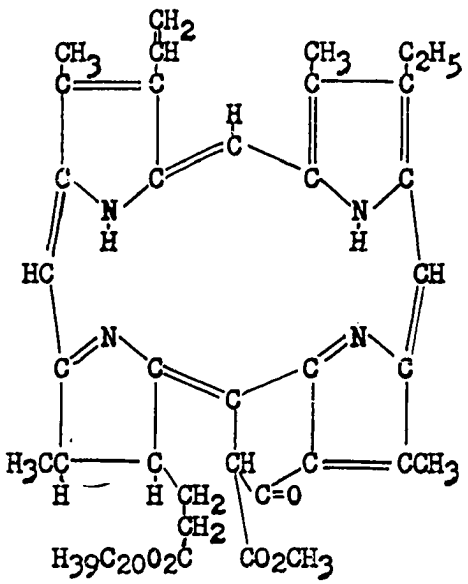
Acetone solution	Methanol solution
very pale yellow	orange
green	green
slight white	dark oil
light greenish yellow	deep red brown
brick red	orange brown
none	white crystals
greenish yellow	yellow
gray pink	green
none	white
deep orange brown	very dark red
orange	orange gray
slight dark	crystals
medium olive	very dark red
orange	orange gray
none	gray
medium greenish brown	very dark orange red
gray red	gray green
white	white crystals and oil

FIGURE 3

THE STRUCTURE OF SOME NATURAL PIGMENTS

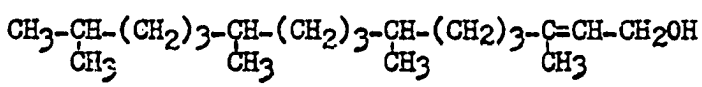


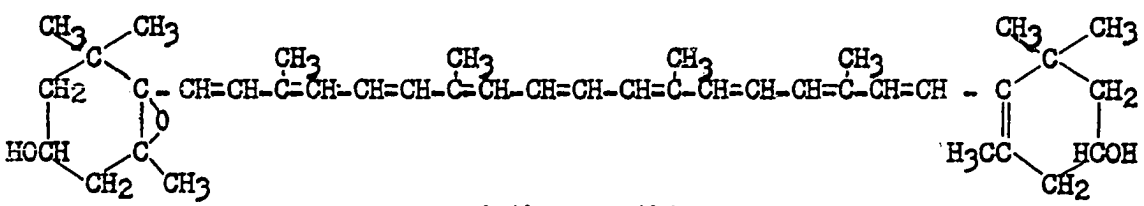
Chlorophyll a



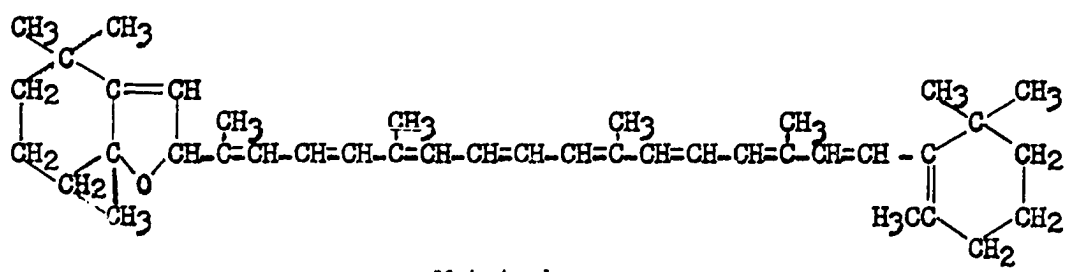
Pheophytin a

The $H_{39}C_{20}O_2$ group is the phytyl group. Phytol is:





Antheraxanthin



Mutatochrome

1. a colorless waxy material.
2. a yellow pigment.
3. a green pigment.
4. an orange pigment.

Ultraviolet and visible absorption spectra of the yellow and orange compounds indicated that they were carotenoids but they could not be identified because they were contaminated with the green pigment and other material. Therefore, the 50 ml. pentane extracts described earlier were treated by the saponification procedure of Goodwin (99). The pentane was removed under vacuum, and the residue was dissolved in 10 ml. of ethanol (more if required), and 1 ml. 60% (w/v) aqueous potassium hydroxide solution was added per 10 ml. of ethanol. This solution was kept over night under a nitrogen atmosphere. The next day the solution was diluted with 4 volumes of water and extracted with equal volumes of diethyl ether until all extractable pigment was extracted. The ether extracts were washed with water to remove soaps and then dried with sodium sulphate. The solutions were now ready to chromatograph on Magnesol columns, and the columns were developed with ether, with ether plus 10% ethanol and finally with ethanol alone.

Generally, the saponification treatment seemed to destroy some of the color in the extracts and only one carotenoid was clearly identified in the saponification mixtures. This compound, mutatochrome, was first found in Plexaura crassa by the following procedure. The ether solution of the saponified pentane extract of Plexaura crassa was put on a Magnesol column. Development of the column with ether produced a single yellow band which was eluted. Some of the yellow solution was distilled under

vacuum to dryness and the pigment was dissolved in pentane and shaken with an equal volume of 90% methanol. This is the phase separation test described by Goodwin (99) and Karrer and Jucker (72). The compound was found to be epiphasic.

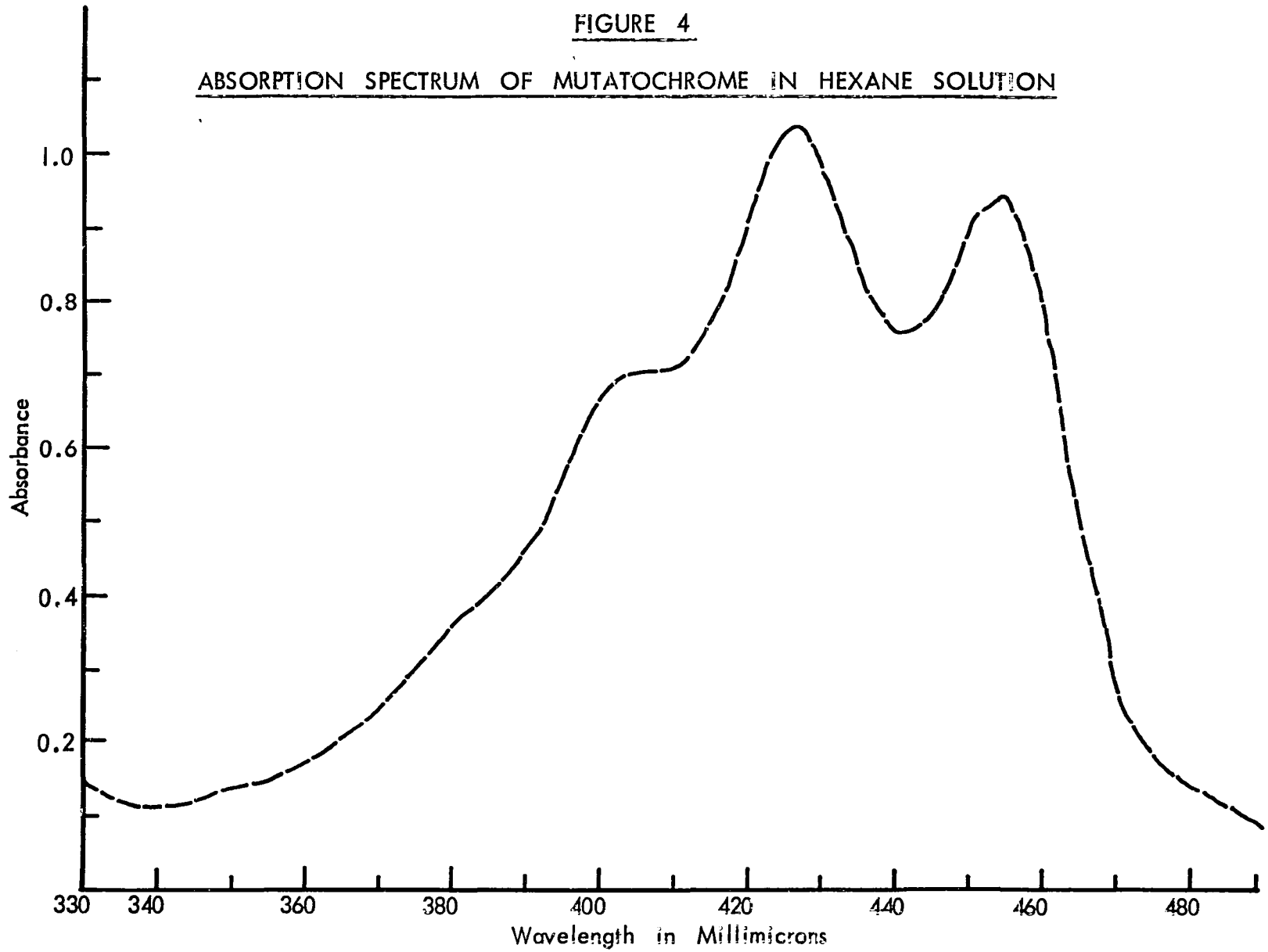
There are few color reactions for the carotenoids. Goodwin (99) has observed that the classical hydrochloric acid color test when tried on some highly purified carotenoids gave variable results. Nevertheless for what the test was worth, it was tried on the ether solution of the pigment. The concentrated hydrochloric acid added to the ether solution slowly became blue in the acid phase and then the blue color faded.

The most reliable property for identification is the absorption spectrum of the compound. The visible absorption spectrum of the yellow pigment in hexane is shown in figure 4 and was determined with the Beckman D. U. Its maxima are at 426 and 455 millimicrons. Flavochrome and chrysanthemaxanthin are the only known compounds other than mutatochrome with similar absorption maxima. Chrysanthemaxanthin is a hypophasic dihydroxy compound and is thereby ruled out. Flavochrome is a 1-3 epoxide and its absorption spectrum should shift on treating a chloroform solution of the compound with hydrogen chloride gas. This shift did not occur. Mutatochrome is the only known carotenoid pigment which shows this behavior. After re-chromatographing the compound on alumina and eluting it with ether its absorption spectrum in chloroform was obtained and the maxima were 433 and 467 millimicrons compared to the reported values of 434 and 469 (Goodwin (99)).

Similar treatment of the pentane extracts of the other gorgonian revealed the presence of this compound in the following species: Muricea

FIGURE 4

ABSORPTION SPECTRUM OF MUTATOCHROME IN HEXANE SOLUTION



muricata, Plexaura crassa, Plexaura esperi, Plexaurella nutans, Plexaurella dichotoma, Eunicea mammosa and Xiphigorgia anceps.

Other pigments were found which do not show a characteristic carotenoid absorption spectrum. One such compound appeared as a yellow band in the chromatography of the ether saponification solution of Muricea muricata. Its absorption spectrum showed a maximum in the ultraviolet at 320 millimicrons. This is closest to the absorption spectrum of the colorless polyenes but the solution did not exhibit the bright green fluorescence characteristic of phytofluene nor did the absorption peaks match phytoene or tetrahydrophytoene. Similar non-carotenoid yellow pigments with absorption maxima in the ultraviolet were found in several of the saponification mixtures. No attempt was made to characterize them.

It was noted earlier that direct chromatography of the pentane extracts sometimes yielded an orange or brown pigment as well as the yellow pigment. Nevertheless, chromatography of the saponification mixtures revealed the presence of only one, mutatochrome. An explanation may be found in Strain's article (87) on the pigments in algae. He observed that saponification destroyed many pigments and consequently he chromatographed his methanol extracts directly without saponification. However, the problem of the isolation of the pigment remained even though the likely explanation for its absence in the saponification mixture was found. The isolation came about in an unexpected manner. The solid material which crystallized out from the pentane extract of Eunicea mammosa was very dark brown in color. The solid dissolved in ether forming a dark brown solution. This was passed through a Magnesol column in an attempt to decolorize the crystalline fraction. Unfortunately for the

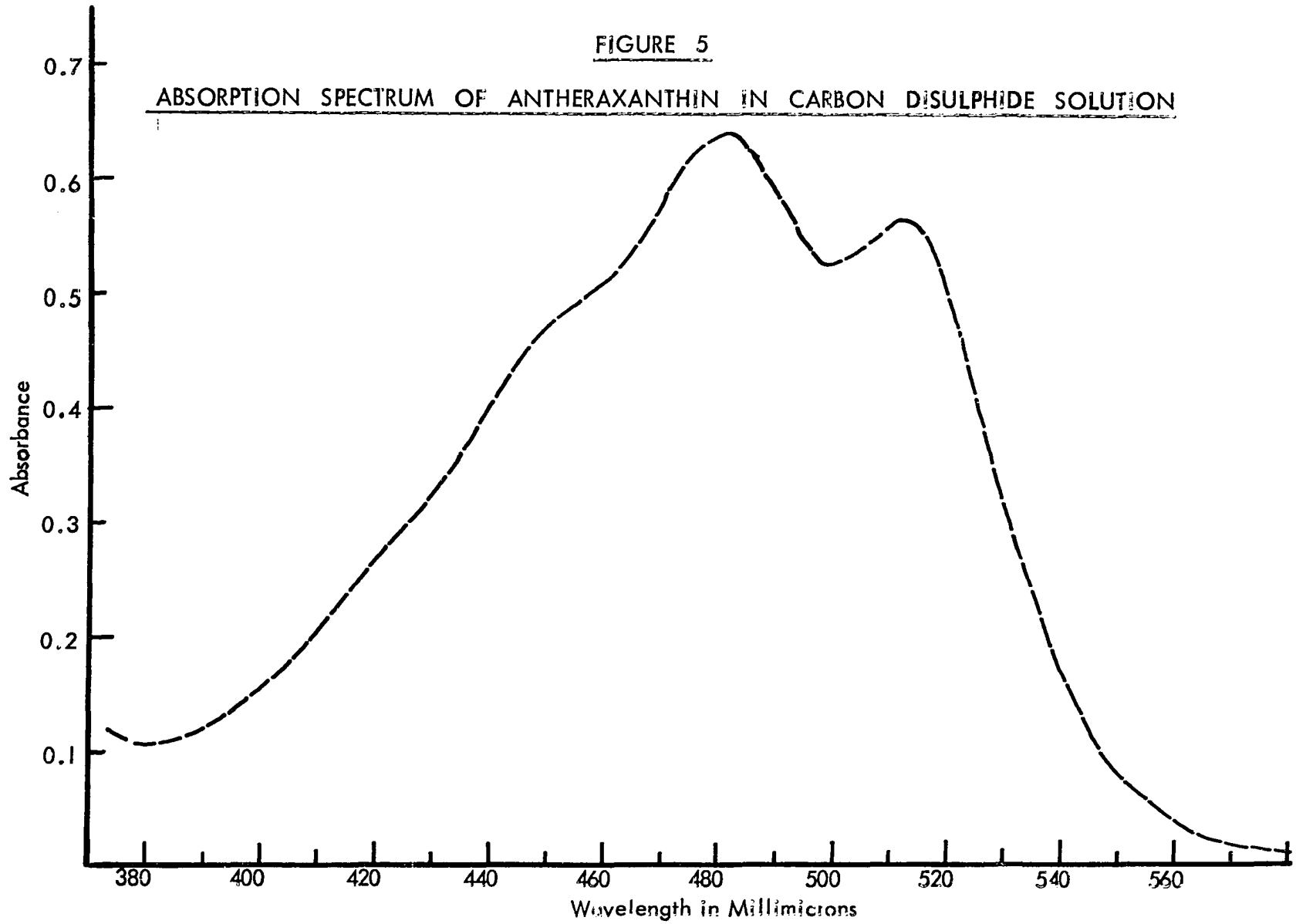
purification process the crystalline substance passed through the column and was eluted with ether with very little separation from a yellow pigment (mutatochrome). An orange brown pigment remained adsorbed at the top of the column. The latter pigment was eluted with ether-ethanol 9:1. The absorption spectrum of this orange pigment in carbon disulfide was that of a typical carotenoid and showed absorption maxima at 482 and 512 millimicrons. These peaks are close to those of antheraxanthin, α-carotene and neo-β-carotene-U. The absorption behavior indicated that the compound was antheraxanthin rather than the other possible compounds. Antheraxanthin, was first isolated from lilies by Karrer and Oswald (100). Their saponification and isolation procedure was used in a modified form to study the orange pigment from Eunicea mammosa.

Two grams of the solid from Eunicea mammosa pentane extract were chromatographed on a Magnesol column and the orange pigment eluted as before. The eluted pigment was dissolved in ethanol and saponified by Goodwin's procedure. This time after standing overnight, the saponification mixture was placed in a separatory funnel, 4 volumes of pentane were added, and then water was added until the ethanol layer became turbid. On standing, a small amount of dark brown solid separated out at the pentane alcohol interface. The alchol layer was removed and the solid was washed with pentane. The pigment was dissolved in benzene and chromatographed on an alumina column. One main band formed and was eluted with benzene ethanol 9:1. The absorption curve of the pigment in carbon disulfide is shown in figure 5. The peaks again correspond to those of antheraxanthin.

The concentrated hydrochloric acid test on an ether solution of

FIGURE 5

ABSORPTION SPECTRUM OF ANTHERAXANTHIN IN CARBON DISULPHIDE SOLUTION

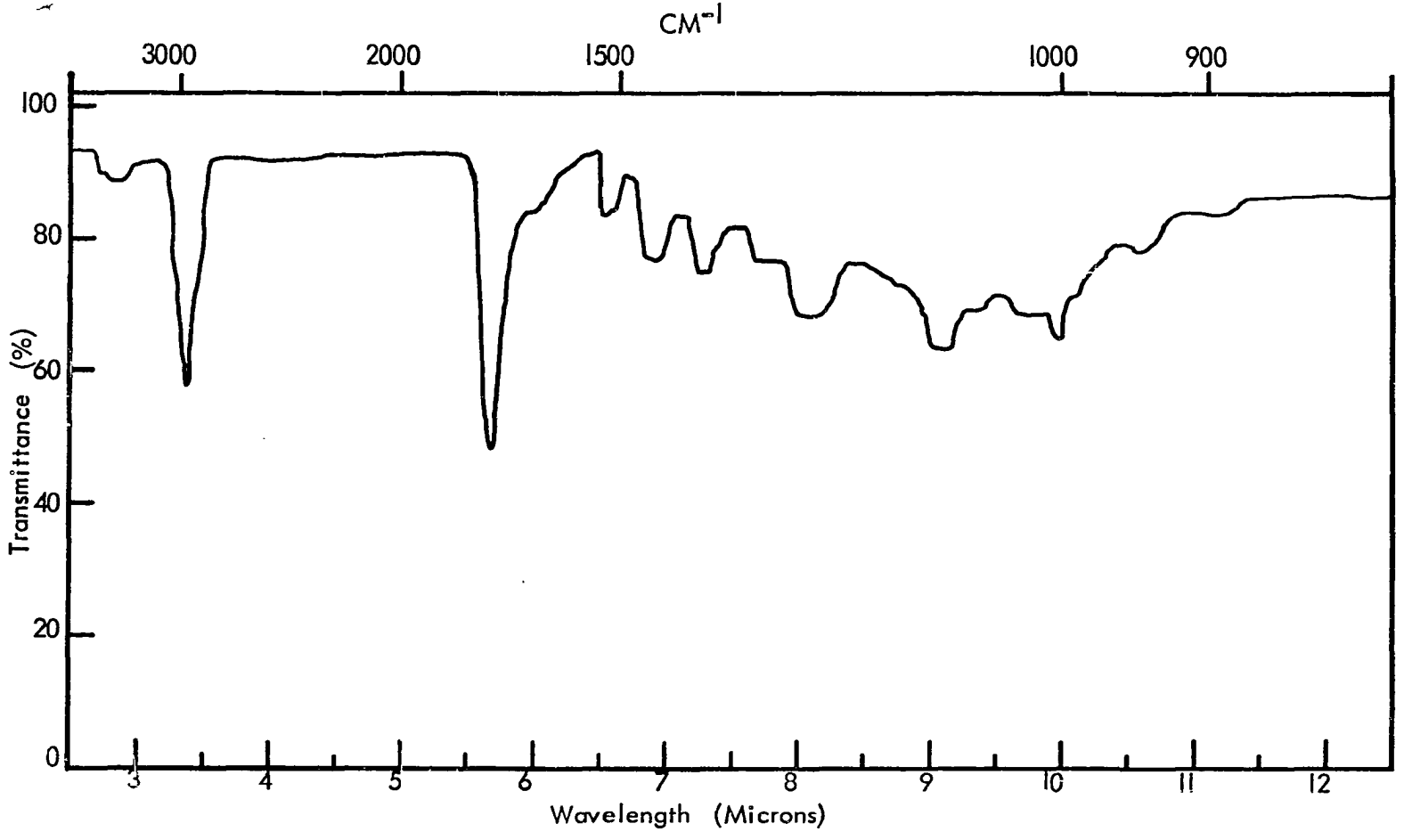


the purified orange pigment gave the same result as that reported for antheraxanthin, i.e., a stable blue color appeared in the hydrochloric acid layer some time after the acid was added to the solution.

The compound was insoluble in pentane or hexane but soluble in ether, methanol and benzene. An infrared curve of the compound obtained by the use of the Perkin Elmer Infracord is shown in figure 6. Finally one of the few specific tests available for carotenoids was tried. Goodwin (99) reported that a 5, 6-epoxide is isomerized to the furanoid form when an epoxide pigment in chloroform solution is treated with a small amount of hydrogen chloride. The change is associated with a shift in the absorption maxima of about 20 millimicrons towards the shorter wavelengths. For this test some of the original orange pigment was obtained from the Eunicea mammosa solid by the usual separation on a Magnesol column. It is not saponified but was dissolved in chloroform and rechromatographed on alumina. This treatment removed some of the impurities and the main band was eluted with chloroform-ethanol 9:1, leaving considerable amounts of a green pigment on the column. Hydrogen chloride gas was passed into the chloroform solution for a very brief time, and then the solution was chromatographed on alumina. This time, two pigments were obtained, one which was eluted with chloroform and was yellow in color, and a second darker pigment which did not move off the column until ethanol was added to the chloroform eluant. The absorption curves of the pigment before treatment with hydrogen chloride and the two pigments produced by the treatment are shown in figure 7. It can be seen that the maxima were shifted towards the shorter wavelengths by the hydrogen chloride treatment, thus indicating that the compound is a 5, 6-epoxide.

FIGURE 6

INFRARED ABSORPTION SPECTRUM OF ANTHERAXANTHIN IN CHLOROFORM SOLUTION



Since the pigment was not saponified, some green porphyrin pigments remained to contaminate the solutions all of which showed some absorption at 640 millimicrons. This points up the problem encountered in studying the carotenoids. Goodwin says that saponification is necessary to destroy these interfering porphyrin pigments. Yet Strain finds that saponification also destroys the carotenoids, and, in fact, it was definitely noted that the addition of alkali to the ethanol solutions of the pentane extracts caused their color to fade appreciably. It is suggested that antheraxanthin was not found in the pentane extracts because it was destroyed by the saponification. However, it was so concentrated in the solid residues that some of it survived the saponification.

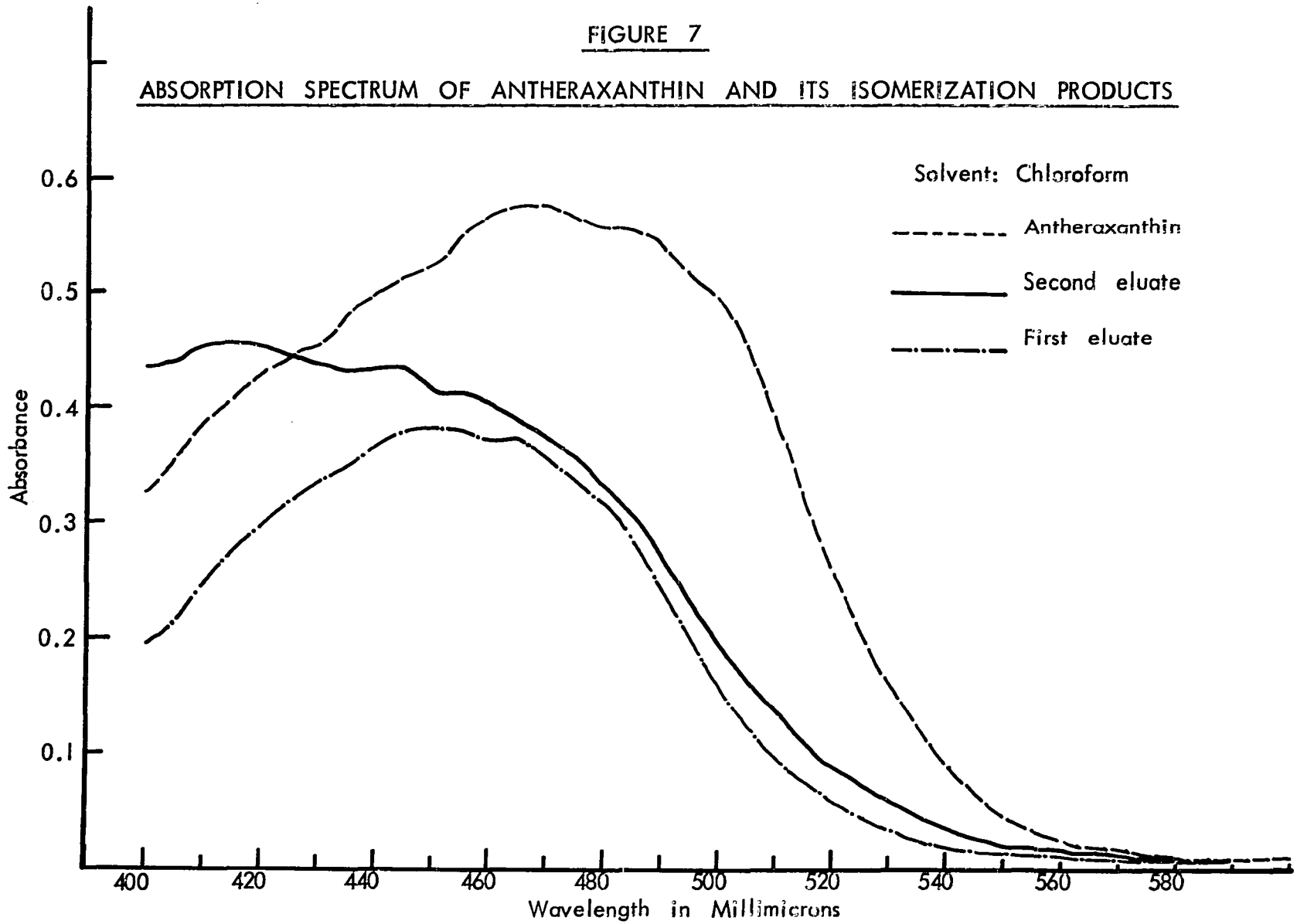
Antheraxanthin was also found in the solids in the pentane extracts of Briareum asbestinum and Plexaura crassa. It is probably present in some other species but may not be found until the more polar solvent extracts are studied.

Isolation of Porphyrin Pigments

Several pentane extracts of the gorgonians fluoresced a brilliant red under ultraviolet light. This fluorescence is characteristic of porphyrin compounds. It was noted that the green pigment or band on the Magnesol column in the preliminary experiments was responsible for the fluorescence. The green zone appeared on the Magnesol column when a pentane extract of Plexaura crassa was used, and the pigment in this zone was eluted from the column with ether and was examined with a wavelength reversion spectroscope. (R. and J. Beck #3505) Three absorption bands were observed with the wavelengths, 666.3, 531.9 and 499.9 millimicrons. The absorption curve in the visible region did not identify the pigment

FIGURE 7

ABSORPTION SPECTRUM OF ANTHERAXANTHIN AND ITS ISOMERIZATION PRODUCTS



but only suggested that it was porphyrin in nature. Ether solutions of the pigment were a clear yellow-green and fluoresced a bright red under ultraviolet light.

The phase test as described by Smith and Benitez (101) was tried. Two ml. of methanolic 30% potassium hydroxide was added to 2 ml. of the ether solution of the porphyrin. A transient yellow color appeared in the alcohol layer, and then changed to green, indicative of chlorophyll _a_ or a derivative.

The hydrochloric acid number test was tried. It is described by Smith and Benitez (101). A series of hydrochloric acid solutions of known strengths was prepared. Five ml. of each concentration was placed in separate test tubes and 5 ml. of an ether solution of the pigment was added to each test tube. The tubes were then shaken and it was observed that the pigment remained in the ether phase until the acid concentrations became 29.9% or greater. Above a concentration of 29.5%, the pigment entered the acid phase, coloring it a rich green. Thus, by definition, the hydrochloric acid number of the pigment is 29.9 corresponding to the acid strength at which the majority of the pigment entered the acid phase. The significance of this result is that the porphyrin is a chlorophyll type compound which has not lost its phytyl group, otherwise its acid number would be below 22. When the acid was diluted with water the pigment went back into the ether phase. This behavior suggested a method for obtaining the porphyrin compounds free from carotenoids. The ether pigment solution was treated with 30% hydrochloric acid which extracted the pigment. The acid phase was recovered by using a separatory funnel. The acid was diluted with four volumes of water and was re-extracted with

fresh ether. The pigment appeared as a clear green color in the ether phase. The acid solution was removed and discarded and the ether phase was washed twice with water, once with 1.0 molar phosphate buffer of pH 6.8 and finally with water. It was then dried with anhydrous sodium sulfate, and the visible and ultraviolet absorption spectra of the pigment in ether solution obtained are shown in figure 8. This is identical with the spectrum of pheophytin a. The observed absorption maxima compared with reported maxima are shown in Table III.

TABLE III

ABSORPTION MAXIMA OF PHEOPHYTIN A

Zscheile and Comar (102)	665	608	559	532	505	470	410	millimicrons	
Smith and Benitez (101)	667	609.5	560	534	505	471	408.5	"	"
Observed	662.5	605	557	530	502	469	407	"	"

The infrared absorption spectrum of the pigment dissolved in carbon tetrachloride was obtained. It is shown in figure 9. It shows absorption bands at the wave numbers 3400, 1620 - 1590, 1365 and 1095 cm^{-1}. These are bands which Weigh and Livingstone (103) report are unique for pheophytin a. The Perkin Elmer Infracord which was used was not working properly in the range below 900 cm^{-1}, otherwise two more bands at 736 and 708 cm^{-1} should have been observed. Other absorption peaks present were those common to a number of the chlorophyll type compounds.

The other gorgonian pentane extracts were also examined for the presence of porphyrins. Twenty-five ml. of the pentane extract of each

FIGURE 8

ABSORPTION SPECTRUM OF PHEOPHYTIN A IN DIETHYL ETHER SOLUTION

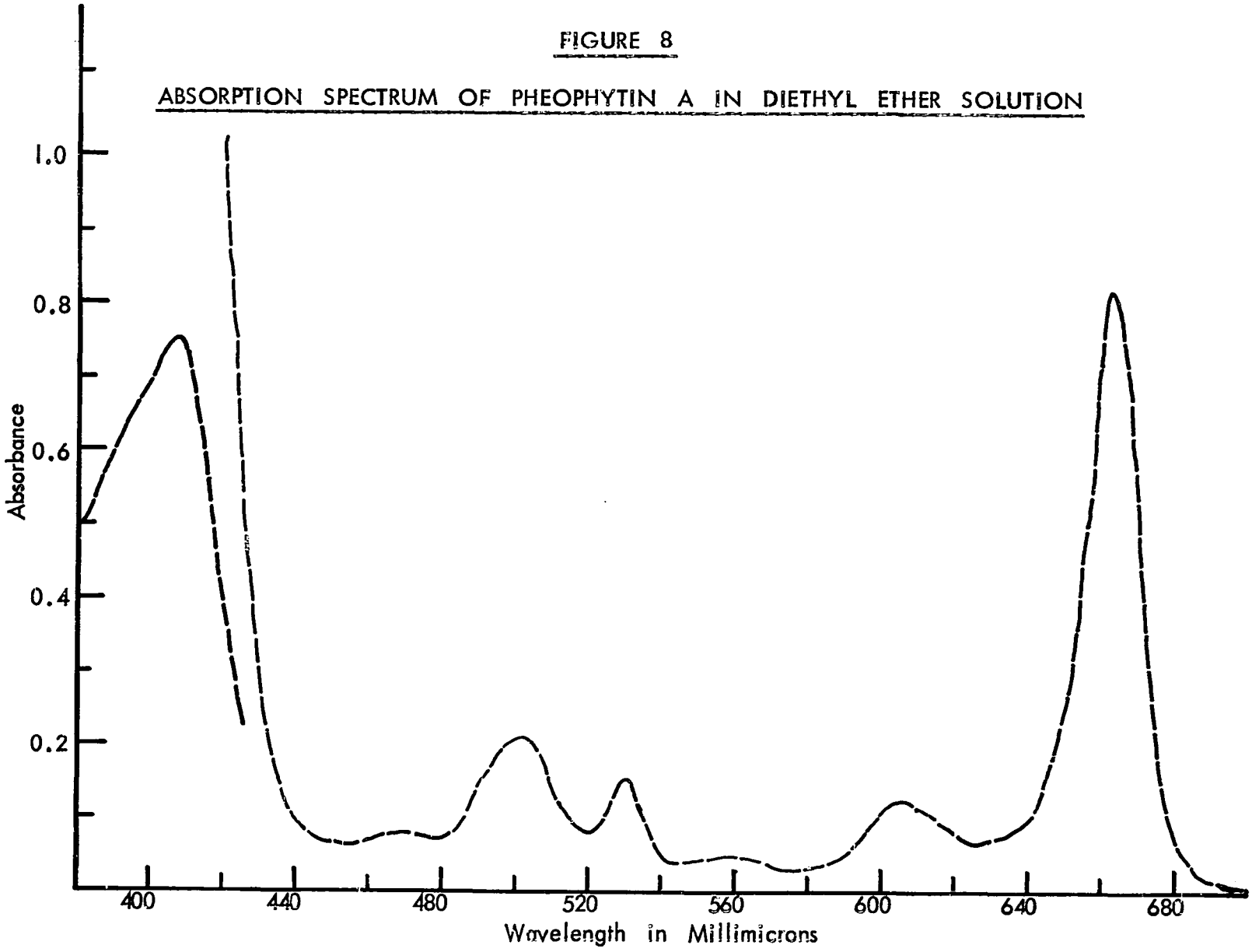


FIGURE 8 (Continued)

ABSORPTION SPECTRUM OF PHEOPHYTIN A IN DIETHYL ETHER SOLUTION

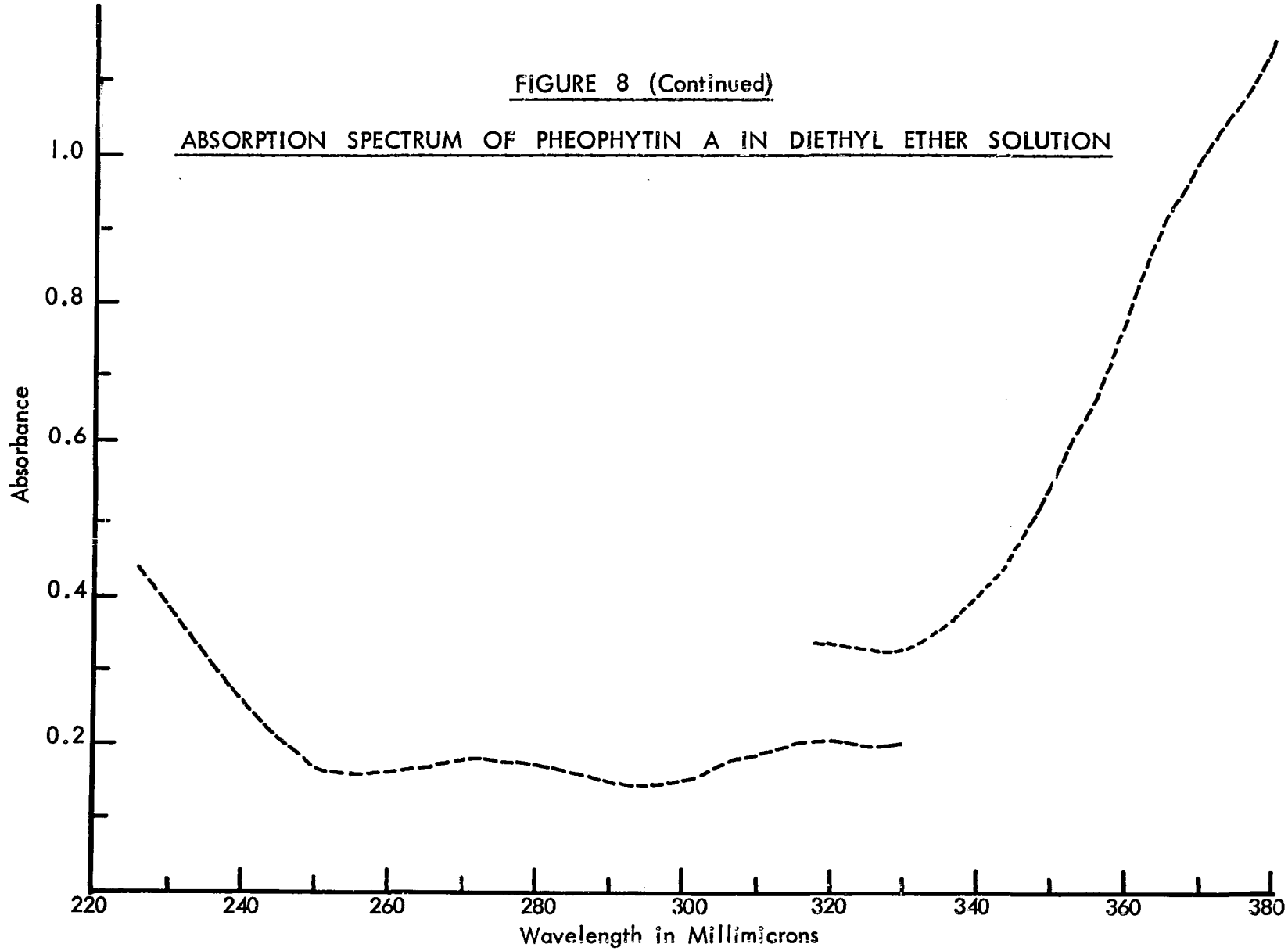
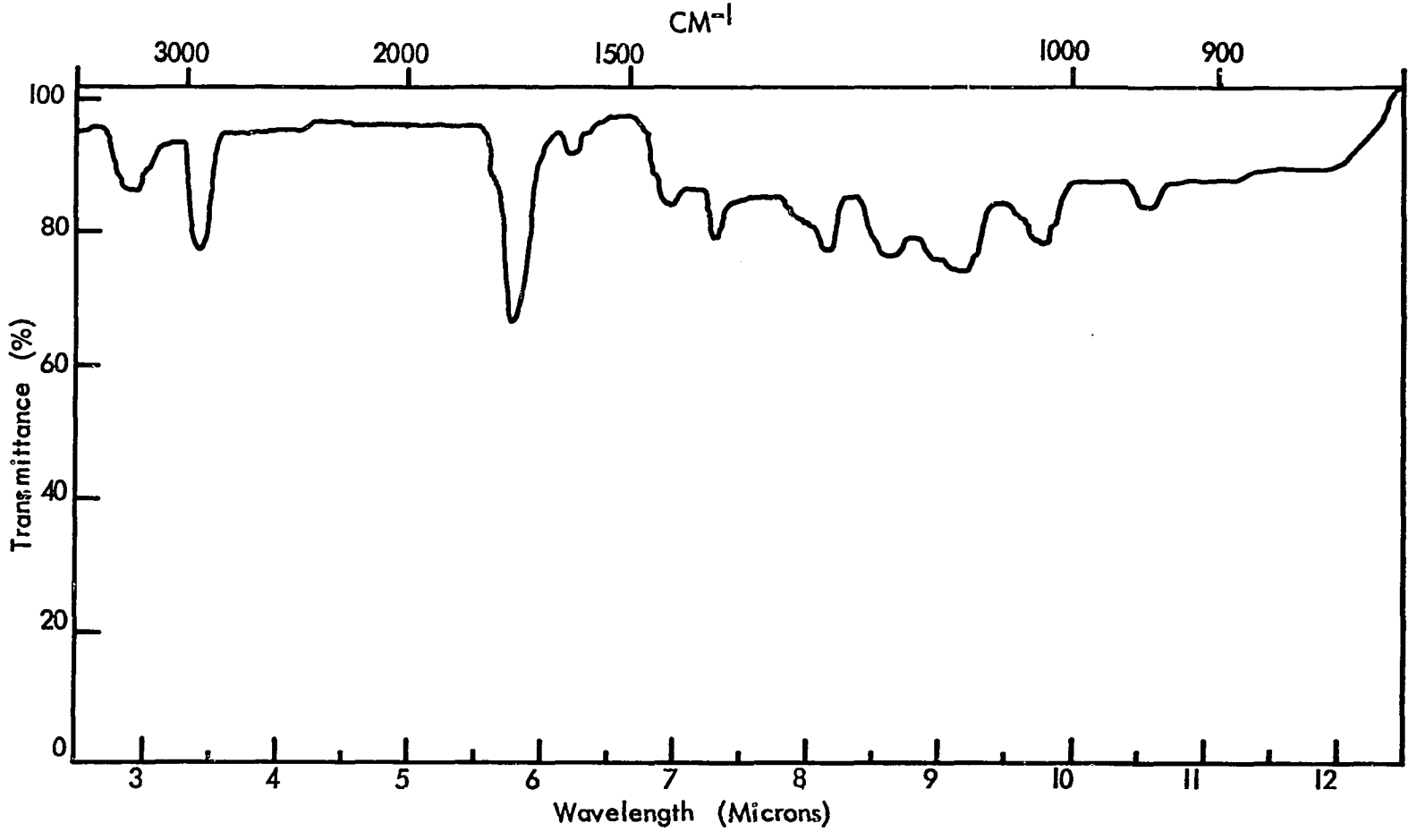


FIGURE 9

INFRARED ABSORPTION SPECTRUM OF PHEOPHYTIN A IN CARBON TETRACHLORIDE



species was distilled to dryness in vacuum and the residues were dissolved in 25 ml. ether. The other solutions were each extracted with 25 ml. hydrochloric acid. More ether was added to obtain a distinct ether phase. The acid phase containing the porphyrins was removed and diluted with six volumes of water. The porphyrins were then re-extracted into fresh ether, the ether phase was removed, washed with water and dried with anhydrous sodium sulphate. The dried ether solutions were chromatographed on Magnesol columns and developed with ether. Usually there was one predominant green band which, when eluted, gave the absorption spectrum of pheophytin a. The extracts from which pheophytin a has been isolated are the pentane extracts of the following gorgonian species. They are listed in decreasing order of pheophytin content.

Eunicea mammosa, Plexaura flavida, Plexaura homomalla, Muricea muricata, Xiphigorgia anceps, Plexaura esperi, Plexaura crassa, Eunica grandis.

The comparisons are visual and are not particularly significant. The pigment may also be present in some of the other species, but the concentration was too small to be isolated in this manner. Some of the species which are not listed did give red fluorescing pentane extracts. If the fluorescent spectra of these solutions could be obtained then it is possible that the porphyrin present could be identified without isolation. The fluorescence maxima of the compounds are very characteristic. For example, the maxima for pheophytin a in ether solution are 672.5 and 715 millimicrons.

Organic Matter Extracted From Gorgonians With Pentane

The pentane extract of each 100 gram gorgonian sample was, as described previously, concentrated to 100 ml. and 1 ml. of this extract was placed in a dry tared test tube, evaporated to dryness under vacuum and weighed. The weight of the dry residue is expressed in Table IV as the percentage of the dry weight of the gorgonian.

TABLE IV

Species	Per cent of dry weight extracted with pentane	Species	Per cent of dry weight extracted with pentane
Eunicea grandis	0.62	Pterogorgia acerosa	0.42
Eunicea tourneforti	0.24	Pterogorgia americana	0.20
Muricea muricata	0.20	Rhipidigorgia flabellum	0.26
Plexaura crassa	2.19	Xiphigorgia citrina	0.18
Plexaura esperi	0.58	Briareum asbestinum	0.45
Plexaura flexuosa	0.06	Eunicea mammosa	0.94
Plexaura Homomalla	0.86	Plexaura flavida	0.19
Plexaurella nutans	0.33	Xiphigorgia anceps	0.23
Plexaurella dichotoma	0.92		

These residues consist of waxes, oils, pigments and crystalline organic matter. Dr. Ciereszko (112) had found colorless crystalline compounds precipitating out from the pentane extracts of Eunicea mammosa and Plexaura crassa. These compounds were termed eunicin and crassin

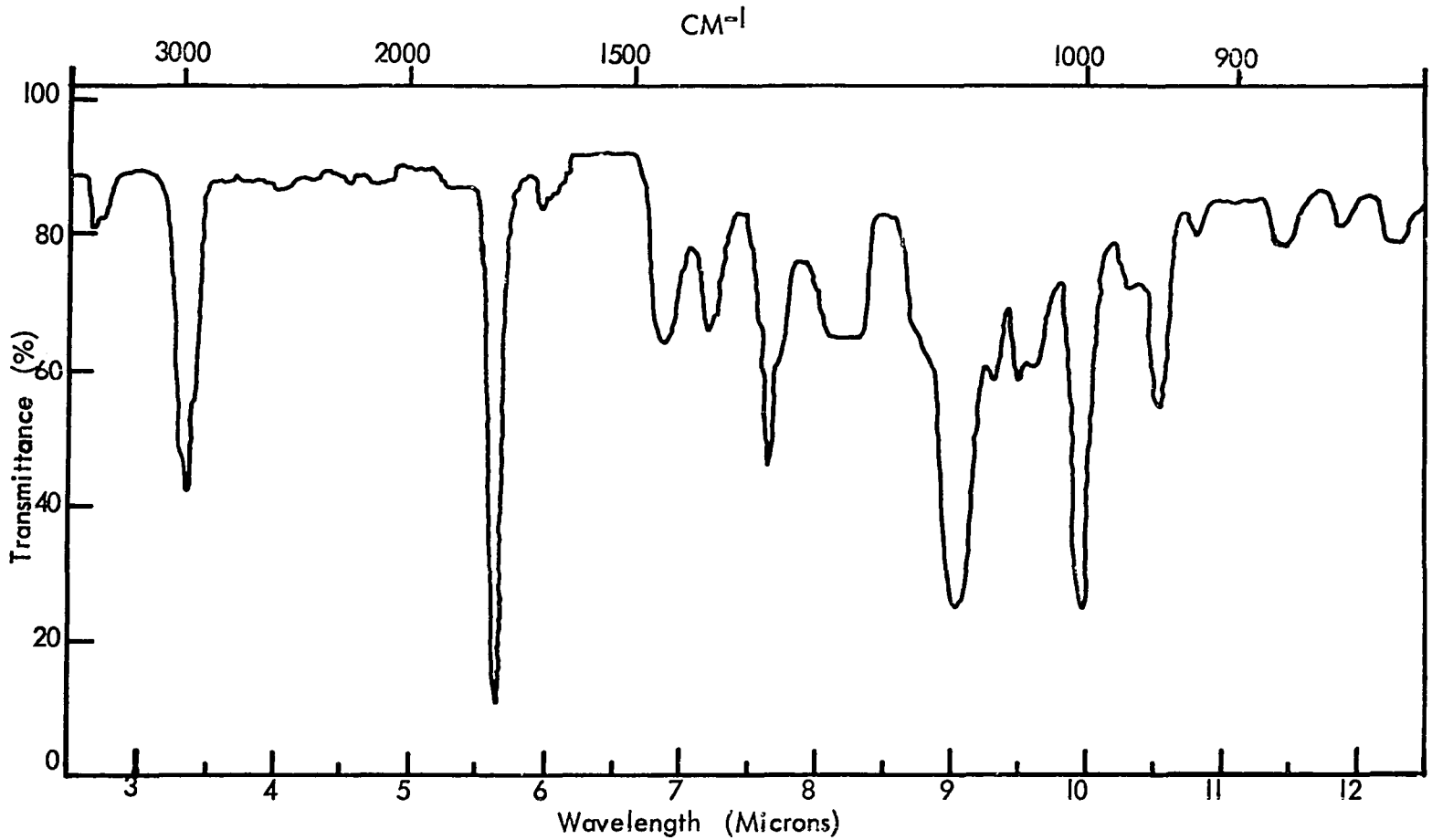
respectively. A third crystalline compound has been found in the Xiphigorgia anceps pentane extract. These compounds will be briefly considered.

The infrared absorption curve of eunicin is shown in figure 10. It shows a strong carbonyl group absorption at 5.6 microns. (or 1175 cm^{-1}). Dr. Weinheimer has attempted to saponify this compound and obtained evidence that it is a lactone. It is optically active, $[\alpha]_D^{27.75}$ = -83.6; c = 0.794 in chloroform and $[\alpha]_D^{23.8}$ = -86.90; c = 1.59 in methanol. The ultraviolet absorption spectrum of eunicin in methanol solution showed only a gradual increase in absorption towards the shorter wavelengths with a slight shoulder in the curve at 230 - 235 millimicrons. The previously described attempts to purify eunicin by passage through Magnesol or alumina columns proved futile, as did attempts to separate the pigments from the compound by counter-current and fractional crystallization methods. The best way to purify eunicin proved to be to treat a hot methanol solution of the eunicin with activated charcoal which adsorbed all the pigment. The hot solution was filtered and the compound crystallized out from solution on cooling. It was then redissolved in ether, and if the ether was allowed to evaporate slowly (by placing the solution in a narrow-necked flask) beautiful, large colorless crystals of eunicin were obtained, melting point 149-151°C (corrected). The compound, dissolved in ethanol, was hydrogenated using 5% palladium on charcoal as the catalyst.

A sample of the compound, 0.3377 grams, was dissolved in ethanol and hydrogenated using 5% palladium on charcoal as the catalyst. The hydrogenation used up 27.85 ml. of hydrogen gas (corrected to 0°C and 760

FIGURE 10

INFRARED ABSORPTION SPECTRUM OF EUNICIN IN CHLOROFORM SOLUTION



mm. mercury pressure). Assuming that one double bond was present and was completely hydrogenated, 0.00124 mole of the compound was present and the molecular weight of the compound calculated from the hydrogenation was 272. From the carbon, hydrogen and oxygen analysis carried out by the Galbraith Laboratories in Knoxville, Tennessee, the closest-fitting empirical formula obtained for eunicin was $C_{15}H_{22}O_3$, molecular weight 255.4. The analysis is shown in Table V.

TABLE V

ANALYSIS OF EUNICIN

	Carbon	Hydrogen	Oxygen (by difference)
Found	71.87%	8.90%	19.23
Calculated as $C_{15}H_{22}O_3$	71.97	8.86	19.17

The compound which crystallized out from the pentane extract of Xiphigorgia anceps was purified in the same way as eunicin, by treatment of the hot methanol solution of the compound with activated charcoal and filtering. The needle-like white crystals which formed on cooling the filtrate were redissolved in methanol and re-crystallized twice. It was exceedingly soluble in chloroform and its infrared absorption spectrum in this solvent is shown in figure 11. The interesting feature of this curve is the strong carbonyl group absorption at 5.65 microns which is a stronger absorption than the $-CH_2-$ group absorption at 3.35 microns. This is the opposite of the infrared absorption curve for the wax from

FIGURE II

INFRARED ABSORPTION SPECTRUM OF COMPOUND FROM XIPHIGORGIA ANCEPS

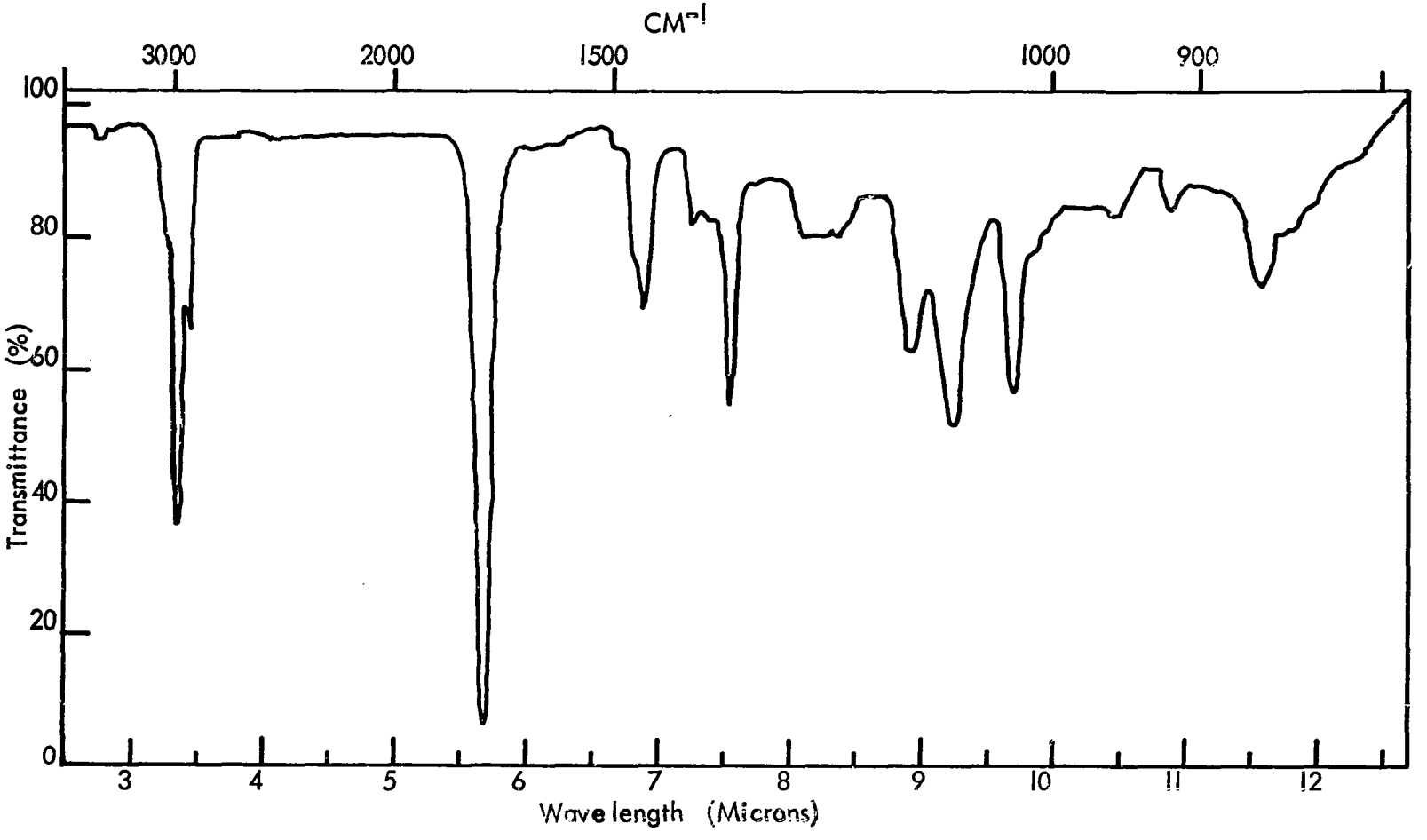
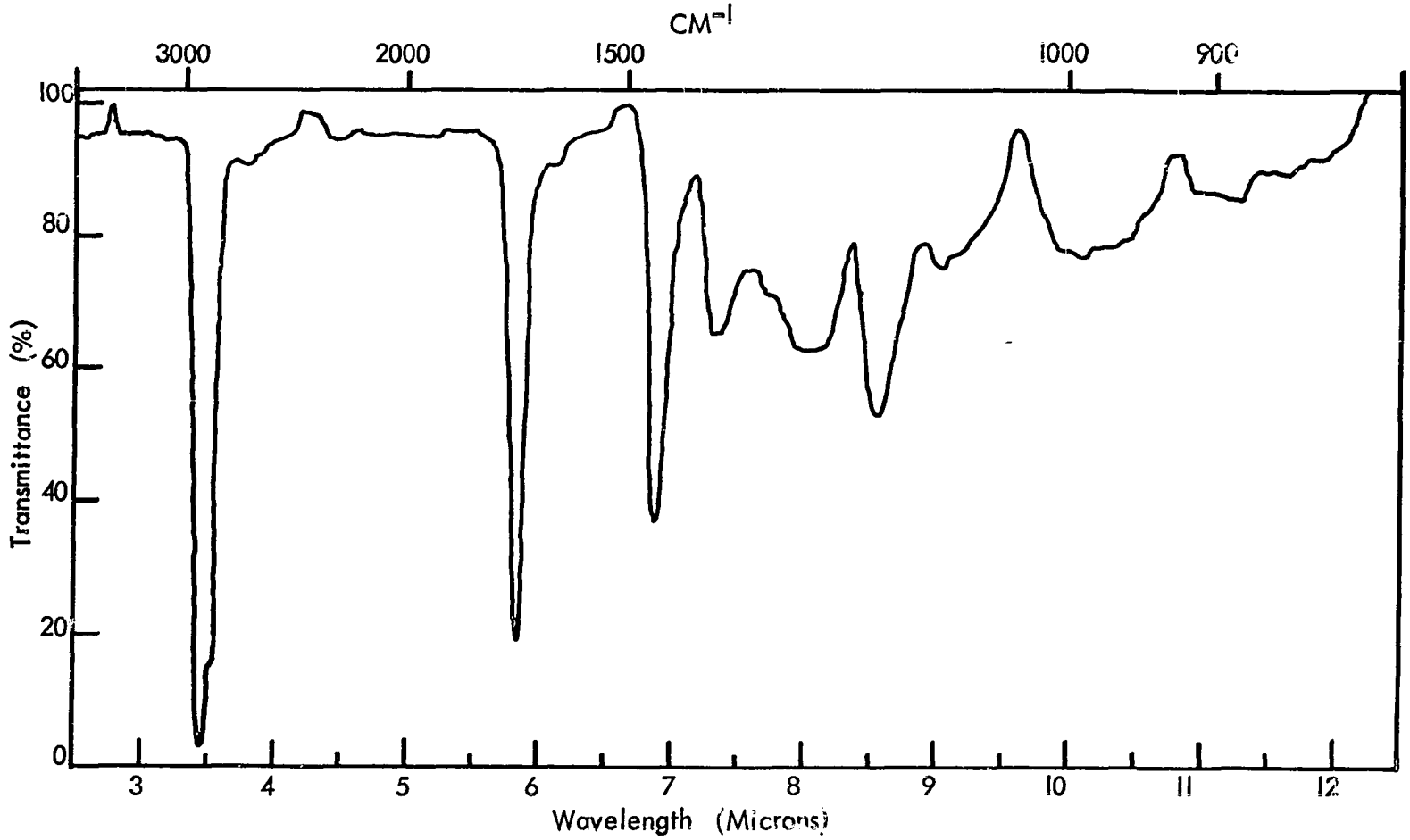


FIGURE 12

INFRARED ABSORPTION SPECTRUM OF WAX FROM PLEXAURA CRASSA



Plexaura crassa where there is a large $-CH_2-$absorption peak and a smaller carbonyl peak since the carbonyl group is diluted by the presence of the long $-CH_2-$ chain. The carbon, hydrogen and oxygen analysis of the compound was done by Galbraith Laboratories and the simplest empirical formula fitting this analysis in $C_{12}H_{18}O_2$. The analysis is shown in Table VI.

TABLE VI

ANALYSIS OF COMPOUND FROM XIPHIGORGIA ANCEPS

	Carbon	Hydrogen	Oxygen (by difference)
Found	74.10	9.27	16.63
Calculated as $C_{12}H_{18}O_2$	74.19	9.34	16.47

If this formula is correct the compound may be a dicyclic ester or diketone.

It may be noted that some of these compounds have been tested for antibiotic activity. Dr. P. R. Burkholder of the Brooklyn Botanic Garden tested crassin and found it possessed some activity against Staphylococcus aureus and also against a human tumor grown in chick embryos. Eunicin was reported active against S. aureus and Clostridium feseri.

Pigments in the Spicules of the Gorgonians

The gorgonian cortex contains calcareous spicules which vary in color from species to species. Those in the cortex at the base of

Plexaura crassa are colored a deep purple. A sample of the spicules of each species was obtained by dissolving the organic matter of the cortex in a hot solution of 10% sodium hydroxide. The spicules settled to the bottom and the supernatant liquid was poured off and the spicules were rinsed with water and dried. A photomicrograph of spicules from Plexaura crassa is shown in figure 13. The photograph was taken on Panatomic X film using a green filter, and the magnification is 82 diameters. The filter made the purple spicules appear nearly black in contrast to the colorless spicules also present.

Attempts were made to extract the purple pigment from the Plexaura crassa spicules with organic solvents. The spicules were ground and treated with ether, acetone, benzene, chloroform, and ethanol, with no effect. An attempt was made to determine the absorption spectrum by suspending the spicules in a solution of like refractive index but this was not possible because the calcium carbonate crystal structure of the spicules has two axes with different refractive indices so that the spicules remained opaque for spectrophotometric studies.

Durivault (92) reported that he had tested spicules while they were dissolving in hydrochloric acid and that they gave positive iron tests. He was studying the yellow and violet-red spicules of Alcyonium palmatum. However, when the Plexaura crassa spicules were tested with potassium thiocyanate and potassium ferrocyanide solutions while they were dissolving in dilute hydrochloric acid, no positive test for iron was obtained. Finally 10 grams of the spicules were placed in just sufficient hydrochloric acid to dissolve them and a light brown solution was obtained which, when tested with potassium thiocyanate, gave just a faint

FIGURE 12

PHOTOMICROGRAPH OF SPICULES FROM PLEXAURA GRASSA

Magnification: 82 Diameters.

positive test for iron. On standing, a brown precipitate settled out of the solution and was filtered off. The brown precipitate was washed with methanol. Some of it dissolved forming a yellow solution. Its absorption spectrum was obtained and like that of the aqueous solution showed no absorption peaks but only a steadily increasing absorption towards the shorter wavelengths.

CHAPTER III

DISCUSSION

Guanidine Bases of Invertebrates

In the past, some rather sweeping generalizations have been made concerning the distribution of phosphagens in the animal kingdom. On the basis of the limited data available, it was concluded that arginine phosphate was the only invertebrate phosphagen and creatine phosphate was the only vertebrate phosphagen. The distribution was also cited as a perfect example of chemical evolution. This theorizing was stimulating, but as comparative biochemistry became more comparative, it also became evident that the early generalizations did not hold true. Roche and his group have been largely responsible for this change in viewpoint. They have demonstrated that the distribution of phosphagens among the invertebrates is erratic, and they have also shown the existence of four new phosphagens, taurocyamine phosphate, glycocyamine phosphate, lombricine phosphate and hirudonine phosphate. It can only be concluded on the basis of present knowledge that the distribution of phosphagens appears random. However, it is felt that there must be chemical differences underlying the morphological differences whereby one classifies animals, and therefore, if there is at present no apparent system in the distribution of phosphagens it is because insufficient data have been accumulated to complete the story. The experimental results reported here are

presented as such additional data, but are in themselves insufficient information on which to generalize further.

The gorgonian zooids can contract into the cortex of the animal when disturbed. This indicates that they possess a contractile mechanism and, presumably, a phosphagen. The most likely explanation for the fact that 7 of the 17 gorgonian species examined contained no guanidine base is that too small samples of the animals were used. The zooids which would contain the phosphagen constitute a very small part of the dry weight of the animal, the major portion being the calcareous cortex and the inner horny protein skeleton. The only guanidine base found in the other 10 gorgonian species examined was creatine and thus creatine phosphate is the likely phosphagen in these species. Three of the species containing creatine also contained traces of what might have been glycocyamine. This would not be unexpected as glycocyamine is considered to be a precursor of creatine. Roche, Thoai and Robin (67) have reported finding traces of creatine in 2 other coelenterates, Anemonia sulcata Penn., and Calliactis parasitica Couch, but they also found arginine in these animals. Roche, Thoai and Hatt (45) found creatine in the alcyonarian Alcyonium palmatum, but again, arginine was also present.

Arginine was found in all the echinoderms studied, the holothurians, Stichopus badionotus, and Synaptula hydriformis and the echinoid, Tripneustes esculentus. These results are in agreement with previous work; Meyerhof had found arginine in Stichopus sp. and Holothuria tubulosa as early as 1928. Needham et al. (58) and Roche, Thoai and Robin (67) have both reported finding arginine in Leptosynapta inharrens. Among the echinoids, two conditions exist. Several have been found to contain

both arginine and creatine, others contain only arginine. Apparently Tripneustes esculentus belongs to the latter class. To date, no one has reported finding creatine alone in an echinoid.

Arginine was found in the tunicate Polycarpa obtecta. There is very little information available about the protochordates. Needham and Needham (11) reported finding arginine in the atrial muscle of Ascidia mentula. Recently Ennor, Griffiths and Morrison (57) studied two tunicates, Pyura sp. and Pyura stolonifora and found only creatine in them. While they did not say that Needham and Needham were wrong, they did suggest that their identification of arginine was based on equivocal evidence. Ennor et al. further stated that if creatine phosphate proves to be the typical tunicate phosphagen then this would be biochemical evidence in favor of their presently accepted phylogenetic classification. Many more species must be examined before the status of the phosphagens in the protochordates is settled.

Pigments of Gorgonians

As has been noted earlier, there is very little information available about the pigments of the gorgonians. In the present work, the examination of these animals has just begun. Mutatochrome and antheraxanthin were identified in some of the species. Mutatochrome was first synthesized from α-carotene by Euler, Karrer and Walker (105) in 1932, but it was not until 1947 that Karrer and Jucker (106) established the identity of mutatochrome with the pigment citroxanthin which they (Karrer and Jucker) (107) had isolated from orange peel in 1944. It does not appear to have been reported as occurring elsewhere. The other pigment, antheraxanthin, was first isolated from lilies in 1935 by Karrer and

Oswald (100). It seems to enjoy a little wider distribution than mutatochrome. Fox, Updegraff and Novelli (108) report finding a compound very similar to antheraxanthin in the ocean mud. More recently, Tischer (109) found antheraxanthin in the fresh water alga Cladophora fracta. Thus the compound is not a stranger to a watery environment.

The green pigment found in so many of the extracts has been identified as pheophytin a. There can be little doubt that it was derived from phytoplankton, but there remains the question of whether or not pheophytin a and the carotenoids were actually extracted from the gorgonians or were they extracted from symbiont algae which may have been present. Recently, Zahl (110) described a method for culturing zooanthellae and this may provide an answer to the question. If the carotenoids and porphyrins in the cultured zooanthellae are examined and differ from those in the gorgonian extracts, it may be assumed that the pigments in the gorgonian extracts were actually part of the gorgonians, whether synthesized de novo or altered after ingestion.

Two final problems remain. Goodwin (71) clearly stresses the importance of saponifying the carotenoid extracts to destroy the porphyrins present. In the present work, it was noted that the unsaponified antheraxanthin remained contaminated by pheophytian a or a similar compound even after being chromatographed three times. On the other hand Strain (87) found saponification destroyed his algal carotenoids and he therefore, chromatographed his methanol extracts directly. Finally there is the example of Heilbron et al. (86) who found that the ester actinoerythrin from Actinia equina possessed a very different absorption spectrum from that of the acid violerythrin, obtained after saponification. Thus identification of the pigments in the ester form may be misleading.

The conclusion seems to be that one must make an arbitrary choice and hope to select the lesser of two evils.

The final question concerns whether or not the drying and storing process altered the carotenoids. This problem can only be checked by comparing the pigments extracted from a stored specimen with those found in a freshly collected animal.

Organic Content of Gorgonians

The organic matter extracted from the gorgonians by pentane is in some cases a considerable portion of the dry weight of the animal. This is of interest from two standpoints. It suggests that here is a rich source of raw materials in which to look for new naturally occurring compounds. To some extent this has been done, and three new compounds have been isolated. Two of these compounds, from Eunicea mammosa and Plexaura crassa, have tentatively been identified as lactones and have been named eunicin and crassin respectively. The third compound was isolated from Xiphigorgia anceps and appears to be an ester or diketone. Of practical interest is the high activity of eunicin against Staphylococcus aureus. Also interesting was the high organic content in some of the animals. This suggests that the gorgonians may have furnished part of the organic matter found in coral reef formations which Link (111) has recently claimed are the sources of some petroleum deposits.

CHAPTER IV

SUMMARY

In recent years it has not been surprising to find creatine present among the invertebrates. It has been reported in trace amounts in the coelenterates Anemonia sulcata and Calliactis parasitica by Roche, Thoai and Robin (67). In the present study, creatine was found in the following nine of seventeen gorgonian species examined: Plexaura flexuosa, Plexaura homomalla, Plexaurella nutans, Plexaurella dichotoma, Pterogorgia acerosa, Pterogorgia americana, Rhipidogorgia flabellum, Eunicea mammosa and Plexaura flavida. Three species, Pterogorgia acerosa, Pterogorgia americana and Rhipidogorgia flabellum appeared to contain traces of glycocyamine, a precursor of creatine. No other guanidine bases were detected. It can be concluded that if the gorgonians contain a phosphagen, it must be creatine phosphate.

Arginine was found in the echinoderms and in the one tunicate species examined. The species studied were the holothurians, Stichopus badionotus, and Synaptula hydriformis, the echinoid, Tripneustes esculentus and the tunicate Polycarpa obtecta. The complete absence of creatine and the presence of arginine in the siphon muscles of Polycarpa obtecta were unexpected and appeared to conflict with the work on tunicates by Griffiths, Morrison and Ennor (64), who found only creatine. These results only emphasize the necessity of making many comparisons

before making a generalization.

A pigment was extracted from Polycarpa obtecta by a 2% solution of hot acetic acid or by hot water. The pigment acted as a pH indicator, turning green in alkaline solutions and red in neutral or slightly acid solutions. In strongly acid solutions, it turned yellow. This latter color change was irreversible. There is evidence that a protein is present but it is not known whether it is only a contaminant or is part of a chromoprotein complex. The pigment is adsorbed by anion exchange resins and behaves as an acid.

The carotenoid pigment, mutatochrome, was found in the pentane extracts of several gorgonians, Muricea muricata, Plexaura crassa, Plexaura esperi, Plexaurella nutans, Plexaurella dichotoma, Eunicea mammosa and Xiphigorgia anceps. Another carotenoid, antheraxanthin, was found in the crystalline precipitate which formed during the pentane extraction of Briareum asbestinum, Plexaura crassa and Eunicea mammosa.

A porphyrin compound, pheophytin a, was found in the pentane extract of nearly every gorgonian. It was isolated from the following gorgonians which are listed in decreasing order of pheophytin a content: Eunicea mammosa, Plexaura flavida, Plexaura homomalla, Muricea muricata, Xiphigorgia anceps, Plexaura esperi, Plexaura crassa and Eunicea grandis. The pentane extracts of some species which were not listed above fluoresced red under ultraviolet light and may have contained pheophytin a, but not in amounts large enough to isolate.

The total organic matter extracted from the gorgonians by pentane was also determined. There was a wide variation and in a few cases as much as one or two percent of the dried weight of the gorgonians was

extracted by pentane. Most of this material appeared to be oils, waxes and pigments but three colorless, crystalline, non-lipid compounds were obtained from three different species. One of these compounds, obtained from Eunicea mammosa, has been named eunicin. It is an optically active, unsaturated compound which behaves like a lactone and from the carbon, hydrogen and oxygen analysis the closest-fitting empirical formula obtained is $C_{15}H_{22}O_3$. A compound extracted from Plexaura crassa has been named crassin. Its behavior also indicates that it is a lactone. Finally, a compound has been found in the pentane extract of Xiphigorgia anceps. It may be an ester or a diketone. The simplest empirical formula calculated from the carbon and hydrogen analysis is $C_{12}H_{18}O_2$. Some of the extracts have shown antibiotic activity.

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