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ISOLATION AND PARTIAL CHARACTERIZATION
OF CARIBBEAN PALYTOXIN

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ISOLATION AND PARTIAL CHARACTERIZATION
OF CARIBBEAN PALYTOXIN

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ISOLATION AND PARTIAL CHARACTERIZATION
OF CARIBBEAN PALYTOXIN

INTRODUCTION

In July of 1960 several pieces of the zoanthid Palythoa caribaeorum (Duchassaing and Michelotti, 1861) (1) were collected at South Reef near Port Royal, Jamaica. The animal material was dried and sent to The University of Oklahoma for investigations on the nature of its lipids. Early in the spring of 1961 it was discovered in a curious manner that this material contained a toxin.

Professor Leon S. Ciereszko and William S. Reeburgh, one of his students, had crushed the dried material in a Waring Blendor and had transferred it to an extractor. During these operations there was dusting of the very dry material into the laboratory. Reeburgh and Ciereszko began to notice sore throats and running noses shortly after handling the material. The severity of these symptoms increased throughout the afternoon. That evening and night, Ciereszko, Reeburgh, and the author, who had been exposed to the dust of the animal for only a short period, suffered from severe chills and fever. The three felt better the next day, but Reeburgh suffered from soreness

of the chest muscles for several days thereafter. Subsequent tests on mice and rabbits revealed that *Palythoa* contained a water-soluble toxin.

In 1965 Professor Ciereszko discovered that another Caribbean species in the same genus, *Palythoa mammillosa* (Ellis and Solander, 1786) (1) is highly toxic. There is some evidence that a third Caribbean species of the genus, *Palythoa grandis* (?), is toxic (2). Professor Ciereszko has collected toxic *Palythoa* on the Great Barrier Reef of Australia and at Eniwetok in the Marshall Islands.

In 1961 Professor Paul J. Scheuer of The University of Hawaii discovered that another animal of the genus *Palythoa*, which is found in warm Pacific waters, also contains a toxin (3). Although the animal is fairly abundant in Hawaiian waters, only the specimens from a tide pool of Maui have proved to be toxic. Since 1961 toxic animals have been found in the waters of other Pacific Islands by Dr. Philip Helfrich (4).

Toxic specimens of both *Palythoa caribaeorum* and *Palythoa mammillosa* have been found in abundant supply in Jamaican waters. Toxic specimens have also been collected at Bimini in the Bahama Islands. The animals found thus far in the Florida Keys and at Bermuda have little toxicity.

Palythoa is a member of the Phylum Coelenterata (Cnidaria), Class Anthozoa, Subclass Zoantharia (Hexacorallia), and Order Zoanthidea. It grows in the Caribbean

Sea in depths from less than one foot to twenty feet. It forms a fleshy crust about one inch thick on coral rock. It normally abounds in clear water where there is considerable movement of the water due to wave action or to currents.

The Jamaican animals contain the toxin in significant amounts only during the summer months. The variation in the level of toxicity may be related to the amount of insolation since these animals contain within their tissues single-celled algal symbionts. These symbionts are zooxanthellae and they are photosynthetic. The intensity of sunlight is greatest in Jamaica during the summer months. It has not been determined, yet, whether the toxin is a product of the animal or of the zooxanthellae, or whether it is derived from the food source. Dr. Thomas F. Goreau of The University of the West Indies has noted that at least some species in the genus Palythoa do not feed (5), so it seems doubtful that the toxin is derived from zooplankton, the food source of most anthozoans. The author has never observed feeding among specimens of Palythoa kept in laboratory aquaria. In addition, no toxicity could be detected in the two samplings of plankton which have been made in the vicinity of toxic animals.

Physiological investigations of crude palytoxin by Harry C. Beall (6) and by Dr. William B. Stavinoha (7) showed that the toxin is a vasoconstrictor. Other

preliminary studies have indicated that palytoxin causes constriction of smooth and striated muscle.

Palytoxin has been isolated and partially characterized. The toxic materials from Palythoa caribaeorum and Palythoa mammillosa appear to be the same. The toxin occurs in the animals in concentrations of approximately 1 part in 25,000 (0.004%).

DISCUSSION OF COLLECTION OF PALYTHOA
AND ISOLATION OF PALYTOXIN

Part I. Collection of Palythoa

The Palythoa caribaeorum and Palythoa mammillosa used in these investigations were collected in Jamaican waters during the summer months of 1965, 1966, and 1967 at South Cay, South Reef, Lime Cay, Drunkenman's Cay, Biddlecombe Shoal, and Brooks Pen, which are in the vicinity of Port Royal and at Discovery Bay and Salt Gut, which are on the northern coast.

Since the animals occur in shallow water, they were collected by using snorkeling equipment, gloves and knives. The divers never reported any ill effects from being in close contact with the animals in the water. Handling the freshly collected animals on the shore, however, often caused severe allergenic symptoms in the author.

The animals were preserved soon after collection by immersion in alcohol or by freezing. Unpreserved animal material quickly lost toxic activity.

Part II. Isolation of Palytoxin

Throughout the isolation procedures the toxicity of fractions was determined by intraperitoneal injection into mice.

Palytoxin was extracted from frozen Palythoa or from Palythoa preserved in alcohol with either aqueous ethanol or aqueous methanol. Palytoxin is not extractable with longer chain alcohols or with the common organic solvents with the exception of dimethylformamide.

In the early experiments frozen animals or alcohol-preserved animals were crushed in a Waring Blendor with ethanol in order to assure intimate contact of the animal material with the solvent. Later it was found that extraction by percolation was just as effective in removing the toxin and was less hazardous. The active extract had a dark brown color and had a relative activity (R.U.) of 20 as compared with the starting material.

The active alcoholic extracts were extracted with pentane in order to remove lipid materials. Pentane removed little of the toxin or pigments from the aqueous alcohol, but it did extract sterols and other lipids (8). Two percent on a dry weight basis of the alcoholic extract is removed by prolonged liquid-liquid extraction with pentane. Palythoa contains 1 percent (based on dry weight) lipid material extractable with hexane.

The alcohol was removed from the active extracts by distillation in vacuo on a rotating flash evaporator at room temperature. Activity was lost if the solution was heated.

The concentrate was then dialyzed against running tap

water. While palytoxin was non-dialyzable, 70% by weight of the solutes of the active solution were dialyzable. The non-dialyzability of palytoxin indicated that it was a large molecule or that it had a shape which prevented its passing through the pores of the dialysis membrane. Relative activity after dialysis was 40.

In the next step of the isolation a new non-ionic polystyrene resin, Amberlite XAD-2, was employed. The resin was designed to remove water-soluble organic materials from aqueous solutions by the formation of hydrophobic bonds. Palytoxin was effectively removed from water solutions by stirring the solutions with the resin beads or by passing the solutions through a column of the resin. At first little activity could be recovered from the resin with aqueous alcohol. Later experiments, however, showed that the palytoxin could be recovered by washing with a homogeneous aqueous solution of methanol and benzene if the Amberlite was soaked in "cellosolve" prior to use.

The methanol and benzene in the toxic solution was removed by distillation on a rotating flash evaporator and the water was removed by lyophilization to give a solid with a relative activity of 1000, a 25 fold increase in activity.

Since palytoxin was soluble in aqueous alcoholic solutions, the "cellosolves" such as 2-ethoxyethanol were considered for use in the purification scheme. Two-phase

solvent systems containing water, ammonium sulfate, and 2-ethoxyethanol had been employed in counter-current distribution studies of ribonucleic acids by Martin and Porter (9) and by Kirby (10). Porter (11) has used such solvent systems for partition chromatography of proteins on Kieselguhr. Only by the addition of salt can two phases be obtained from 2-ethoxyethanol and water which are otherwise completely miscible. Use of one of these 2-phase systems showed that over 95% of the toxin appeared in the upper organic layer, while some of the pigmented materials remained in the lower aqueous phase. It was further found that on proper dilution of the upper toxic phase with water, 2-butoxyethanol ("butyl cellosolve") and hexane, another 2-phase system developed. The lower phase of this system contained relatively little of the brown pigment and most of the palytoxin. In order to eliminate some of the pigmented materials, both these two-phase distributions were employed in the purification scheme.

The major impurity at this stage was the ammonium sulfate introduced in the above steps, but it was easily removed after lyophilization of the active solution by dissolving the toxin in dimethylformamide and filtering off the ammonium sulfate on a sintered glass funnel. The dimethylformamide was distilled on a rotating evaporator which was equipped with a dry ice-acetone condenser. Using the dry ice condenser made it unnecessary to heat

the active solution above room temperature even though the boiling point of dimethylformamide is 153°C. at 760 mm. Hg. Relative activity after the two-phase distributions and removal of ammonium sulfate was 2000.

Because palytoxin could not be recovered from Avicel (microcrystalline cellulose), carbon-Celite, silicic acid, Florisil, alumina, and charcoal columns, dextran gels were employed for column chromatography of palytoxin.

Palytoxin could be easily eluted from the Sephadexes which are cross-linked gels of polydextran. Sephadex LH-20, a methylated dextran which swells in organic solvents, was found to give good separations although repeated gel filtrations were necessary to give a chromatographically pure product.

The toxin was only slightly retarded by the dextran gels when chromatographed in water, water-methanol, methanol, or dimethylformamide. Such behavior indicates a molecular weight of four to five thousand which is the range of the exclusion limit of Sephadex LH-20.

Ionophoresis of palytoxin showed it to be cationic. This suggested the possibility of using ion-exchangers in the purification, but the usual acid resins were not employed because the changes in pH are sufficient to destroy palytoxin which is sensitive to both acidic and basic media. Even in a weakly acidic solution of ammonium sulfate, palytoxin is denatured within a few hours at room temperature.

Carboxymethyl cellulose, a cation-exchange gel, retained palytoxin when a water solution of the toxin was put through a column of the gel. The toxin was easily eluted with 2M sodium chloride. No activity was lost and some of the pigmented impurities were removed. The ion exchange step was employed in the purification scheme because it was simple and it eliminated one of the slower gel filtrations on Sephadex LH-20.

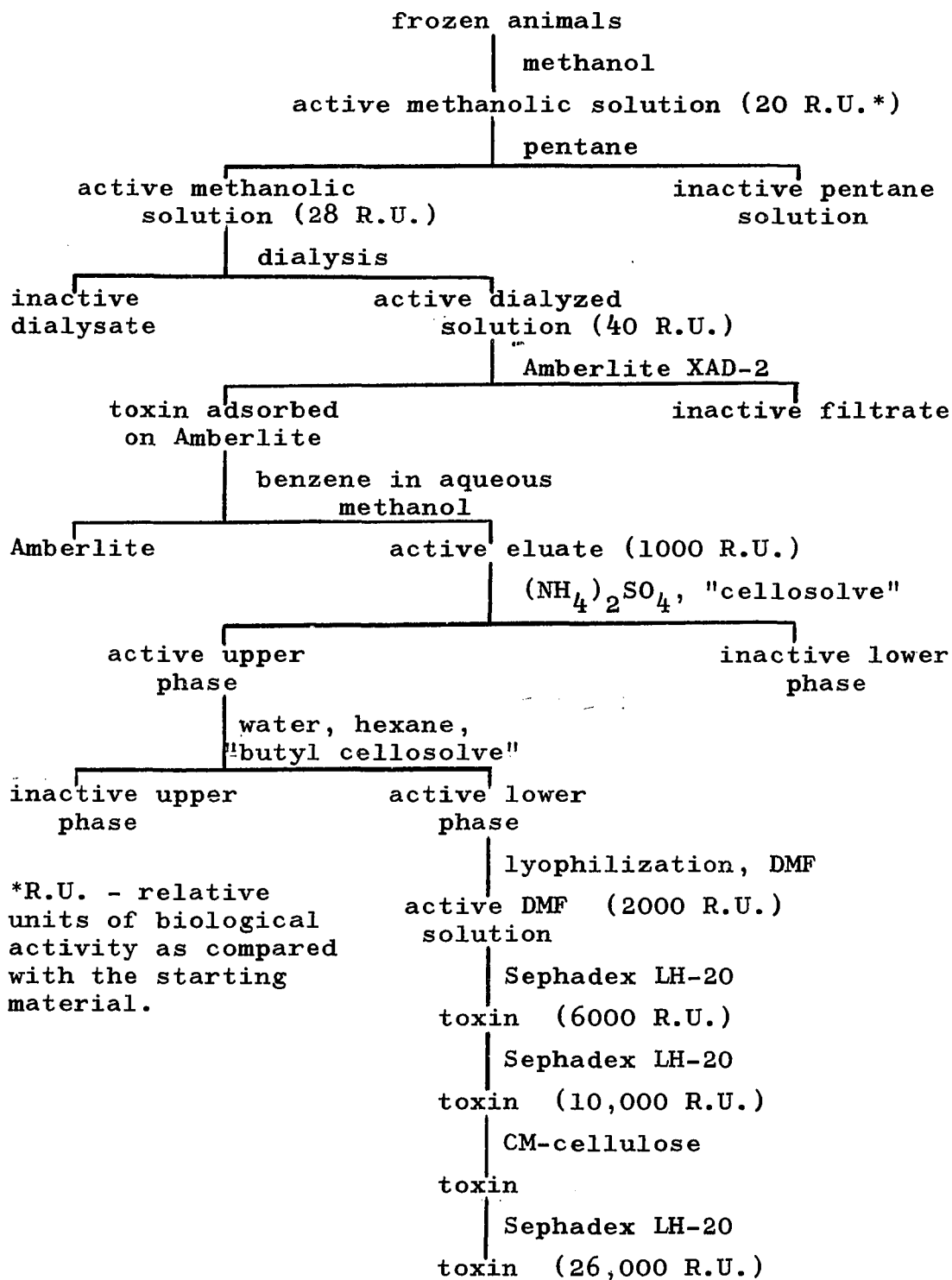
A final chromatography on Sephadex LH-20 was used to separate Palytoxin from the remaining natural impurities and from the sodium chloride which was introduced in the ion-exchange experiment. The final product showed a 26,000 fold increase in activity over that of the starting material.

Figure 1 shows an outline of the isolation procedure for palytoxin.

The final purified toxin from each of the two animals showed single spots with identical Rf values in two thin layer chromatography systems. Single spots of the same Rf were also obtained by ionophoresis in two different buffer systems. The ultraviolet absorption spectra were the same but there were differences in the infra-red absorption spectra in the region of 1525 cm.^{-1} .

FIGURE I

ISOLATION OF PALYTOXIN



DISCUSSION OF CHARACTERIZATION STUDIES

The elemental analysis of palytoxin suggested its empirical formula to be $C_{12}H_{21}O_5N$. Early studies with palytoxin from Palythoa mammillosa showed that the toxic material yielded a residue when burned. The residue indicated the presence of an inorganic moiety, but qualitative tests for both sulfate ion and phosphate ion were negative. A recent elemental analysis of palytoxin isolated from Palythoa caribaeorum and concentrated by ultrafiltration showed that the material yields no residue. Carbon, hydrogen, oxygen, and nitrogen total 99.79%.

The nitrogen content of 5.32% is much smaller than that found in proteins which average approximately 16% nitrogen. The large ratios of carbon and hydrogen to oxygen and nitrogen indicate the presence of a lipid moiety.

The nondialyzability of palytoxin and its behavior on dextran gels indicate that its molecular weight is approximately 5000. Ultrafiltration using a membrane with an exclusion limit of 10,000 molecular weight, however, showed that palytoxin would not pass the membrane and indicated that its molecular weight may be greater than

10,000. No sedimentation boundary was observed on ultracentrifugation. This observation argues for a molecular weight of less than 3000, although such behavior in the ultracentrifuge might be expected for a high molecular weight compound if a lipid moiety decreased the density of the molecule to make it buoyant in an aqueous medium.

In hydrolysis experiments with small amounts of palytoxin from Palythoa mammillosa the presence of cholesterol was indicated by thin layer chromatography and by gas chromatography. The results of our experiments lead us to consider that relatively small polar molecules with hydrophobic moieties might form micelles and behave as polymers in aqueous media. Such reasoning is supported by the facts that palytoxin forms "soapy" solutions and is surface active.

Gas chromatography of the ether soluble materials resulting from the hydrolysis showed several components. One had the same retention time as that of cholesterol. Since it has been shown (8) that Palythoa contains several 3- β -hydroxy sterols, we might speculate that some of the components are those of a sterol mixture, and that palytoxin is a mixture--a family of toxins which differ only in the nature of the sterol moiety. Such a family of closely related compounds would likely chromatograph on thin layers as a single spot as do the sterol mixtures from Palythoa and from some other marine coelenterates.

Thin-layer electrophoresis at pH 7 and at pH 9 showed that palytoxin is cationic.

The ultraviolet spectrum of palytoxin (Figure 2) shows peaks at 232 $m\mu$ ($E_{1cm.}^{1\%} = 127$) and 262 $m\mu$ ($E_{1cm.}^{1\%} = 72.6$). Counter-current distribution of palytoxin in a two-phase solvent system of water, "cellosolve," and $(NH_4)_2SO_4$ showed definitely that the toxicity corresponds with the compound which absorbs at 232 $m\mu$ and 262 $m\mu$.

Because pyrimidine and purine bases also show peaks around 260 $m\mu$ and because of the ratio of nitrogen to the other elements in palytoxin, the presence of a nitrogen base in palytoxin was considered a possibility. The ultraviolet absorption peaks of the pyrimidine and purine nucleosides, however, are not appreciably altered by acidic and basic media (13), while the ultraviolet spectra of palytoxin in acidic media and basic media (Figures 3 and 4) after 12 hours are drastically altered from that in neutral water solution as shown in Figure 5. After palytoxin stands for several hours at room temperature in 0.1 N HCl (pH 1) its ultraviolet absorption peak at 262 $m\mu$ diminishes from an absorbance of 0.52 in neutral solution to 0.08 while the peak at 232 $m\mu$ is not appreciably altered. After palytoxin stands for 12 hours at room temperature in 0.01 N NaOH (pH 12), however, its absorption peak at 262 $m\mu$ is diminished to an absorption shoulder and the peak at 232 $m\mu$ is obliterated although the solution in the basic

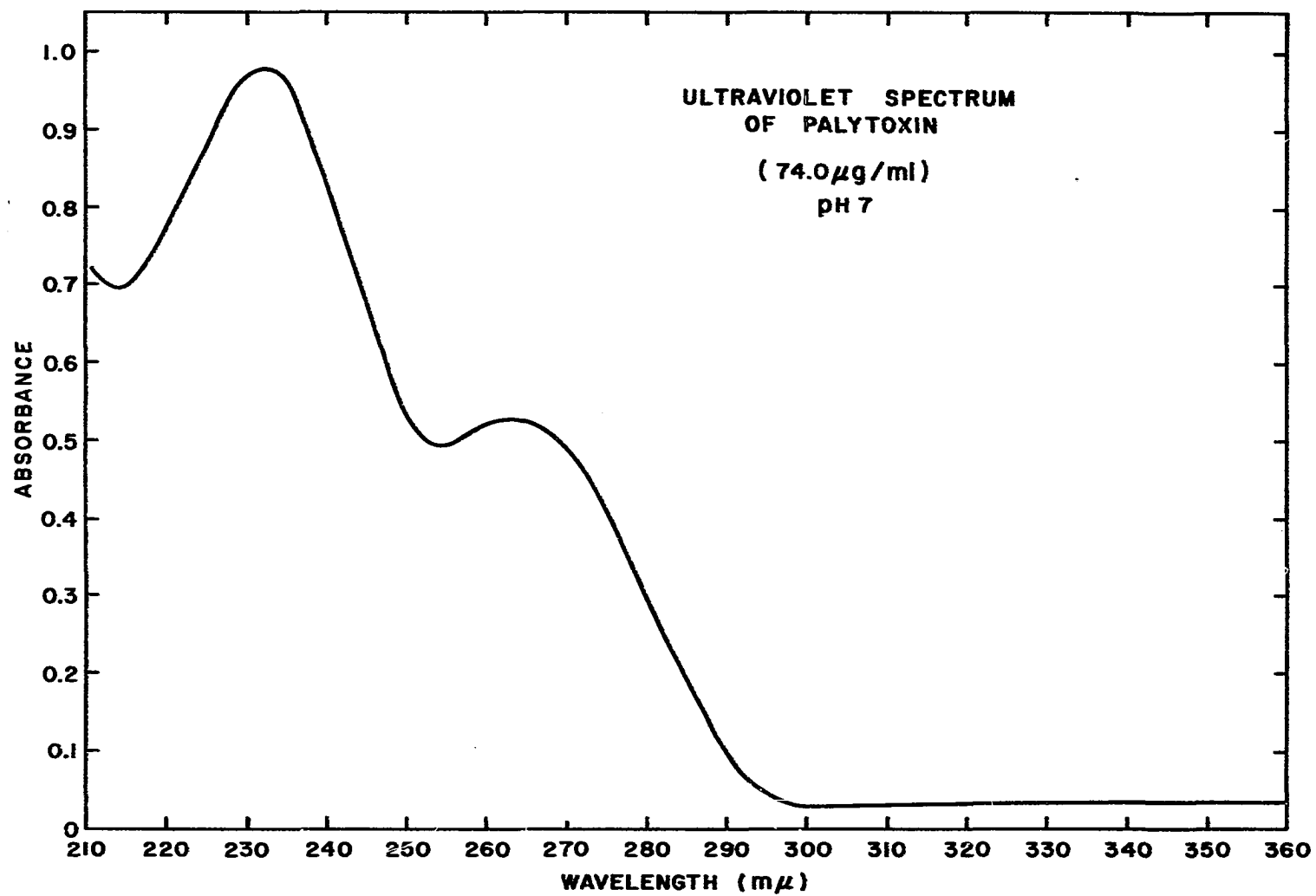


Figure 2

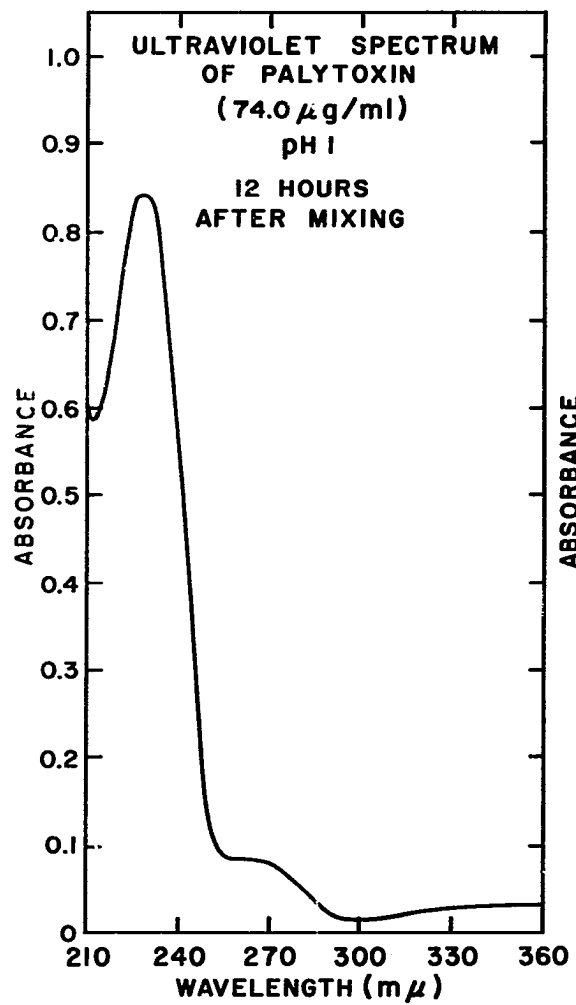


Figure 3

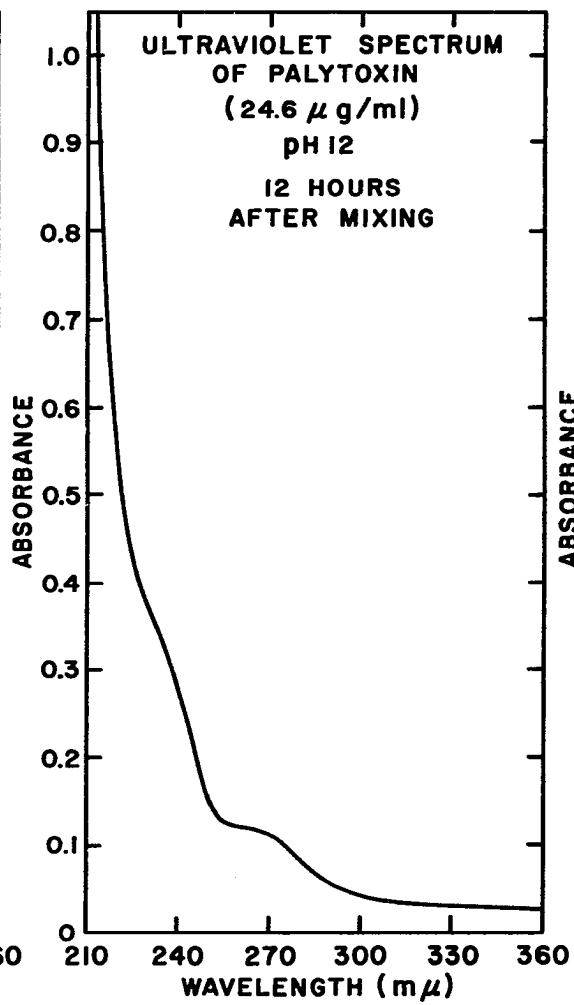


Figure 4

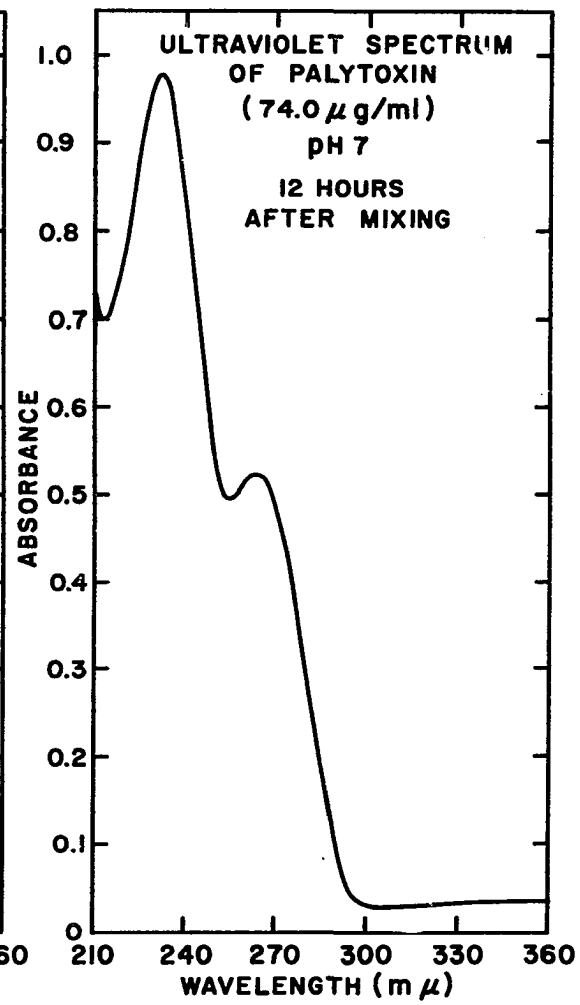


Figure 5

medium is "black" below 240 $m\mu$ at the original concentration. To discern the shape of the absorption spectrum in 0.01 N NaOH below 240 $m\mu$ the solution must be diluted as is shown by comparing Figure 4 with Figure 7. Figures 6 and 7 show the absorption spectra of palytoxin in basic and acidic media, respectively, immediately after mixing. The acidic medium does not immediately diminish the peak at 262 $m\mu$ as does the basic medium. The concentrations are the same as that used in preparing the spectrum shown in Figure 5.

Heteroannular dienes and simple compounds possessing an electron lone pair absorb below 260 $m\mu$ as do α, β - unsaturated ketones which absorb in the region of 235 $m\mu$ (14). Homocyclic and heterocyclic ring compounds with conjugated double bond systems such as the purine and pyrimidine bases may absorb in the region of 260 $m\mu$. By assuming a minimum molecular weight for palytoxin of 777, the smallest theoretical molecular weight which accommodates a cholesterol moiety, one can calculate a molar extinction coefficient of 5.64×10^3 in neutral solution for the peak at 262 $m\mu$. The theoretical molecular weight was calculated from the data of the elemental analysis. This extinction coefficient has a value of the same order of magnitude as those of the pyrimidine bases (15) as shown in Table I.

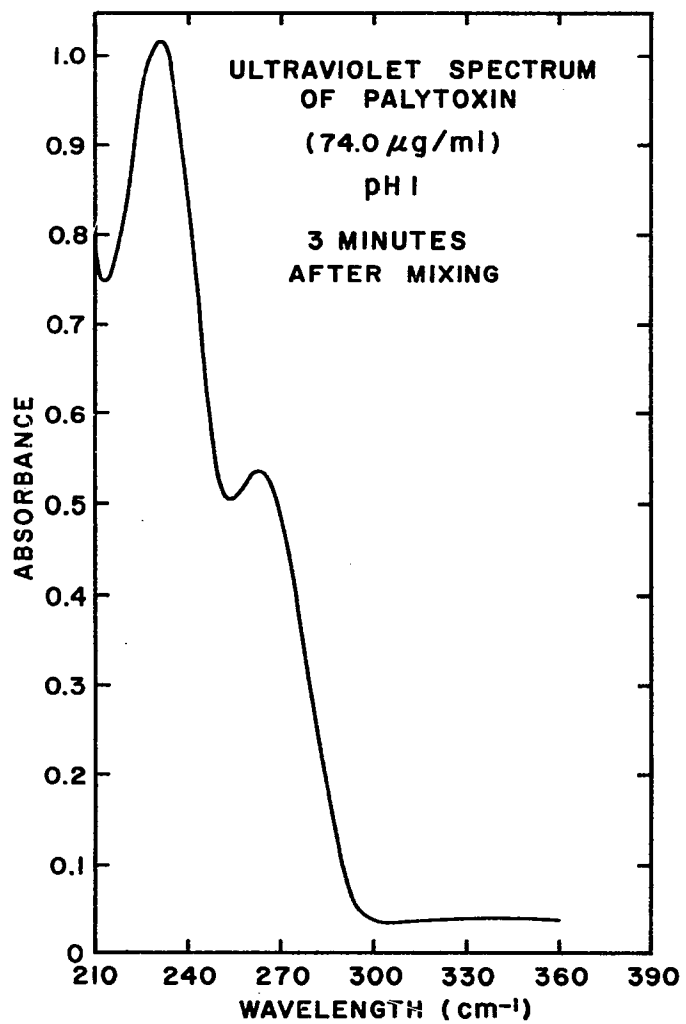


Figure 6

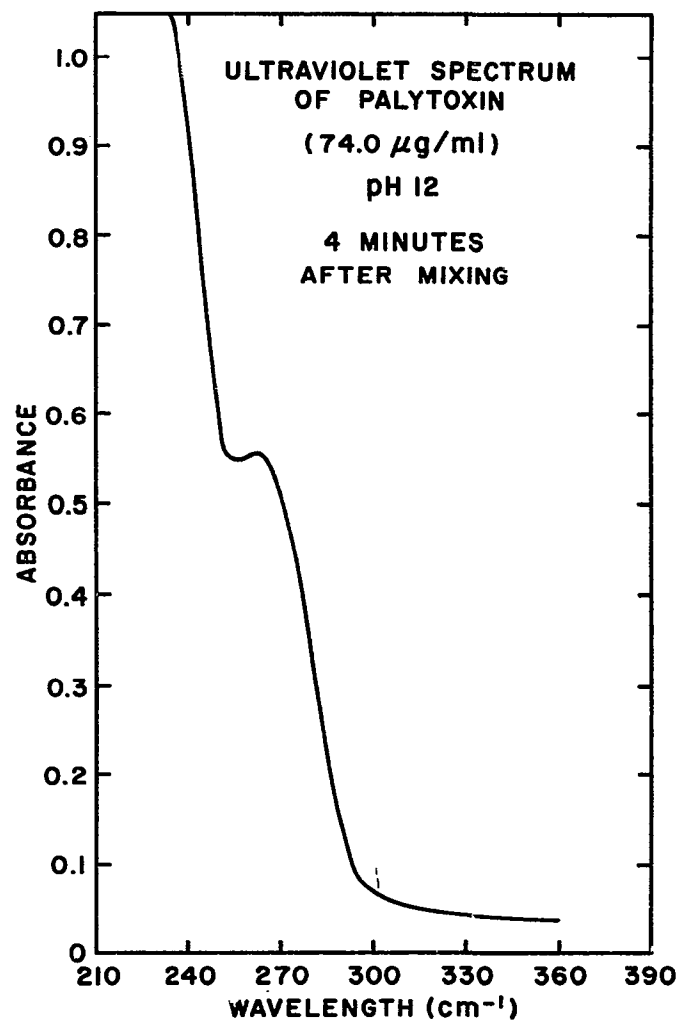


Figure 7

TABLE I

Nitrogen Base	Wavelength of Maximum Absorbance (m μ)	Molar Extinction Coefficient at pH 7 (X 10 ⁻³)
Adenine	260.5	13.35
Cytosine	267	6.13
Guanine	246	10.7
Hypoxanthine	250	10.6
Thymine	264.5	7.89
Uracil	260	8.2
Palytoxin	262	5.64*

*Based on the smallest theoretical molecular weight which accommodates a cholesterol moiety.

The ribonucleosides have larger extinction coefficients and the wavelengths of maximum absorbance are shifted slightly (15) from those of the free bases as can be seen in Table II. There are similarities between the ultraviolet absorption spectrum of palytoxin at pH 7 and those of the ribonucleosides but there are also marked differences between them in acidic and basic media.

TABLE II

Ribonucleoside	Wavelength of Maximum Absorbance (m μ)	Molar Extinction Coefficient at pH 7 (X 10 ⁻³)
Adenosine	259	15.4
Cytidine	271	9.1
Guanosine	252.5	13.6
Inosine	248.5	12.2
Uridine	262	10.1
Palytoxin	262	5.64*

*Based on the smallest theoretical molecular weight which accommodates a cholesterol moiety.

The infra-red spectra of palytoxin are shown in Figures 8 and 9. The differences in the spectra probably arise from impurities in the palytoxin from Palythoa mam- millosa which is not quite as active (24,000 R.U.) as the palytoxin from Palythoa caribaeorum (26,000 R.U.). They show strong broad absorption at 3400 cm^{-1} . Absorption in this region is attributed to the oxygen-hydrogen stretching vibrations of hydroxyl groups. Absorption in this region can also arise from nitrogen-hydrogen stretching vibrations (16). The absorption between 2800 cm^{-1} and 3000 cm^{-1} results from carbon-hydrogen stretching vibrations. The absorption at 1650 cm^{-1} may result from double-bonded carbon-nitrogen vibrations (17). Cytidine and uridine both show similar absorptions in this region (Figures 9 and 10) although the center of the absorption band for uridine is shifted to 1775 cm^{-1} . The broad band at 1050 cm^{-1} probably is the result of carbon-oxygen stretching vibrations and oxygen-hydrogen in-plane deformations of a primary or secondary alcohol, although there is usually an associated sharp band between 1260 cm^{-1} and 1350 cm^{-1} (17). The weak broad peak at 1515 cm^{-1} may arise from the deformation vibrations of the charged nitrogen function. The region of the spectrum between 1200 cm^{-1} and 1600 cm^{-1} shows absorption but no peaks. Figure 11 shows the spectrum of a mixture of the disodium salt of uridine-3'-(2')-phosphate, tetrahydrate and

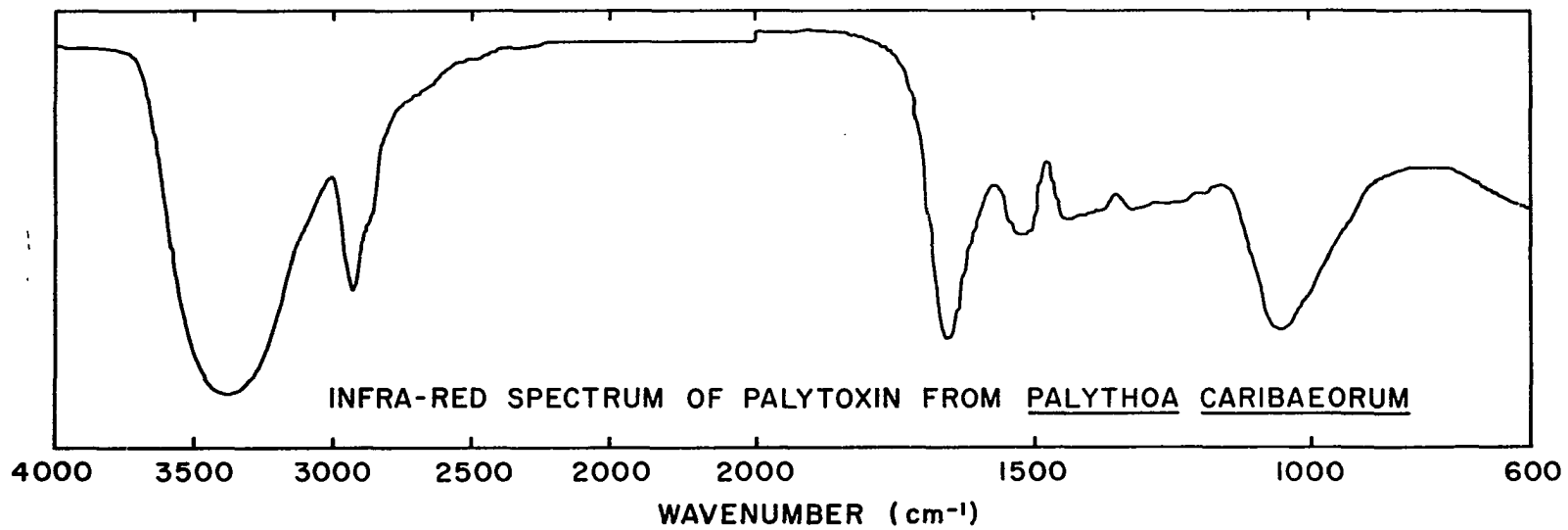


Figure 8

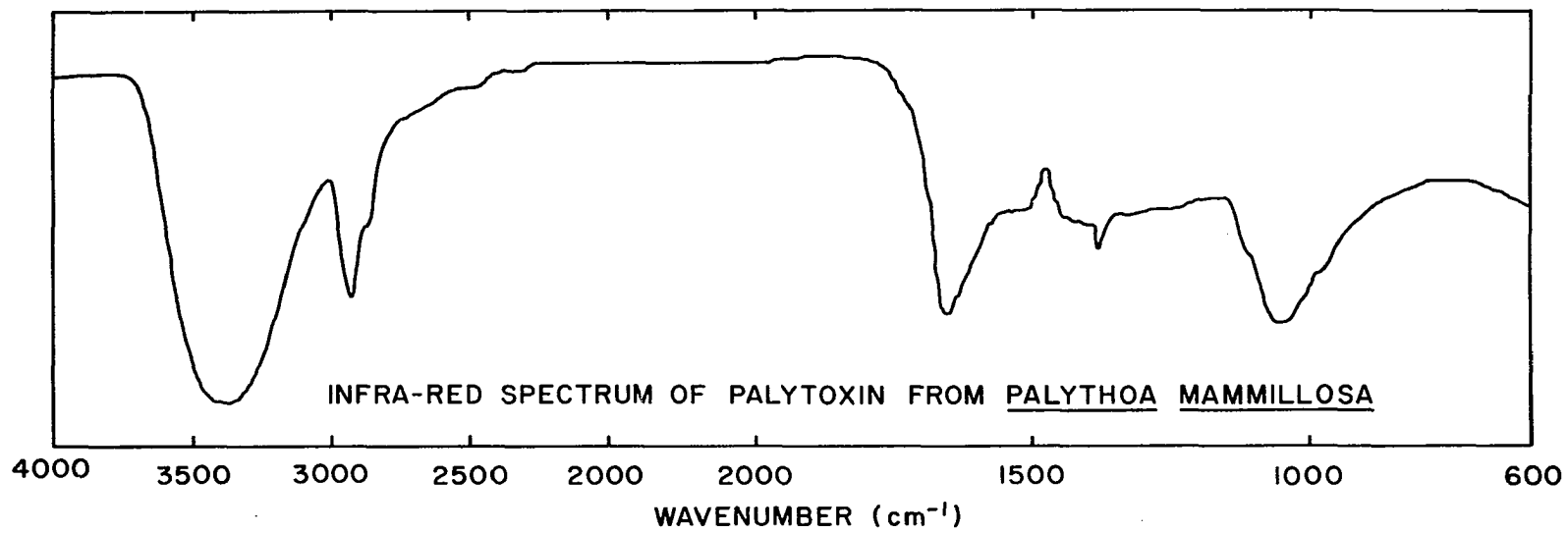


Figure 9

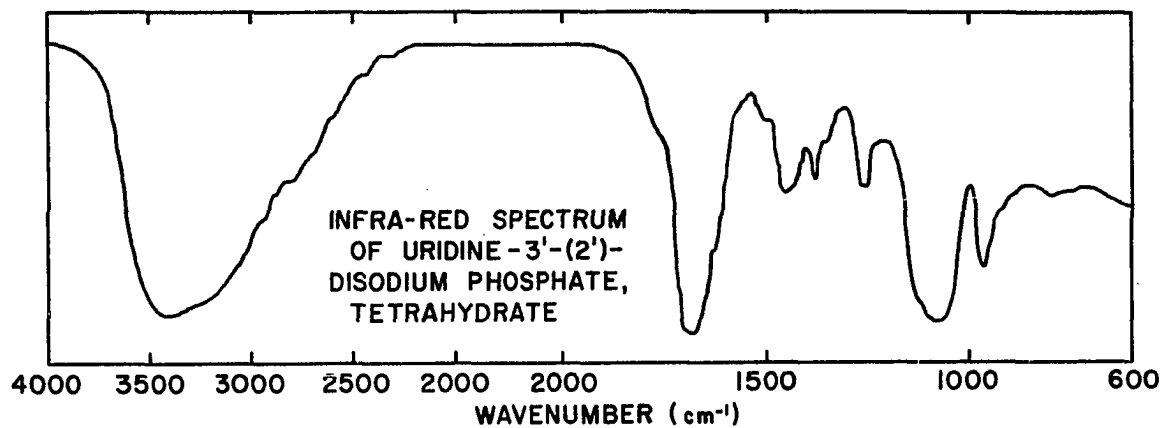


Figure 10

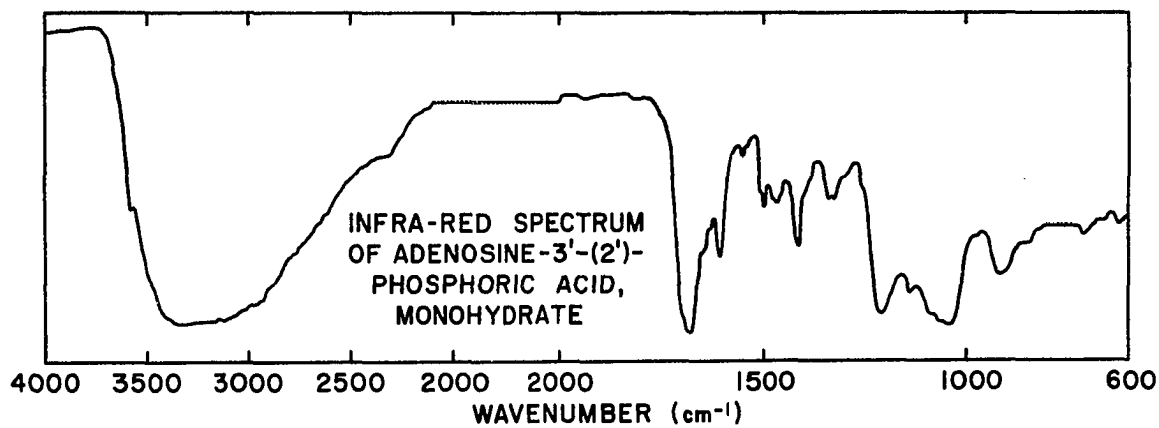


Figure 11

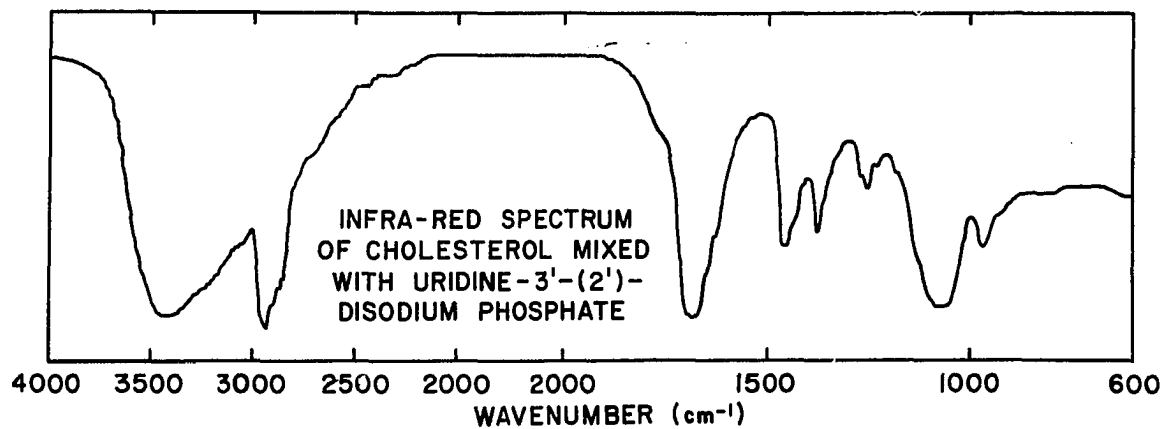


Figure 12

cholesterol. Except for the region from 1200 cm^{-1} to 1600 cm^{-1} it is similar to that of palytoxin. This may indicate the presence of similar residues in palytoxin. The infra-red data are at least not incompatible with the presence of steroidal and pyrimidine-like moieties.

Free pyrimidine nucleosides have been isolated from the marine sponge Cryptotethia crypta and identified by Bergmann and Feeney (18), Bergmann and Burke (19, 20) and Bergmann and Stempien (21). Bergmann's group identified the 3- β -D-arabofuranosides of thymine and uracil and 9- β -D-ribofuranosyl-2-methoxyadenine. It is interesting that the sugar moiety in two of these compounds is not the usual ribose or deoxyribose but is arabinose.

Palytoxin gives a negative Molisch test for carbohydrates but gives a positive reaction with Schiff's reagent after oxidation with periodate. The positive reaction with Schiff's reagent after oxidation indicates the presence of adjacent hydroxyl or amine groups. Cytidine, a pyrimidine nucleoside, which contains a carbohydrate moiety also does not give a definitely positive Molisch test. This is probably because the compound is difficult to hydrolyze unless the heterocyclic ring is reduced (12, 22, 23) although uridine, a similar compound, does give a positive Molisch test.

Thin-layer chromatography of the water-soluble products from the acidic hydrolysis of palytoxin indicated the

presence of trace amounts of xylose. The presence of only small amounts of xylose in the hydrolysis mixture is compatible with the presence of a pyrimidine nucleoside moiety because these nucleosides are difficult to hydrolyze.

Untreated palytoxin will restore the pink color of rosaniline when treated with Schiff's reagent. It does not give the purple color which is the response from dialdehydes, however. This does suggest the presence of an aldehyde function, although organic bases can also restore the pink color by reaction with the sulfurous acid of the Schiff's reagent (24). A negative test for the aldehyde function was obtained with Tollen's reagent.

On a thin layer of Avicel palytoxin gives a light lavender color reaction with ninhydrin reagent for amines and amino acids. Glucosamine and uridine also give a faint lavender color while glucose gives no color and the amino acids, arginine, histidine, lysine, phenylalanine, tyrosine, and valine give a purple color. In a similar experiment with ninhydrin-collidine reagent (25) for amines and amino acids, palytoxin gave no color, neither did glucose and uridine while the amino acids and glucosamine reacted to give a variety of colors. Palytoxin gave a negative color reaction with Morgan-Elson reagent (26) for amino sugars. These data suggest that palytoxin has no amino sugar residue.

Palytoxin does not melt but decomposes slowly above 200°C.

EXPERIMENTAL

All solvents were redistilled before use. Sephadex LH-20 (Pharmacia Fine Chemicals Inc.) was used for the gel filtrations. CM-cellulose C-50 (Pharmacia) was used for the ion-exchange experiments. Rohm and Haas Amberlite XAD-2 resin and Union Carbide dialysis tubing (1 7/8 in. S.S.D.C.) were used. Thin layer chromatography was done on 5 X 20 cm. and 20 X 20 cm. glass plates coated with the adsorbents named in the text.

Gas chromatographic analyses were performed on a F and M Research Chromatograph Model 810. The infra-red spectra were produced on a Beckman IR-8 spectrophotometer using potassium bromide pellets. The ultraviolet spectra were recorded on a Beckman DK-1 spectrophotometer in 1 cm. cells. For the counter-current distribution experiment the 120 tube steady state distribution machine of Quickfit and Quartz was used. Thin-layer electrophoresis studies were done with a 100 volts dc power source on 1 X 3 in. Phoroslides separation-support strips (Millipore Corp.) in a Phoroslides electrophoresis chamber. A Diaflo Model 50 (Amicon Corp.) ultrafiltration cell and ultrafilters from the same company were employed for the filtration experiments.

Elemental analyses were done by the Alfred Bernhardt Laboratories, Mulheim, Germany.

Part I. Isolation of Palytoxin

Methanolic Extraction of Palytoxin. Pieces of frozen animals approximately 10 X 10 X 2.5 cm. were placed in 8 liter percolators and allowed to stand overnight in methanol. The next day the liquid was drained from the bottom of the percolators. Each batch of animal material was extracted a second time with fresh methanol and the two extracts were combined.

Removal of Lipids from the Methanolic Extracts. The toxic methanolic solutions were extracted with pentane in a 4 liter liquid-liquid extractor (Ciereszko, 27) until only a little color could be extracted into the pentane layer.

Dialysis of Toxic Extracts. The methanol was distilled from the extracts on a flash evaporator at room temperature after which the remaining aqueous solution was placed in cellulose tubing and dialyzed for 12 hours against running tap water.

Concentration of Palytoxin with Amberlite XAD-2 Resin. The active aqueous solutions were passed through columns of Amberlite XAD-2 which had been pretreated by soaking for several hours in 2-ethoxyethanol. The resin was filtered just before use. A 5 X 70 cm. column of Amberlite was used for 4 liters of extract. The extracts were put

through the columns 2 or 3 times to insure that most of the toxin had adhered to the Amberlite. The columns were washed with distilled water until little color was being removed. The toxin was eluted with a solution of water, methanol and benzene (400:50:13.5).

In an alternate method 300 to 500 ml. of active solution were stirred with 250 g. of Amberlite for several hours after which the resin was filtered, washed with water, and soaked for 2 hours in a solution of water, methanol and benzene (400:50:13.5). The resin was filtered; the filtrate contained the toxin.

Distribution of Palytoxin Between Two Liquid Phases.

The methanol and benzene were removed from the toxic solutions by distillation on a flash evaporator at room temperature. In the resulting aqueous solution was dissolved 15 g. ammonium sulfate per 55 ml. After the addition of 2-ethoxyethanol (32 ml./15 g. ammonium sulfate), the mixture was shaken vigorously in a separatory funnel. On standing for 15 minutes the solution separated into two layers. The lower layer was withdrawn and discarded. The upper phase had the following materials added: water (14 ml./32 ml. of toxic solution), hexane (23 ml./32 ml.), and 2-butoxyethanol (1 ml./1ml.). Two phases resulted and the lower aqueous phase was removed and refrigerated. The upper phase was washed with a small volume of water which was withdrawn and combined with the other aqueous

layer. The toxic aqueous solution was lyophilized and the resulting residue was triturated with dimethylformamide. The insoluble ammonium sulfate was removed by filtration. The dimethylformamide was distilled at room temperature in a flash evaporator which was fitted with a dry ice-acetone condenser.

Gel Filtration of Palytoxin. The toxic material was dissolved in a minimum of methanol and water (1:1 by volume) and chromatographed in the same solvent on a 5 X 90 cm. column of Sephadex LH-20. The flow rate was adjusted to 1ml./min. and fractions were collected each hour. Each fraction was subjected to bioassay and the toxic fractions were combined and lyophilized.

Palytoxin was only slightly retarded by the molecular sieves and began coming off the column about 50 ml. after the void volume of 500 ml. The void volume was determined by chromatography of blue dextran which has a molecular weight of 2,000,000.

The succeeding gel filtrations on Sephadex LH-20 were done in a similar manner.

Ion-Exchange Chromatography of Palytoxin. A solution of palytoxin in water was passed through a 2.5 X 90 cm. column of CM-cellulose G-50 (sodium form). Since palytoxin is cationic it was retained on the column while some of the contaminating pigments were washed through. The column was thoroughly washed with water after which the

toxic material was eluted with 2 M NaCl. The toxic fractions were combined and lyophilized.

Part II. Characterization Studies of Palytoxin

Thin-Layer Chromatography of Palytoxin. Ten micrograms of palytoxin were spotted on an Avicel Plate. The chromatogram was developed in n-propanol-ethyl acetate-water (7:1:4) and visualized by spraying with anisaldehyde reagent (28). After the plate was heated for 5 minutes at 95°C. a single spot appeared ($R_f = 0.66$). A single spot was also seen if the plate was visualized by spraying with trichloroacetic acid in chloroform (29).

Single spots were observed on Avicel with R_f values of 0.55 when the chromatograms were developed in dimethylformamide-ethyl acetate-water (15:16:10). The same visualization procedures as used above were employed.

Chromatography of palytoxin on silica gel G and Avicel in n-amyl alcohol-pyridine-water (7:7:6) produced homogeneous trailing from the origin to an R_f of 0.6. On plates of Kiesselgur G in the same solvent system, palytoxin appeared as an homogeneous streak from the solvent front to R_f 0.75. Attempts to find effective solvent systems other than the two above for chromatography of palytoxin on Avicel were unsuccessful. Trailing on thin-layer chromatograms in many solvents might be expected from large ionic molecules which have nonpolar moieties.

Thin-Layer Electrophoresis of Palytoxin. Approximately 5 $\mu\text{g.}$ of palytoxin were spotted in the center of a 3 inch cellulose acetate strip which had been soaked in Tris buffer (pH = 1.0, I = 0.05). The strip was placed in the electrophoresis chamber which contained the same buffer solution. A 100 volt electrical field was applied for 20 minutes after which the strip was dried and visualized with anisaldehyde reagent (28). A single spot which was observed a few millimeters from the center of the strip showed that palytoxin had migrated toward the cathode.

Similar results were obtained using the same voltage and a neutral $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer (I = 0.1).

Counter-Current Distribution of Palytoxin. One hundred and fifty milligrams of partially purified palytoxin (6000 R.U.) were dissolved in 20 ml. of the upper phase of a mixture composed of 15% $(\text{NH}_4)_2\text{SO}_4$, 30% "cellosolve," and 55% water. The solution was divided in two 10 ml. volumes and put in the first two tubes of the steady-state distribution machine with an equal volume of the lower phase. The remaining 118 tubes contained 10 ml. of each phase of the solvent mixture. The machine was programmed for 181 upper phase transfers and 179 lower phase transfers. An agitation period of 3 minutes and settling period of 3.5 minutes were used. After the 360 cycles were completed, the upper phase was collected in 120 fractions and the lower phase in 55 fractions. - Ten

per cent of the original activity was detected in upper phase fractions 115 through 120 and 1 through 9. These were the only significantly toxic fractions and the only fractions which absorbed in the ultraviolet. The most toxic fractions showed two peaks in the ultraviolet at 262 μ and 232 μ . Only 1% of the palytoxin could be detected in the lower phase fractions which were opposite the toxic upper phase fractions.

Although the mild acidity of the solvent system destroyed most of the biological activity of palytoxin, the experiment showed that the ultraviolet peaks at 232 μ and 262 μ were those of palytoxin. Earlier studies with a small hand-operated machines indicated that this might not be true.

Comparison of Spot Tests on Palytoxin and Other Compounds. Two μ g. of palytoxin were spotted on each of three Avicel plates adjacent to 1 μ g. of each of the following: glucose, glucosamine, and uridine and adjacent to 2 μ g. of each of the amino acids, arginine, histidine, lysine, phenylalanine, tyrosine and valine. One plate was sprayed with a 3% solution of ninhydrin in n-propanol, one with ninhydrin-collidine reagent (25) and one with Morgan-Elson reagent (26). Table III shows the colors which developed.

TABLE III

Color Reactions with Three Spray Reagents

<u>Spray Reagent</u>	Ninhydrin	Ninhydrin-Collidine	Morgan-Elson
Compound			
arginine	purple	grayish-red	yellow
glucose	none	none	none
glucosamine	lavender	pink	yellow
histidine	purple	gray	
lysine	purple	pink	none
phenylalanine	purple	green	yellow
tyrosine	dark lavender	brown	orange
uridine	light lavender	none	none
valine	purple	violet	pale yellow
palytoxin	light lavender	none	none

It can be seen that uridine and palytoxin give the same responses to each of the spray reagents and that they give no color response with ninhydrin-collidine reagent for the detection of amines and amino acids or with Morgan-Elson reagent for detection of amino sugars and only a weak response to ninhydrin reagent for amino acids.

Ultrafiltration of Solutions of Palytoxin. Twenty milliliters of a solution of palytoxin were placed in the Diaflo filtration cell. The cell was fitted with a UM-2 ultrafilter (1000 molecular weight cutoff). The cell was sealed and a pressure of 50 p.s.i. was applied with

compressed nitrogen. The solution was stirred while it filtered at a rate of 1 ml./min. All but 2 ml. of the solution was filtered. None of the palytoxin passed the membrane.

The experiment was repeated with a UM-1 ultrafilter (10,000 molecular weight cutoff) and again none of the palytoxin passed the membrane.

The experiments indicated that palytoxin may have a molecular weight greater than 10,000.

Test for Phosphate Ion in Palytoxin. Ten μ l. of the concentrated hydrolysate of palytoxin (p. 37) were spotted on an Avicel plate and sprayed with a solution prepared by combining 3 ml. of a 5% ammonium molybdate solution, 7 ml. of 5 N HCl and 90 ml. of acetone. After the plate was thoroughly dry it was sprayed with a freshly prepared reducing solution (30). The blue color of a positive test did not appear on the plate.

Since carbon-phosphorus bonds are not hydrolyzed by dilute acid and because the nature of the residue of palytoxin was unknown, it seemed advisable to check for the presence of a phosphonic acid moiety by the method below.

The method for total phosphorus determination of Fleischer, Southworth, Hodecker, and Tuckerman (31) was used as a qualitative test. In a filter paper 1.7 mg. of palytoxin was burned in oxygen in a Schöniger combustion flask containing 25 ml. of 4 N H_2SO_4 . After combustion

the acidic solution was boiled for 35 min. then diluted to 50 ml. To 2.5 ml. of this solution was added 2 ml. of a colorimetric reagent prepared by adding 4 g. of ascorbic acid to a solution of 20 ml. 1% bismuth subcarbonate in 7 N H_2SO_4 and 20 ml. 2.5% ammonium molybdate tetrahydrate. The test solution was diluted to 10 ml. and compared with a blank solution in a Beckman DU spectrophotometer. The solution did not absorb at 660 $m\mu$, the wavelength at which phosphomolybdate ion absorbs. The negative test for phosphorus was confirmed on a second sample of palytoxin.

Treatment of 0.8 mg uridine-2'-(3')-phosphoric acid (disodium salt) in a similar manner did produce a solution which absorbed at 660 $m\mu$ as did standard solutions of NaH_2PO_4 .

Schiff's Test on Palytoxin. Fifty micrograms of palytoxin, glucose, uridine and potato starch were spotted side by side on an Avicel plate. The plate was sprayed with Schiff's reagent (32). A pink color appeared where palytoxin was spotted. This suggested the presence of an aldehyde function although other functional groups can restore the pink color of Schiff's reagent (24).

In a similar experiment in which the plate was first sprayed with a 1% aqueous solution of sodium metaperiodate and dried at room temperature, dark purple spots appeared where palytoxin and uridine were spotted. The purple color rather than the pink is the positive test for dialdehydes.

The positive test after oxidation indicated that the structure of palytoxin accommodates adjacent hydroxyl groups which can be oxidized to aldehyde groups with periodate.

Aldehyde Test on Palytoxin with Tollens' Reagent. In order to determine whether palytoxin itself has an aldehyde functional group, the following test (33) was done. Fifteen mm. of Tollens' silver nitrate reagent and 5 mm. of a 2% solution of palytoxin were drawn into one end of a capillary of 1 mm. bore. The other end was sealed and the contents were centrifuged back and forth several times and then allowed to stand. A black precipitate of metallic silver or a mirror on the glass did not appear. This showed that palytoxin does not have reducing properties and it therefore is not an aldehyde.

A similar experiment with a 1% solution of anisaldehyde produced a silver mirror inside the capillary tube.

Molisch Test for Carbohydrate on Palytoxin. Two drops of a 1% solution of palytoxin was put into a narrow 3 inch semimicro centrifuge tube to which 1 drop of a 5% solution of α -naphthol in 95% alcohol was added. The contents of the tube were mixed thoroughly. Two drops of concentrated sulfuric acid were layered at the bottom of the tube. No red or pink color developed as it did in control tests with glucose and uridine. Even on a larger scale test, however, cytidine gave only a very faint pink color and in some

repeat tests gave no color at all. Repeating the test on palytoxin gave no definite positive reaction.

Hydrolysis of Palytoxin. Three ml. of 1 N HCl containing 3.5 mg. of palytoxin from Palythoa mammillosa were refluxed for 3 hours. After the solution had cooled it was extracted with ether in a 10 ml. liquid-liquid extractor, lyophilized, and taken up in 0.5 ml. of water. This solution and others prepared in a similar manner were used for the sulfate ion test and for the thin-layer chromatography described below.

The palytoxin (26,000 R.U.) from Palythoa caribaeorum was available only for preliminary experiments.

Test for Sulfate Ion (34) in the Hydrolysate of Palytoxin. Three drops of the concentrated hydrolysate of palytoxin (see above) were mixed on a spot plate with 1 drop of saturated potassium permanganate solution. One drop of the mixture was placed on a paper strip (Carl Schleicher and Schuell Co.) which had been impregnated with barium chloride and dried for 7 min. at 75°C. The excess barium chloride was removed by bathing the paper in a beaker of distilled water followed by rinsing under the tap. The paper was placed in 1 N oxalic acid which removed any unused KMnO_4 as well as any MnO_2 which precipitated in the paper through oxidation of the cellulose. Since no violet or pink ring remained on the paper, the test for sulfate ion was negative. A positive test

depends on the coprecipitation of permanganate with barium sulfate to form a stable violet color in the paper. The method is capable of detecting as little as 2.5 μg . of sulfuric acid.

In a similar test on a 1% solution of sodium sulfate, a violet ring remained in the filter paper.

Thin-Layer Chromatography of the Hydrolysate of Palytoxin. Fifteen microliters of the concentrated hydrolysate (p. 37) were spotted on an Avicel plate which had been spotted with 5 μg . of each of the following sugars: mannose, glucose, galactose, rhamnose, fructose, ribose, arabinose, xylose and fucose. The plate was developed for a distance of 15 cm. in n-propanol-ethyl acetate-water (7;1;2), dried, sprayed with aniline-oxalic acid reagent (35) and heated for 30 minutes at 95°C. The reducing pentoses (ribose, arabinose, and xylose) and one component of the hydrolysate gave pink spots while the other sugars with the exception of the keto sugars gave brown spots. The keto sugars gave no color at all. According to Partridge (35) the pink color is specific for reducing pentoses. The faint pink spot of the hydrolysate had the same Rf (0.35) as that of D-(+)-xylose. The chromatogram showed several other components of the hydrolysate and they all had Rf values less than 0.35. None gave a color reaction with the spray reagent which was similar to those given by the sugars. The major spot of the hydrolysate appeared white.

on the tan background. It had an Rf of 0.29 and appeared dark purple against a greenish tan background under ultraviolet light.

In another experiment formic acid-methylethyl ketone-t-butyl alcohol-water (3:6:8:3) was used as the solvent system and again the Rf of xylose and that of the pink spot of the hydrolysate were the same.

In a third experiment 15 μ l. of the hydrolysate were spotted at the same place as 0.5 μ g. of xylose in the corner of a 20 X 20 cm. Avicel plate. The plate was developed 12.3 cm. in n-propanol-ethyl acetate-water (7:1:2.5), dried and developed 13 cm. in the same direction and in the same solvent. After it was dried again the plate was turned 90° and developed 15 cm. in formic acid-methyl ethyl ketone-t-butyl alcohol-water (3:6:8:3). The plate was dried and visualized with aniline-oxalic acid reagent. A single pink spot on the plate indicated that xylose is possibly a product of the hydrolysis of palytoxin.

Chromatography of the Ether-Soluble Portion of the Hydrolysate of Palytoxin. The ether-soluble material from the acid hydrolysate of palytoxin (p. 37) was dissolved in 0.5 ml. of benzene and 30 μ l. of the solution were spotted on a thin-layer plate of silica gel H adjacent to a 4 μ g. spot of cholesterol. The plate was developed in benzene-ethylacetate (60:20), dried and sprayed with 5% concentrated sulfuric acid in 95% ethanol. Cholesterol

showed an Rf of 0.42 and the hydrolysate gave a corresponding spot. The hydrolysate also showed spots near the origin and a major spot with an Rf of 0.71.

Some of the same material was chromatographed in the gaseous phase on a 6 ft. X 1/8 in. column of Gas Chrom Q (30-100 mesh) which was coated with SE-30 (3% concentration) at an oven temperature of 230°C. and an injection port temperature of 270°C. The detector temperature was 310°C. and the flow rate of the helium carrier gas was 50 ml./min. Eight peaks were seen in the chromatogram. The major peak had a retention time of 37.0 min. Under the same chromatographic conditions, cholesterol had a retention time of 36.8 min. which is in good agreement with that of the major peak of the hydrolysate.

Similar results were obtained with the ether extractables from a basic hydrolysis in which 3.4 mg. of palytoxin were heated in a boiling water bath for 100 minutes in 1 ml. of 0.1 N sodium hydroxide. In this case 12 peaks were seen in the chromatogram and the four major ones had the following retention times: 34.4 min., 46.7 min., 51.6 min., and 92.6 min. When cholesterol was chromatographed immediately afterward it had a retention time of 34.8 min., which again is in close agreement with that of one of the hydrolytic products. The difference in the retention time of cholesterol in the two experiments was probably due to differences in the flow rate of the helium

since the experiments were not done on the same day.

The chromatography data indicated that cholesterol may be one of the hydrolytic products.

Liebermann-Burchard Test for Sterols on the Hydrolysate of Palytoxin. The ether extractables from the acidic hydrolysis (p. 37) of palytoxin from Palythoa mammosa gave a positive Liebermann-Burchard test (36) for sterols, although the colors were not the same as those given by cholesterol. One drop of hydrolysate in chloroform was mixed with 1 drop of acetic anhydride on a spot plate. One drop of concentrated sulfuric acid was carefully added. The chloroform "crept" on the surface of the plate and was green in color while the remaining solution was orange. When the test was done on cholesterol in a similar manner the colors were blue and pink respectively.

SUMMARY

Palytoxin has been isolated and found to be an unstable, cationic compound which has an apparent molecular weight of several thousand. It is nonproteinaceous and its suggested empirical formula is $C_{12}H_{21}O_5N$. The presence of cholesterol and xylose in the hydrolysate of palytoxin has been indicated by comparative chromatography. The ultraviolet and infra-red spectra and the elemental ratio suggest the possibility of a pyrimidine or purine moiety. The smallest molecular formula which accommodates these features is $C_{36}H_{63}O_{15}N_3$. The three nitrogen atoms per molecule would allow a pyrimidine ring as well as an amine group which would explain the cationic nature of palytoxin.

If xylose is present in the hydrolysate, it is possible that it is an artifact produced by the decarboxylation of D-glucuronic acid or L-iduronic acid under acidic hydrolytic conditions as shown by Mann and Tollens (37). In this case cholesterol might be attached by an ester bond to the rest of the palytoxin molecule. Such a linkage would account for the loss of biological activity in both basic and acidic media since esters are hydrolyzed in both. The lack of a carbonyl absorption in the

infra-red, however, argues against this possibility.

The characterization studies are preliminary at this stage but they suggest several lines of approach for further chemical investigations of palytoxin when larger amounts of purified material are available.

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