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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

INDOLE COMPOUNDS FROM MARINE COELENTERATES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

DOCTOR OF PHILOSOPHY

BY

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1969

INDOLE COMPOUNDS FROM MARINE COELENTERATES

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INDOLE COMPOUNDS OF MARINE COELENTERATES

INTRODUCTION

The indoles of coelenterates have received very little attention though indole compounds have been very extensively investigated from medical and mammalian biochemical viewpoints. What attention coelenterate indoles have received has been directed specifically towards serotonin. This dissertation is a step towards remedying this situation.

Phillips stated that the discharge of isolated nematocysts of Metridium senile probably contained serotonin and possibly N,N-dimethylserotonin (bufotenine) (1). Mathias, et al, using biological assay, found no serotonin in Metridium or in Anemonia sulcata (2,3). They did find less than 2.0 μg/gm of serotonin in freeze-dried Physalia sp. (Portuguese Man-of-War), about 5 μg/gm in Actinia equina (an anemone), and about 10 μg/gm in the tentacles, 30 μg/gm in the body wall, and 600 μg/gm in the coelomic cavity tissues of Calliactis parasitica. Welsh reexamined the data for Metridium using chemical rather than biological assay and found serotonin at levels of 0.24-0.47 μg/gm fresh tissue in the tentacles, 0.02-0.09 μg/gm in the body wall, and 0.60-1.30 μg/gm in the gut tissues (4). He also found 0.74-1.3 μg/gm

in Sagartia luciae, (an anemone), 1.5 μg/gm in Hydra oligactis, and possibly bufotenine in Physalia sp. Kline and Weissbach found that 5-hydroxyindoleamines were released from Hydra littoralis by electrical stimulation (5). Histochemical studies by Wood and Lentz localized serotonin in the nerve endings of a Hydra, and in tentacle cells, peristome, pharynx, and especially the nematocysts of Metridium senile (6). Finally Blanquet has found no serotonin in isolated, undischarged nematocysts from Aiptasia pallida, though he states it is possible that it might have leaked from the nematocysts during washing procedures (7).

In summary, serotonin has not appeared consistently when looked for, and the amounts present from species to species and tissue to tissue are variable. Except for two mentions of bufotenine, no other indoles have been observed in coelenterates.

CHAPTER I

MATERIALS AND METHODS

Materials

Animals

The Zoanthids *Palythoa caribbeorum* and *P. mammillosa* for isolation of individual indole compounds were collected at Port Royal, Jamaica, in August, 1966, and frozen. They were stored in the freezer at -25°C. until used. No apparent losses of indole content occurred over a period of two years. The *Palythoa* sp. used for quantitation were obtained at Port Royal, Jamaica in July, 1968, and frozen. They were also stored at -25°C.

Specimens for the indole survey were alcohol preserved (*Palythoa* sp. and other Zoanthids and Anemones), dried whole (Gorgonians and some Alcyonaceans), or dried and ground (Alcyonaceans and one *Metridium senile*). The Alcyonaceans were obtained at Eniwetok atoll using facilities of the Eniwetok Marine Biological Laboratory. The deep water Gorgonians were obtained by Dr. David H. Attaway in approximately 60 feet of water at Discovery Bay, Jamaica. The other Gorgonians were obtained by Dr. A. J. Weinheimer's

group in the vicinity of Miami, Florida. Anemones and Zoanthids came from Eniwetok; Puerto Peñasco, Mexico; Friday Harbor, Washington; and Port Royal, Jamaica.

Chemicals

Solvents were analytical grade reagents redistilled prior to use with the exceptions of ethanol, USP, and ether, which were not redistilled, and hexane which was not analytical grade. For the chromatograms for quantitative determinations, the butanol was redistilled from sodium hydroxide pellets, and the isopropanol twice redistilled. Acetone was distilled from potassium permanganate and dried over sodium sulfate. Methylethylketone was distilled and dried over sodium sulfate.

Fisher reagent grade 1-nitroso-2-naphthol was purified by chromatography on silicic acid in benzene.

Ehrlich's aldehyde (p-dimethylaminobenzaldehyde) was obtained from Eastman.

Ascorbic acid was obtained from Matheson, Coleman, and Bell.

Tryptophane and serotonin creatinine sulfate were obtained from Nutritional Biochemicals Co. and tryptamine obtained from Mann Research Laboratories, Inc. The serotonin-O-sulfate ester was synthesized from serotonin creatine sulfate from Pierce Chemical Corp. and practical grade chlorosulfonic acid from Eastman Chemical Co., by the procedure given below.

All chromatographies were done on Avicel microcrystalline cellulose from American Viscose Co., technical grade for columns and superfine for thin-layer and preparative plates.

Methods

The serotonin-O-sulfate ester was synthesized according to the procedure given by Kishimoto, et al, with some modefications (8). The procedure used was to dissolve 2 gm serotonin creatinine sulfate in 10 ml concentrated sulfuric acid cooled in an ice bath. Chlorosulfonic acid was added drop by drop until foaming ceased upon further addition. Hydrogen chloride was removed by applying a vacuum from a water aspirator for 15 minutes. The reaction mixture was then poured onto 100 ml frozen distilled water and allowed to melt completely. The solution was neutralized with barium carbonate and filtered. The filtrate was passed through Amberlite IR-120 (a sulfonic acid ion-exchange resin) in acid form, neutralized by adding Amberlite IR-45 (a weak base ion-exchange resin) to pH 4-5, and then concentrated to about 15 ml on a rotary evaporator with a Dewar condenser cooled by dry-ice and acetone mixture. The solution became brownish colored from degradation products and from the IR-45. This color was not completely removed by chromatography on Avicel using methanol, nor by precipitation with acetone which precipitated a brownish powder that appeared by infrared

absorption spectrography to be a mixture of serotonin, the sulfate ester, and degradation products. The acetone precipitated material in a potassium bromide pellet from the spectrography was redissolved in water, concentrated along with the acetone supernatant, rechromatographed in methanol on Avicel, and saved as a frozen aqueous solution for ultraviolet absorption studies and spectrophotofluorimetry. An earlier tenth-scale synthesis product (very brown and hydroscopic) was used for comparison purposes in chromatography. The low yields of impure material that I had (approximately 1-2 mg of the tenth-scale product and 2-4 mg of acetone precipitated powder, which is less than 0.5 per cent of theory) were also obtained by the Japanese workers who had a 3 per cent yield at best and state that they had several failures in this synthesis and that brown colored products formed.

For chromatography the following solvents were used: 1) butanol mixture composed of n-butanol, acetic acid, and water (4:1:1), 2) isopropanol mixture composed of isopropanol, concentrated ammonia, and water (8:1:1), and 3) methylethylketone mixture composed of methylethylketone, acetone, formic acid, and water (40:2:1:6). The first two solvent systems have received considerable use for indoles and are quite useful for the polar indoles (9,10,11,12,13). They appear to make separations on the basis of strength of adsorption to cellulose under different pH conditions.

The third solvent system was developed by L. Reio (14), and in my hands was not nearly as useful because the water content was exceedingly critical and because separation appears to be based on partition between water-formic acid in the cellulose phase and methylethylketone as a mobile phase, so that all indoles of interest to me had similar R_f values because of their low solubility in methylethylketone.

Columns for chromatography in the isolations were made by slurrying technical grade Avicel in the solvent to be used, and pouring the slurry to a 20 cm height in a 4 cm diameter glass column with a fritted glass disk covered by 2-3 cm of fine glass beads, and equipped with a variable bore stopcock. A Calbiochem timed-change fraction collector was used.

Preparative thin-layer chromatography plates were prepared by slurrying 100 gm superfine Avicel in 450 ml distilled water for 25 seconds at high speed in a Waring Blendor, then spreading the slurry on 40 X 20 cm glass plates to a thickness of 400 microns. No thicker layer could be spread because the plates would crack badly upon drying.

Thin layer plates for the indole survey, for checking fractions for indole content, and for densitometry were made by slurrying superfine Avicel as above and spreading it 250 microns thick on 20 X 20 cm plates. The sample was

spotted 2 cm from each edge at the lower left-hand corner of the plate which was developed first in isopropanol solvent from left to right and then in butanol solvent from bottom to top.

Indoles were detected by spraying with Ehrlich's aldehyde, which was a 1 per cent solution of p-dimethylaminobenzaldehyde in methanol and concentrated hydrochloric acid (1:1). The plates were sprayed until translucent then heated at 75° C for 3-5 minutes and examined immediately.

Colors given by indole compounds with Ehrlich's reagent are somewhat specific; tryptophane gives a red-violet changing to yellow-green, tryptamine a red-violet changing to blue-violet, serotonin a blue changing to deep blue, and serotonin-O-sulfate a red-violet changing to deep blue.

The 5-hydroxyindoles were detected with a spray of 1 per cent 1-nitroso-2-naphthol in ethanol followed by nitrous acid in hydrochloric acid (15). This reagent is very specific, giving a violet spot on a pale yellow background only for 5-hydroxyindoles.

Ultraviolet absorption spectra from 400 mμ to 230 mμ were determined on a Beckman DB-1 recording spectrophotometer using solutions made up in 0.1 N hydrochloric acid and in 0.1 N sodium hydroxide.

Fluorescence spectra were determined on an Aminco-Bowman spectrophotofluorimeter with an Electrovolt x-y recorder.

Samples for quantitation were made by freeze-drying seven random samples from separate colonies of frozen Palythoa mammillosa or caribbeorum of greater than 2 gm size. The samples were prepared from alcohol preserved Zoanthids by separating the liquid and solids, measuring each, then taking the same proportion of each and recombining as a sample for lyophilization. The lyophilized samples, weighed to the nearest two milligrams, were ground and extracted with two volumes of 80 per cent ethanol for two to four hours. Ten microliters of solution were taken to spot for chromatography prior to densitometry.

Densitometries were performed on two-dimensional thin-layer chromatograms on a Photovolt densitometer with recorder. The graphs were integrated using a Keuffel and Esser planimeter rather than the integrator with the densitometer, because of poor zeroing characteristics of the integrator. A yellow filter was interposed into the light beam to enhance sensitivity by cancelling the yellow color of excess Ehrlich's reagent and contrasting the blue of the coupled indoles. The only drawback to this is that the yellow color of tryptophane would have been better for this than the red-violet, since it could have been measured with a blue filter. However, this would lead to dense backgrounds from the Ehrlich's reagent and long waits as the yellow color develops slowly.

Standard curves for serotonin, tryptamine, and tryptophane were made by spotting on thin-layer plates known amounts of solutions of the above to produce spots ranging from 0.5-25 mμMoles, developing the plates in one dimension with butanol solvent, spraying, and reading. Because the conditions of the detecting process (heat and strong mineral acid) lead to extensive hydrolysis of the serotonin-O-sulfate and because no primary standard of pure material was available, the serotonin-O-sulfate was read as serotonin.

Samples for the survey of indole content were prepared by grinding two grams of dried animal, extracting with 80 per cent ethanol, and spotting 30 μl at the corner of a thin-layer plate. In the case of alcohol preserved specimens, 30 μl of the alcohol supernatant were spotted on the thin-layer plates and developed in two dimensions.

CHAPTER II

ISOLATION AND QUANTITATION OF INDOLES

The analytical procedure for indoles for indentification consists of four parts: solvent extraction, chromatography, spectral identification, and quantitation. The main idea was to isolate the indoles of interest as a group until they were sufficiently concentrated to give clean separation on preparative thin-layer chromatography plates. The analyses were made in a darkened room using low-actinic, aluminum-foil-covered, or black-painted glassware to protect compounds from light.

Solvent Extraction

The solvent extraction consisted of grinding 300 gm frozen P. mammillosa or caribbeorum in two volumes of 80 per cent ethanol in a Waring Blendor, then stirring the mixture under carbon dioxide for 45 minutes with ascorbic acid added to a concentration of 0.5 per cent. Ethanol was chosen as solvent on the basis of suggestions in the literature (16,17,18,19), and ascorbic acid was used to protect the serotonin for reasons discussed below. The slurry was filtered, and the residue extracted three more

times, once with 60 per cent ethanol and twice with 80 per cent ethanol, then discarded. The filtrates were combined and concentrated to a volume equal to approximately half of the original weight on a rotary evaporator using a chilled water condenser. The aqueous residue which contains the indoles was extracted with five volumes hexane, then with five to eight volumes ether. The organic extracts were discarded. The aqueous residue was then lyophilized. The lyophilized residue was taken up in approximately 50 ml methanol and the mixture filtered to remove salts and ascorbic acid. The filtrate was concentrated to dryness on a rotary evaporator with a Dewar condenser. All concentrations beyond this point were done on a rotary evaporator using a Dewar condenser cooled with dry ice in acetone.

Chromatographic Separation

The methanol soluble concentrate from above was dissolved in 5-10 ml 50 per cent aqueous methanol and an equal volume of the butanol solvent mixture was added. A precipitate formed. The mixture of solid and liquid was transferred to an Avicel column and chromatographed with butanol mixture. A void volume of 100-125 ml was discarded, and fractions of 20-30 ml were collected until indoles no longer came off the column. Fractions were tested by thin-layer chromatography on Avicel plates using isopropanol mixture. Elution patterns for the indoles of

the two Palythoa species are shown below. As can be seen three main groups of fractions may be made, tryptamine, serotonin-tryptophane, and serotonin-O-sulfate.

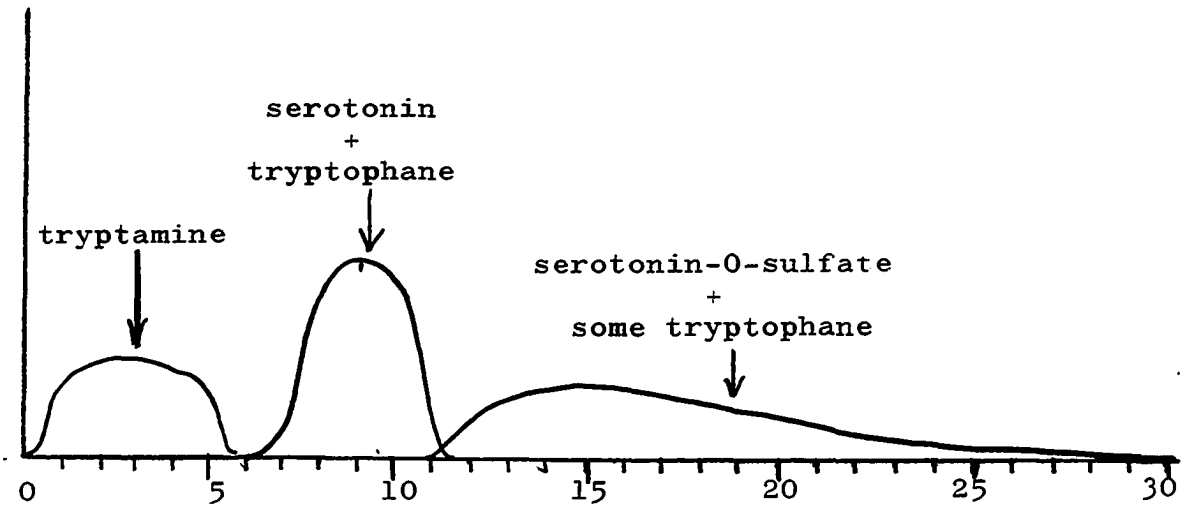


Fig. 1--Elution pattern for Palythoa mammillosa indoles from an Avicel column with butanol mixture. Peak heights are in arbitrary units. Numbers are fraction tube numbers.

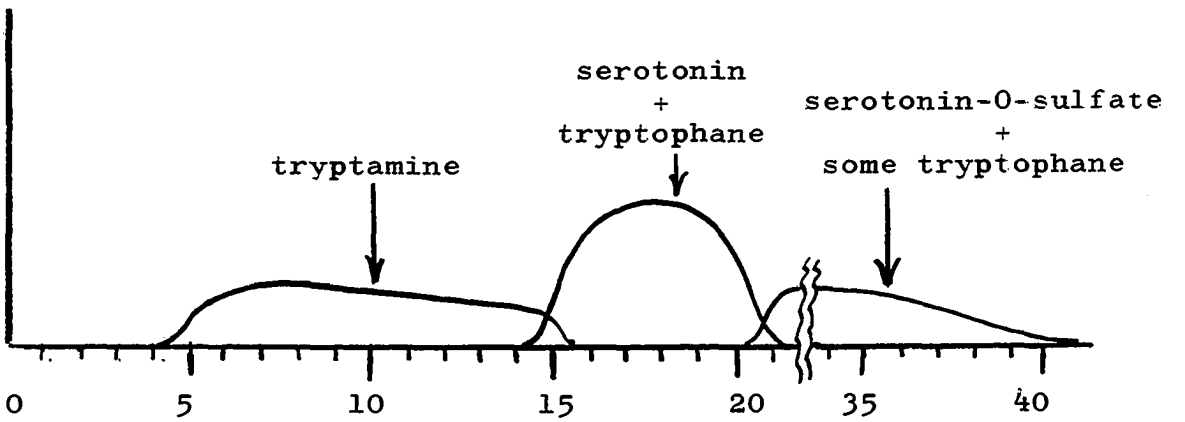


Fig. 2--Elution pattern for Palythoa caribbeorum indoles from an Avicel column with butanol mixture. Peak heights are in arbitrary units. Numbers are fraction tube numbers.

Each of the fractions was concentrated to dryness; the tryptamine and serotonin-tryptophane fractions were separately taken up in methanol and water, an equal volume of isopropanol solvent added, and each fraction placed on an Avicel column and chromatographed with isopropanol solvent, to yield a tryptamine fraction from one column and serotonin and tryptophane fractions from the other. The fractions were subjected to preparative thin-layer chromatography in butanol mixture and then in isopropanol mixture.

The serotonin-O-sulfate fractions were taken to dryness on a rotary evaporator, dissolved in 10 ml water and passed through an Amberlite IR-120 column in acid form. The pH of the eluate (pH 1) was adjusted to 3-4 by adding IR-45 and stirring. The solution was then taken to dryness, taken up in methanol and water (1:1) and chromatographed on preparative thin-layer plates in methanol.

All of the above fractions were subjected to a final chromatography along with the synthetic serotonin-O-sulfate and known serotonin, tryptophane, and tryptamine on preparative thin-layer plates using the methylethylketone mixture. The samples were then eluted and taken for spectral analysis.

During the final chromatographic separation, what had appeared to be fairly clean indole compounds would again give bands corresponding to indoles supposedly removed in

earlier steps, e.g. tryptamine would separate from serotonin or vice versa. The probable cause is that since these compounds are in strong competition with other types of compounds for solvent molecules and adsorbent sites, the similarities due to the large indole nucleus tend to force them together as like compounds in early chromatographic steps despite supposedly different polarities. In the case of the contamination of the serotonin-O-sulfate by serotonin, apparently a certain amount of hydrolysis occurs, even at neutral pH. This is accelerated in base to the extent that the compound disappears in 15 minutes at pH 12-14, and all that is recovered is serotonin.

The purification of the very polar tryptophane and serotonin-O-sulfate is quite difficult because amino acids and other possible zwitterions will co-chromatograph with them in the butanol and isopropanol systems. The serotonin-O-sulfate could not of course be purified in the basic isopropanol system.

A major difficulty in isolation of these compounds is their instability. Tryptophane is unstable in ultraviolet light and sunlight. The products of the photolysis are such compounds as tryptamine, and other indole compounds found in plants. This destruction may be stopped by ethanol, egg white, or cabbage juice (20). Tryptophane has also been converted to serotonin by ultraviolet (21). Indolacetic acid can be degraded into skatole, indolealdehyde, and indolecarboxylic acid (22), indolealyoxylic

acid, and anthranilic acid (20). Heat also leads to problems, as a temperature of 66-70°C for the short period of time used to dry spots for chromatography can cause 25 per cent losses (23). *Serotonin is very sensitive to* oxidation, at least in blood containing systems. The addition of ascorbic acid prevents this oxidative destruction (24). In the presence of oxygen and ferrous ion, ascorbic acid will hydroxylate aromatic compounds and convert tryptamine into serotonin (25,26). This reaction requires the presence of oxygen or peroxide. In my isolation scheme, dry ice was used to generate an inert atmosphere while indole materials were in the presence of ascorbic acid and metal ions, and after the lyophilization procedure when an inert atmosphere was not provided the ascorbic acid was absent, being left as insoluble in methanol. Protection from light at all stages, and working at room temperature or lower were the other precautions used to prevent losses. When such precautions were not taken, a complete loss of indoles occurred prior to the chromatographic steps.

Spectral Analysis

Ultraviolet absorption spectra of authentic tryptamine, and of tryptamine isolated from P. mammillosa and caribbeorum dissolved in 0.1 N hydrochloric acid were identical with a small side peak at 285-287 mμ, the maximum at 275-277 mμ, and a shoulder at 268-270 mμ. Both

Palythoa tryptamines dissolved in 0.1 N sodium hydroxide gave spectra identical with those determined in acid.

Ultraviolet spectra of authentic serotonin and both isolated serotonin samples in acid showed a strong shoulder at 295 mμ, with the maximum at 275 mμ. In base they showed a peak at 318 m , and a second stronger peak at 272-275 mμ.

Ultraviolet spectra of isolated tryptophane and serotonin-O-sulfate did not correspond to the spectra of known tryptophane or of synthetic serotonin-O-sulfate. Therefore, spectrophotofluorimetry was used to determine the identity of these isolated compounds. Fluorescence spectra were determined by manually finding an activating wavelength that gave maximum fluorescence intensity, fixing this wavelength and automatically scanning the fluorescence spectrum. The activation spectra were determined by fixing the fluorescence wavelength of which the intensity was being measured and automatically scanning the activation spectrum for the wavelength that stimulates maximum emission. The results are summarized on Table 1.

Authentic tryptophane and tryptophane isolated from both Palythoa sp. gave superimposeable fluorescence curves in acid. The same result was found for the comparison of the samples of serotonin-O-sulfate in acid solution. The slight shift of the P. mammillosa is within apparent variations of the samples.

In base the fluorescence spectra for known tryptophane

TABLE 1

RESULTS OF SPECTROPHOTOFLUOREMETRY

Sample	Fluorescence Spectra		Activation Spectra	
	Fluorescence Maximum	Activating Wavelength	Activation Maximum	Fluorescing Wavelength
Tryptophane				
in 0.1 N HCl				
Authentic	360-380	295		
P. caribbeorum	360-380	295		
P. mammillosa	360-380	295		
in 0.1 N NaOH				
Authentic	380-400	308	250s, 305	380
P. caribbeorum	410-430	308	305-315	370
			310s, 340	380
			310s, 340	395
P. mammillosa	375-400	308	240s, 300	370
			240s, 300	380
			240s, 300	390
Serotonin-O-sulfate				
in 0.1 N HCl				
Synthetic	370-380	310		
P. caribbeorum	370-380	310		
P. mammillosa	363-377	310		
in 0.1 N NaOH				
Synthetic	410-427	310	310	375
			310, 360s	395
			310, 360s	400
			310, 360s	410
P. caribbeorum	382-395	310	310, 360s	370
			310, 360s	380
			310, 360s	395
P. mammillosa	384-409	310	305-310	375
			305-310	385
			305-310	400

Values in millimicrons
"s" denotes a shoulder on the main peak

and the isolated tryptophanes were not in complete agreement; therefore activation spectra were determined. For the 380 mμ fluorescence peak of authentic tryptophane, a sharp activation peak with a low shoulder at 250 mμ was found at 305 mμ.

Tryptophane isolated from P. mammillosa gave a very broad fluorescence peak from 375-400 mμ in base, and activation spectra were run for fluorescence at 370, 380, and 390 mμ. In all cases a sharp peak with a low shoulder at 240 mμ and maximum at 300 mμ was found. This tryptophane sample was apparently contaminated with traces of serotonin since in base the fluorescence maximum for serotonin is 380-395 mμ for activation at 340 mμ, and as the fluorescence peak choice was shifted to longer wavelengths, a small, low shoulder appeared at approximately 340 mμ on the activation curve.

The base absorption spectrum of P. caribbeorum tryptophane showed a broad, messy peak with shoulders at 372, 382, and maximum at 390-408 mμ. Activation spectra showed a broad based peak at 305-315 mμ for 370 mμ fluorescence, a tall broad peak with high sharp shoulder at 310-320 mμ and maximum at 340 mμ activation for 380 mμ fluorescence, and a slight broadening at 310 mμ on a 340-350 mμ activation peak for 395 mμ fluorescence. This is interpreted as indicating a heavy serotonin contamination of P. caribbeorum tryptophane. A fluorescence spectrum for this

sample of tryptophane in base with 307 mμ activation gave a broad peak at 410-430 mμ that did not correspond to known tryptophane.

Literature values for tryptophane do not agree with the values I obtained for authentic tryptophane. One article gives a 365 mμ fluorescence for 285 mμ activation at pH 11 (28). The purpose of the paper was to develop quantitative procedures, and the wavelengths chosen are not necessarily those of maximum sensitivity, since in many biological systems there may be substances that interfere at the most sensitive wavelengths.

The fluorescence spectrum for serotonin-O-sulfate in basic solution with 310 mμ activation was a broad peak from 410-427 mμ for the synthetic sample and peaks from 384-409 mμ and 382-395 mμ for P. mammillosa and caribbeorum, respectively. Activation spectra for synthetic material for fluorescence at 375, 395, 400, and 410 mμ showed a sharp maximum at 310 mμ with a rise developing at 360 mμ to a low plateau as the fluorescence wavelength increased. This material was contaminated with ion-exchange resin material which may account for this secondary peak and peak broadening.

The activation spectra for P. caribbeorum showed a sharp peak at 310 mμ with a shoulder developing into a low secondary peak at 360 mμ. This shoulder may be due to a contaminant of some sort. The final fluorescence spectrum

for the serotonin-O-sulfate in base from this animal, showed a peak at 395-410 mμ for activation at 310 mμ.

Activation spectra for P. mammillosa material for 375, 385, and 400 mμ fluorescence showed a sharp peak at 305-310 mμ. The final fluorescence spectrum showed a peak at 390-410 mμ for activation at 310 mμ.

The fluorescence spectra for acid solutions of the compounds were identical for each compound regardless of the source. The activation spectra for basic solutions of the compounds were identical for each compound with the exception of tryptophane from P. caribbeorum. This material showed a response to 310 mμ activation wavelength, and its fluorescence spectrum in acid was identical with that of authentic tryptophane.

The R_f values for the four compounds of interest in butanol mixture and isopropanol mixture on Avicel are listed in Table 2, along with literature values and values for authentic samples (11,12).

The R_f values of fractions obtained from Palythoa have fairly close correspondence with those of authentic samples of the same compounds. Furthermore, co-chromatography of animal extracts with genuine 5-hydroxytryptophane, tryptophane, and tryptamine confirmed the presence of tryptophane and of tryptamine and ruled out the possibility of 5-hydroxytryptophane as the material where the serotonin-O-sulfate was shown to be. The occurrence of

serotonin was confirmed by its R_f values and its reaction with 1-nitroso-2-naphthol. As mentioned before, these compounds react uniquely with the Ehrlich's aldehyde spray, further confirming their presence.

TABLE 2

R_f VALUES OF ISOLATED INDOLES

Compound	Butanol Solvent			Isopropanol Solvent		
	Animal	Known	Lit.	Animal	Known	Lit.
Tryptamine HCl	67-74	60-62	69	81-88	85-89	78
Tryptophane	25-36	37	37-42	25-40	30-37	34-36
Serotonin	38-41	32-36	40	54-69	60-73	59
Serotonin-O-sulfate	07-18	10-20	--	33-45	30-40	--

Animal refers to values of material isolated from either P. mammillosa or caribbeorum.

Known refers to authentic samples or the synthetic serotonin-O-sulfate.

Butanol solvent is butanol, acetic acid, water (4:1:1).

Isopropanol solvent is isopropanol, concentrated ammonia (8:1:1).

Quantitation of Indoles

The results from densitometric quantitation of the indoles of seven samples of P. caribbeorum, seven of P. mammillosa, one of a "green-centered" Zoanthid from Puerto Peñasco, Mexico, and one of a small Zoanthid from Puerto Peñasco, are listed in Table 3. The results are expressed in micrograms of substance per gram dry weight of animal or parts per million.

TABLE 3

QUANTITATIVE DETERMINATIONS OF INDOLES

Sample	Serotonin-O-sulfate	Tryptophane	Serotonin	Tryptamine
P. caribbeorum	68	20	164	84
	87	20	274	51
	107	12	136	41
	87	trace	177	71
	117	24	532	194
	76	12	122	60
	86	trace	66	60
average	90	13	210	80
P. mammillosa	118	trace	88	80
	144	25	425	42
	830	16	487	35
	300	trace	206	34
	102	16	201	116
	96	16	167	50
	99	17	179	52
average	241	13	250	58
"Green-centered" Zoanthid	trace	trace	trace	97
Small Zoanthid	trace	trace	151±40	175

Values in micrograms per gram.

Trace indicates that a spot could be seen at the proper location but the densitometer would not register its presence.

The figures demonstrate one of the reasons why proof of the presence of tryptophane was so difficult. In both P. mammillosa and caribbeorum the tryptophane content ranges from trace-24 μg/gm with averages 13 μg/gm, trace being about 2-8 μg/gm. Correcting for the two-thirds water content, the 300 gm samples taken for isolation contain approximately 1.3 mg total tryptophane.

There is some doubt about the 830 μg/gm figure for P. mammillosa serotonin-O-sulfate, since the presence of other indoles very close on the plate poses scanning problems, and visually the serotonin-O-sulfate spot was not almost three times more dense nor three times larger than that of the next sample in Table 3. If this sample is not included, the average for the other six values, 143 μg/gm, is close to that of P. caribbeorum. Total serotonin-O-sulfate in each sample taken for isolation is approximately 10 mg. Considering that this compound separates with salts and the bulk of the amino acids, that it cannot be chromatographed in basic solvents, that it readily hydrolizes, and that the removal of salts and amino acids envolves a sulfonic acid ion-exchange resin, 10 mg of material is not a great quantity with which to be working.

The serotonin content averages 210 μg/gm for P. caribbeorum and 250 μg/gm for P. mammillosa, ranging in both animals from 100 μg/gm to about 500 μg/gm.

Though tryptamine is present in the same or lesser amounts than serotonin-O-sulfate, it is much easier to isolate because it is stable and is readily separated from other compounds because of its relatively low polarity.

More samples would be desirable in the Zoanthid evaluations, since serotonin-O-sulfate and tryptophane levels are below the detection threshold of the densitometer.

Sources of error in the quantitation are numerous.

The densitometer uses a 2 cm slit length, and it is very difficult to make sure only the compound of interest is being scanned. To develop the plates further runs the risk of destruction of the compounds by the acid or base, e.g. hydrolysis of serotonin-O-sulfate, oxidation of serotonin. The response of the densitometer to almost invisible variations in plate thickness and spray density is often greater than to the compounds, and a yellow filter does not completely alleviate the problem.

The sample size chosen has a tryptophane and serotonin-O-sulfate content below the range of best instrument sensitivity. However, the serotonin and tryptamine contents are right in the middle of this range. The problem with these two compounds, expecially serotonin, is that one is approaching (and in two cases for serotonin exceeding) the chromatographic resolution of the system. This leads to trailing with its concomitant problems in scanning separation. A smaller sample size would remove tryptophane levels completely below detection levels, and to increase sample size on a separate plate for a tryptophane determination would not work because the serotonin trails into the tryptophane spot in the isopropanol solvent.

The use of a planimeter to integrate the curves is better than machine integration, especially at low values. The precision is 0.1 cm^2, and for very low concentrations of material this could create an uncertainty of 50 per cent.

For serotonin and tryptamine the uncertainty from this source would be 3-10 per cent.

CHAPTER III

A SURVEY OF INDOLES OF COELENTERATES

The survey of indoles included Zoanthids, Anemones, Gorgonians and Alcyonaceans, all members of the class Anthozoa. The first two orders, Zoanthidea and Actiniaria, are in the subclass Zoantharia, and the second two, Gorgonacea and Alcyonacea, are in the subclass Octocorallia.

The samples of deep water Gorgonians had been stored one year at the time of the survey; the other samples of Gorgonians had been collected the previous two weeks; the Alcyonaceans had been stored one year, but had been immediately ground and placed in air-tight polyethylene bags in the dark after drying; the Zoanthids and Anemones were alcohol preserved with the exception of a ground Metridium senile specimen which had been stored in an air-tight container.

The results of the survey of indoles in the Zoanthids and Anemones is listed in Table 4. Indole indentification was made on the basis of relative R_f values and color of spots. Quantities were estimated by comparison with spots of known amounts of serotonin. Figure 3 shows relative locations on thin-layer plates of the indoles indentified

TABLE 4

INDOLES OF ZOANTHIDS AND ANEMONES

Animal		Indole Number										
		1	2	3	4	5	6	7	8	9	10	11
Zoanthids												
Palythoa tuberculosa	(E)		+					+		+		
Palythoa mammillosa	(J)	++	++		+	+	+	+++	++++	+		
Palythoa caribbeorum	(J)	+	+				+	+	+	+		
Epizoanthus scotinus	(F)		+++							+		
"Green-center" Zoanthids	(M)		+	±	±			±		++	±	
"Small" Zoanthids	(M)	+	+		+		+	++	+	+		
Anemones												
Anemone from Ursula	(E)		±							+		+
Green Anthopleura elegantissima	(F)		±	±	±					+		+
Brown Anthopleura elegantissima	(F)		+		++					+		
Metridium senile	(F)		+++					++		++		+
Stoichactis helianthus	(J)		+			four others not identifiable				+	±	±
Anemone from Mexico	(M)			+	+					+	±	+

Letters in parentheses denote location of collection, E=Eniwetok, F=Friday Harbor, Washington, J=Port Royal, Jamaica, M=Porto Peñasco, Mexico

Scale is: ± = 0.1 μgm; + = 0.1-0.5 μgm; ++ = 0.6-1.0 μgm; +++ = 2.0 μgm; ++++ = 3.0 μgm per spot

Indole numbers: 1=serotonin-O-sulfate; 2= tryptophane; 3=5-hydroxytryptophane; 4= indoleglycerol phosphate; 5=unknown; 6=indoleglycerol; 7=serotonin; 8= 5-hydroxyindoleacetaldehyde; 9=tryptamine; 10 and 11=unknown.

by number on the Table for Zoanthids and Anemones.

As can be seen, all Zoanthids and Anemones contain free tryptophane and tryptamine. Serotonin occurs in all Zoanthids, but only in one Anemone, Metridium senile. The other indoles had no discernible pattern of occurrence, though more indoles seemed to occur and in greater quantities in the Zoanthids.

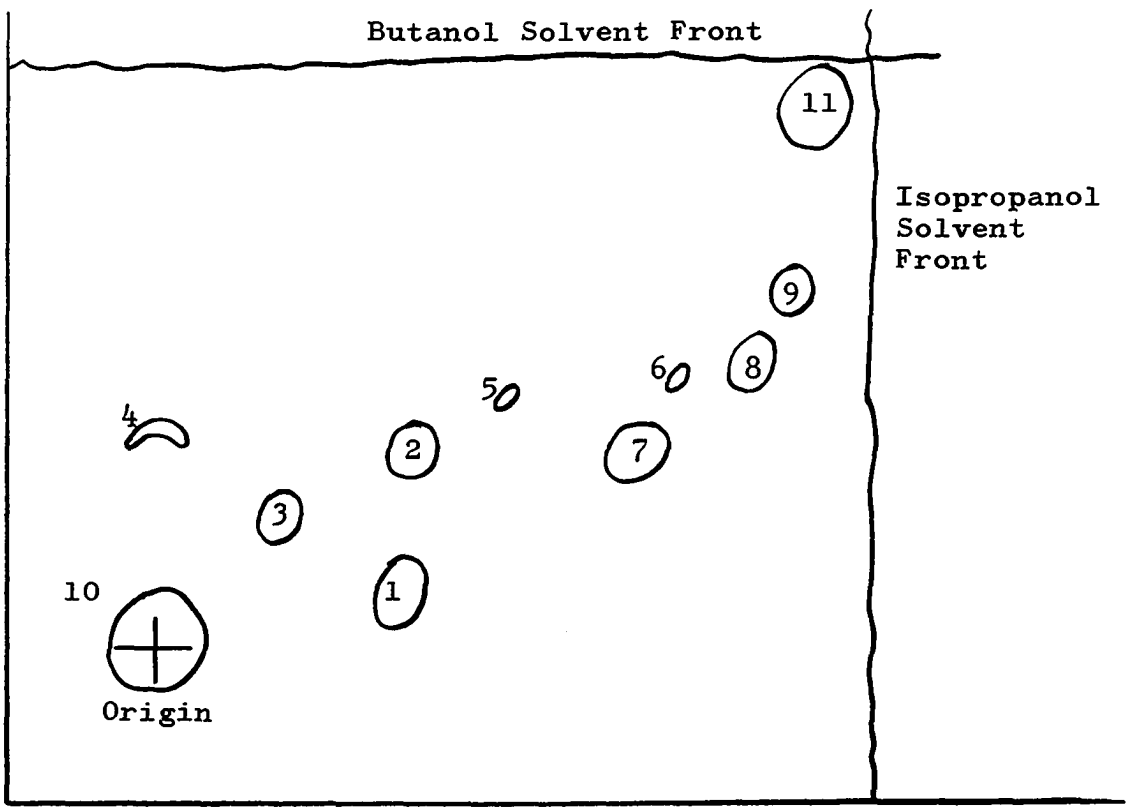


Fig. 3--Chromatographic Pattern for Zoanthid Indoles Identification is positive only for nos. 1,2,3,7, and 9 (1,2,7, and 9 have been isolated, 3 was compared to an authentic sample). 1= serotonin-O-sulfate; 2= tryptophane; 3= 5-hydroxytryptophane; 4= indoleglycerol phosphate; 5= unknown; 6= indoleglycerol; 7= serotonin; 8= 5-hydroxy-indoleacetalhyde; 9= tryptamine; 10 and 11= unknown.

Chromatography of the Alcyonacean samples showed the almost exclusive occurrence of non-polar indoles, as the

Ehrlich's reagent reactive spots appeared in the upper left third of the thin-layer plates, with occasional spots lower down. In all cases streaking and poor resolution prevented identification. In the 26 species of Alcyonaceans examined, the color reactions were highly variable and the resolution very poor. Identifications of like compounds from different samples could not be made, and therefore no table is presented. Figure 4 shows the main areas where Ehrlich's aldehyde positive areas appeared. This figure is greatly simplified, and in no way could be considered a summary, but merely partially representative.

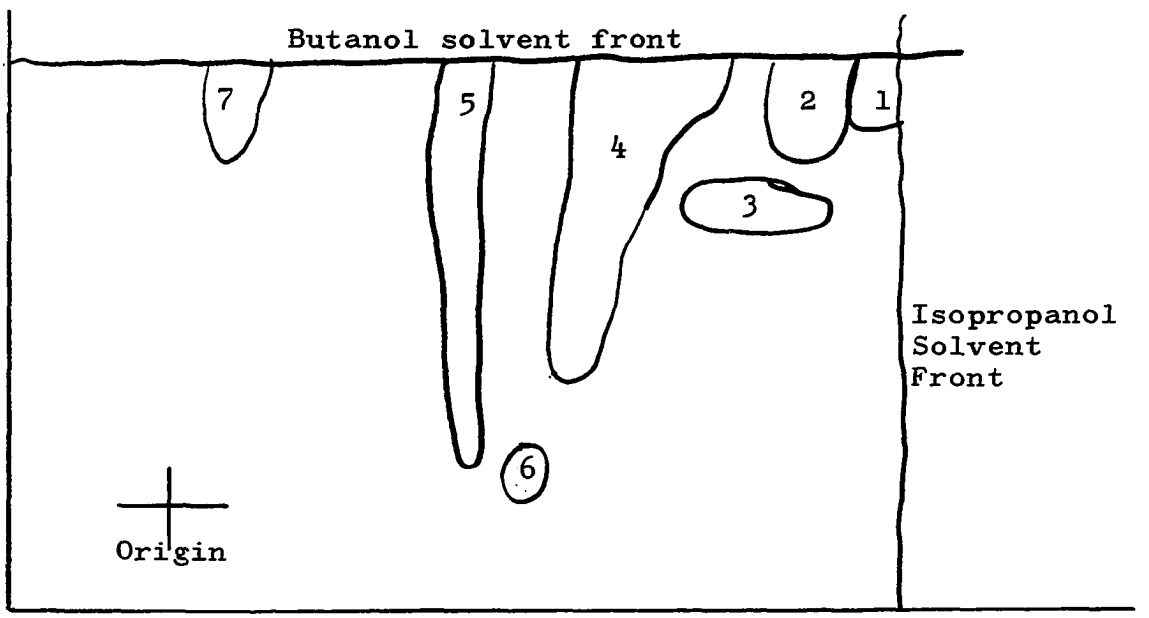


Fig. 4--Chromatographic Patterns for Alcyonaceans
1 was generally a trace of pale blue, and occasionally pink or violet; 2 was mostly pink to red-violet and sometimes blue-violet; 3 was pink to red-violet but often that area had up to four spots in it; 4 was the most variable and on occasion smeared down to the origin; 5 varied in location as did 6 and 7, and colors were from blue-violet to too faint to determine.

The indoles occurring in Gorgonians are listed in Table 4. As can be seen from Figure 5 and Table 5, the non-polar indoles predominate as in the Alcyonaceans which are in the same suborder.

The separation of indoles in Gorgonians was much more distinct than for Alcyonaceans. These indoles are present at concentrations of 10-15 parts per million which is in the lower ranges compared to the Zoanthids. Spot number 3 may not represent an indole at all since it gives a much pinker color reaction than do most indoles.

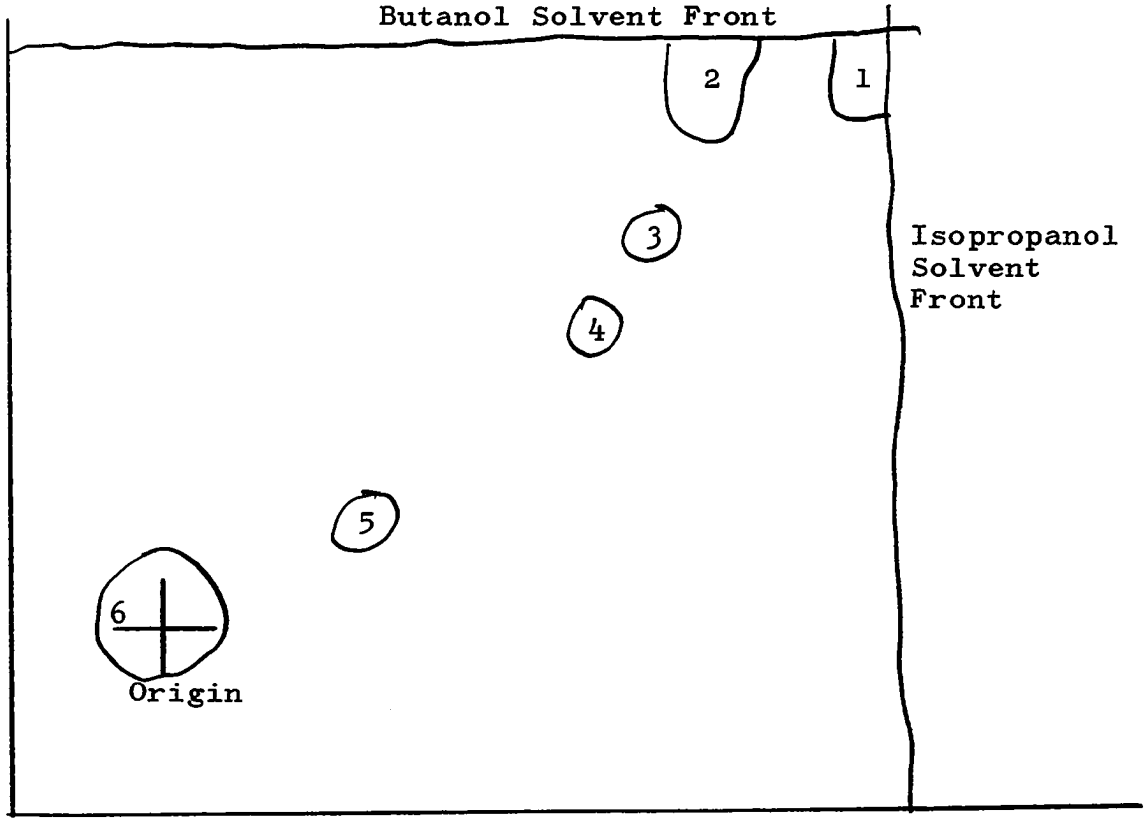


Fig. 5--Chromatographic Patterns for Gorgonians
No identifications may be attached to the numbers except possibly number 5 is tryptophane.

TABLE 5

INDOLES OF GORGONIANS

Animal	Indole Number 1	2	3	4	5	6
Deep Water Gorgonians						
# 1	+	++	+	±	±	+
2	+	++	±			
3	+	++				
4	+	++	±			
5	±	+				
7	±	+++		+	±	+
9	+	++			++	+++
13	++		+	+		
15	+	+				
18	+	±			±	
Shallow Water Gorgonians						
Eunicea mammosa	+	+	+	±	±	
Eunicea Knighti	±		+			
Eunicea asperula	+					
Eunicea calyculata		+	+	±		
Eunicea palmeri	+	+	+	+	+	
Eunicea tourneforti	+					
Red Leptogorgia setacea	+		+			
Yellow Leptogorgia setacea	+		+		±	
Pseudopterogorgia acerosa	+	+				
Pseudopterogorgia americana	+	+	±			
Pseudoplexaura porosa	+	±	±	±		
Pseudoplexaura flagellosa	+	+	+	±		
Plexaura flexuosa	+	++	±			
Plexaura homomalla	±				±	
Plexaurella grisea	+	±	±			
Plexaurella dichotoma	+		±	±		
Plexaurella fusifera	±		±			
Plexaurella nutans	+				±	
Muricopsis flavida			+	+	+	

Deep water Gorgonians were obtained in 60 feet of water at Discovery Bay, Jamaica. Shallow water Gorgonians were obtained at Miami, Florida.

Scale is: ± = 0.1 μgm; + = 0.1-0.5 μgm; ++ = 0.6-1.0 μgm; +++ = 2.0 μgm per spot.

Indole numbers correspond to those in Figure 5, but no identification is made for them.

In animals it has been demonstrated that tryptophane is hydroxylated to 5-hydroxytryptophane, then decarboxylated to serotonin (29). Chadwick and Wilkinson have shown that rat liver can sulfate the serotonin to serotonin-O-sulfate (27). The interesting thing in Palythoa sp. is that apparently no 5-hydroxytryptophane is present. There are two explanations for this: either the amounts of 5-hydroxytryptophane are too small to detect in this species (a Zoanthid and two Anemones do have this compound, which implies that this pathway is operating in these animals), or the animal decarboxylates tryptophane to tryptamine, then hydroxylates it to serotonin as suggested by the large amounts of tryptamine found. However, plants are known to decarboxylate tryptophane to tryptamine, and since these animals contain symbiotic algae, the tryptamine may come from algal decarboxylation of tryptophane.

The function of serotonin in these animals may be as a neuromediator. This suggestion is supported by the finding that histochemical techniques located serotonin in the nervous system of Hydra sp. and not in the nematocysts (6). If this is the case, the serotonin-O-sulfate could well be an inactive form that occurs after neural stimulation or else the storage form prior to stimulus transmission. This is supported by the finding that serotonin-O-sulfate is 100X less active on gut tissue than is serotonin (8).

SUMMARY

Serotonin, tryptamine, tryptophane, and serotonin-O-sulfate were isolated from Palythoa caribbeorum and P. mammillosa, and their identities established by spectral methods. The compounds were shown to be present at levels of 24 parts per million or less for tryptophane, 50-200 parts per million for tryptamine, 60-550 parts per million for serotonin, and 70-300 parts per million for serotonin-O-sulfate. In two Zoanthids from Mexico, serotonin-O-sulfate and tryptophane were found in trace amounts, serotonin levels in trace amounts in the "Green-centered" Zoanthid and 151 parts per million in the small Zoanthid, and tryptamine levels at 97 and 175 parts per million for the "Green-centered" and small Zoanthids, respectively.

A survey of the occurrence of indoles was conducted using thin-layer chromatography. Eleven different compounds giving colored spots with Ehrlich's reagent were found in Zoanthids and Anemones, though not all were found in all species. Several indoles were found in Alcyonaceans, but these could not be identified because of the large variations in color reactions and R_f values. The Gorgonians showed only six different indoles, though none were

identified. The indoles of Aoanthids and Anemones were more polar than those of Gorgonians and Alcyonaceans. All 67 species examined appeared to contain indole compounds.

LITERATURE CITED

1. Phillips, J. H., Nature, 178, 932 (1956)

2. Mathias, A. P., Ross, D. M., and Schachter, M., Nature, 180, 658 (1957).

3. Mathias, A. P., Ross, D. M., and Schachter, M., J. Physiol., 151, 296 (1960)

4. Welsh, J. H., Nature, 186, 811 (1960).

5. Kline, E. S., and Weissbach, H., Life Sci., 4, 63 (1965) [C.A., 62, 15122a (1965)]

6. Wood, J..G., and Lentz, T. L., Nature, 201, 88 (1964)

7. Blanquet, R., Comp. Biochem. Physiol., 25, 893 (1968)

8. Kishimoto, Y., Takahashi, N., Egami, F., J. Biochem. (Tokyo), 49, 436 (1961)

9. Stowe, B. B., and Thimann, K. V., Arch. Biochem. Biophys., 51, 499 (1954)

10. Armstrong, M. D., and Robinson, K. S., Arch. Biochem. Biophys., 52, 287 (1954)

11. Armstrong, M. D., Shaw, K. N. F., Gortatowski, M. J., and Singer, H., J. Biol. Chem., 232, 17 (1958)

12. Durkee, A. B., and Sirois, J. C., J. Chromat., 13, 173 (1964)

13. King, L. J., Parke, D. V., and Williams, R. T., Biochem. J., 98, 266 (1966)

14. L. Reio, Chromatographic Reviews, 3, 104 (1961)

15. Udenfriend, S., Weissbach, H., Clark, C. T., J. Biol. Chem., 215, 337 (1955)

16. Davis, V. E., Huff, J. A., and Brown, H., Clin. Chem. Acta, 9, 427 (1964)

17. Davis, V. E., et al, Clin. Chem. Acta, 9, 419 (1964)

18. Tyler, V. E., Science, 128, 718 (1958)

19. Bulard, C., and Leopold, A. C., Compt. Rend. Acad. Sci., Paris, 247. 1382 (1958)

20. Melchior, G. H., Planta, 50, 262 (1957)

21. Doepfner, W., and Cerletti, A., Experientia, 14, 376 (1958)

22. Mayr, H. H., Planta, 46, 512 (1956)

23. Wier, J. K., and Tyler, V. E., Jr., J. Pharm. Sci., 52, 419 (1963)

24. Davis, R. B., J. Lab. Clin. Med., 54, 344 (1959)

25. Udenfriend, S., Clark, C. T., Axelrod, J. and Brodie, B. B., J. Biol. Chem., 208, 731 (1954)

26. Brodie, B. B., Axelrod, J., Shore, P. A., and Udenfriend, S., J. Biol. Chem., 208, 741 (1954)

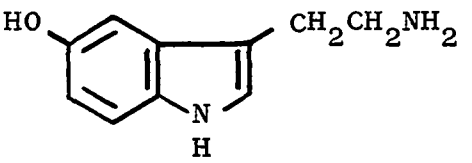
27. Chadwick, B. T., and Wilkinson, J. H., Biochem. J., 76, 102 (1960)

28. Duggan, D. E., Bowman, R. L., Brodie, B. B., and Udenfriend, S., Arch. Biochem. Biophys., 68, 1 (1957)

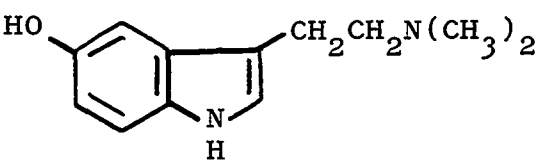
29. Kveder, S., and McIsaac, W. H., J. Biol. Chem., 236, 3214 (1961)

APPENDIX I

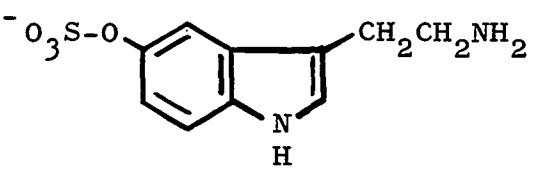
STRUCTURE OF INDOLES DISCUSSED



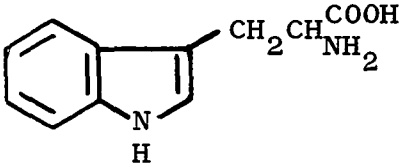
Serotonin



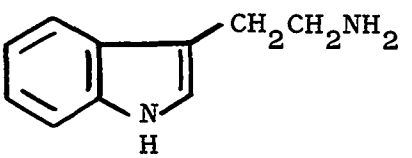
Bufotenine



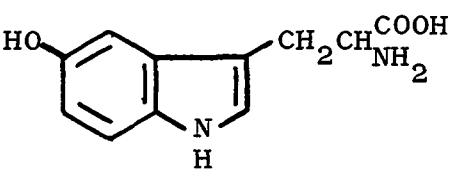
Serotonin-O-Sulfate



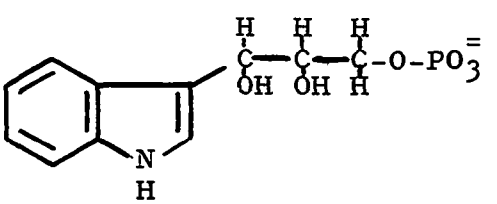
Tryptophane



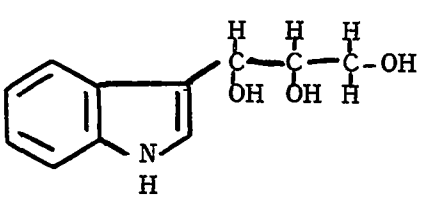
Tryptamine



5-hydroxytryptophane



Indoleglycerolphosphate



Indoleglycerol