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THE CHEMICAL NATURE OF THE TOXIC SECRETIONS
OF THE BOXFISH (OSTRACION LENTIGINOSUS SCHNEIDER)

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To
Gloria

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THE CHEMICAL NATURE OF THE TOXIC SECRETIONS
OF THE BOXFISH (OSTRACION LENTIGINOSUS SCHNEIDER)

By David Bradley Boylan

A thesis submitted to the Graduate School of the University
of Hawaii in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

ABSTRACT

An interesting metabolite may have come to light when it was noted that the secretions collected from a disturbed Hawaiian boxfish, (Ostracion lentiginosus Schneider) were extremely toxic to other reef fish. The toxin has been named pahutoxin since pahu is the Hawaiian name for the boxfish.

A simple bioassay developed by Thomson using brackish water mollies as test fish proved useful in devising an efficient isolation scheme. Initial extraction of the crude toxic secretions with butanol was followed by chromatography of the butanol residue on silicic acid and picric acid-coated anion exchange resin. The resulting product crystallized from acetone and exhibited an optical

rotation $[\alpha]_D^{20} + 3.08^\circ$ (methanol) and a melting point of 75°C .

Nuclear magnetic resonance and infrared spectra demonstrated the presence of a trimethyl quaternary nitrogen, a large aliphatic portion, and the presence of one or more ester functions. Hydrolytic degradation studies provided several key products that allowed the total structure of pahutoxin to be postulated as choline 3-acetoxypalmitate (I).

Proof of the structure rested in the synthesis of racemic pahutoxin by a modified Reformatsky reaction. The product, 3-hydroxypalmitic acid, was acetylated with acetyl chloride and esterified with choline to give a compound which was spectrally identical with natural pahutoxin.

By the same synthetic procedure the 3-acetoxy esters of pelargonic (C_9), lauric (C_{12}), myristic (C_{14}), and palmitic (C_{16}) acids were synthesized and their biological activities were compared with the activities of natural pahutoxin. Decrease in aliphatic chain length, with all other structural features unchanged, was accompanied by a marked decrease in toxicity and hemolytic ability. The parallelism of these two activities suggested that either hemolysis is the cause of toxicity to fish or it is closely related to it.

I. INTRODUCTION

Biotoxins have been observed in a great variety of marine organisms. However, researches in this area have been largely limited to the zoological and pharmacological aspects of the toxins and very little is known concerning their chemical nature. Among the notable recent or current research efforts on the chemistry of marine biotoxins are ciguatera toxin found in the red snapper (Lutjanus bohar Forskal) or the moray eel (Gymnothorax javanicus (Bleeker));¹ saxitoxin from the Alaskan butter clam (Saxidomus giganteus Deshayes) and the California mussel (Mytilus californianus Conrad);² holothurin from the sea cucumber (Actinopyga agassizi Selenka);³ nereistoxin from a marine annelid (Lumbriconereis heteropoda Marenz);⁴ and tetrodotoxin from the puffer fish (Spheroides rubripes Torafugu).⁵ But, even less information is known about the chemistry of physiologically active marine metabolites such as alarming substances, i.e., compounds communicating danger,⁶ sex attractants, and repellent substances or substances used to repel would-be attackers.⁷

Such a metabolite was suggested when Clark and Gohar⁸ noted in 1953 that it was dangerous to put the Red Sea boxfish into small aquarium tanks containing other fishes, as this often resulted in the death of many of the fish inhabitants of the aquaria.

Brock,⁹ while collecting reef fish on Oahu, observed that certain reef fish when confined with the Hawaiian boxfish (Ostracion lentiginosus Schneider) died within a few minutes. Further observations led Brock to assume that the boxfish when highly excited secreted a poisonous substance into the water which killed other fish in the vicinity.

In 1962 a bioassay for the boxfish toxin was developed by Thomson¹⁰ using brackish water mollies (Mollienesia lalipinna Le Seur) which could be collected in abundant quantities the year round. The sensitivity of these fish to the toxin allowed a semiquantitative estimation of its concentration. Thomson further found that a crude toxin could be collected by immersing the distressed boxfish in distilled water. This toxic extract could then be heated to precipitate proteins and deactivate enzymes without an appreciable loss in toxicity.

An investigation of closely related fish in the family Tetraodontidae revealed that the cowfish (Lactoria fornasini Linnaeus) under stress secreted a substance lethal to other fish. However, the puffer fish (Arothron hispidus Linnaeus) gave off a toxic substance whose biological activity more nearly paralleled that of tetrodotoxin than that of boxfish toxin.¹⁰ The New Guinea frog fish or toad fish, a distant relative belonging to the family Batrachoididae, is reported by Whitely¹¹ to squirt

out a liquid or slime which fouls the water killing other fish. However, the report is vague and the possible relation between this toxin and the boxfish toxin remains in doubt.

Despite the ability of the boxfish to secrete a substance extremely lethal to other fish, several reports have appeared in the literature concerning the use of the boxfish as a seafood. In David Malo's "Hawaiian Antiquities" translated in 1898, he mentioned that the pahu or boxfish was kapu to Hawaiian women and reserved for male consumption only.¹² Buddle¹³ reported that the cowfish and boxfish are among a group of fish some of which are eaten or even esteemed by the inhabitants of Singapore. Other reports suggest that the boxfish and related species are quite poisonous and should be avoided as a food. Such inconsistencies can probably be attributed to a second type of poisoning that evolves through the food chain.¹⁴ This is supported by Halstead's findings in 1954 that the boxfish (Ostracion cubicus Linnaeus) contained a ciguatera-type toxin.¹⁵

The biological activity and the chemical nature of the crude toxic secretions of the boxfish paralleled very closely those of holothurin A isolated from the sea cucumber,³ and of the steroidal saponin isolated from the starfish.¹⁶ These similarities prompted Thomson¹⁰ to draw the false conclusion that the boxfish toxin was a steroidal saponin.

A detailed chemical investigation of the pure toxic component derived from the crude secretions of the boxfish (Ostracion lentiginosus Schneider) appeared therefore interesting. Synthesis of the toxic principle and of a series of homologues and examination of their biological activities would provide structure-activity relationships as well as a basis for elucidation of the mechanism of biological action. Such studies would enlarge our knowledge of the chemistry of marine organisms and contribute to an expanding field of research.

Moreover, such a pharmacologically active compound might be of medicinal interest or might even provide a repellent against marine predators.

Clearly then, full structural elucidation of this toxin coupled with biological studies should provide valuable new information on the chemistry and biological activity of marine organisms.

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Grateful acknowledgment is also made to the following people:

Dr. D. A. Thomson whose studies of the boxfish provided useful procedures for collection and biological testing of

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II. EXPERIMENTAL SECTION

All melting points are uncorrected and were determined on a Fisher-Johns melting point apparatus.

Elemental analyses were performed by Berkeley Analytical Laboratory, Berkeley, California.

Ultraviolet (UV) spectra were recorded on a Beckman DK-2 ratio recording spectrophotometer.

Infrared (IR) spectra were recorded on a Beckman DK-2 ratio recording spectrophotometer.

Infrared (IR) absorption spectra were determined with a Beckman IR-5 automatic recording spectrophotometer either in chloroform or carbon tetrachloride solution using matched cells with 0.4 mm path lengths. Infrared absorption maxima are designated as strong (s), medium (m), weak (w), broad (b), and sharp (sh).

Nuclear magnetic resonance (NMR) spectra were determined with a Varian Model A-60 Analytical NMR Spectrometer. The sweep width was 500 c.p.s. in all cases, and the maxima are recorded in delta (δ) values, referring to tetramethylsilane as $\delta = 0$. The center position and character of all peaks are described and their relative intensities are reported in parentheses.

Optical rotation measurements were determined with the ETL-NPL Automatic Polarimeter Type 143A.

Mass spectrometry data were supplied by Varian Associates (M66 Prototype).

Adsorbents used in chromatographic separations include silicic acid (Mallinckrodt - 200 mesh size) prepared according to a procedure outlined by Wren,¹⁷ cellulose (Brinkman Co.), polyamide powder (Brinkmann Co.), alumina G (Brinkman Co.), and polyethylene powder (Dow Chemical Co.). Gel filtration experiments were performed using Sephadex G-25 (Pharmacia, Uppsala, Sweden). Dowex 1-X4 chloride ion exchange resin (Dow Chemical Co.--100 to 200 mesh) was also used.

The presence of a tertiary or quaternary nitrogen was determined by testing with Dragendorff's spray reagent.¹⁸

A. Procurement of Toxic Material

Most of the boxfish were collected from Waikiki and Kaneohe reef areas; however, the presence of a toxin was confirmed in samples collected on Kauai, Maui, and Tahiti. Following the method outlined by Thomson,¹⁰ the fish were netted and immediately placed in a container with a small amount of distilled water. The water-soluble toxin discharged by the fish was collected and the fish were returned to their environment. This process is referred to as milking since the unharmed fish regenerates the toxin and can be remilked at a later date. The foamy mucous substance was heated in order to denature

enzymes responsible for rapid hydrolysis of pahutoxin. The samples were then stored in a refrigerator to limit further decomposition.

After becoming familiar with the chemical nature of the toxin, it was possible to develop a superior method. The crude secretions were extracted immediately with 1-butanol. Since the toxin was easily extracted into butanol, possible hydrolytic decomposition was reduced to a minimum.

Toxicity studies using brackish water mollies (Mollienesia latipinna Le Seur) as test fish¹⁰ provided a semiquantitative method for the determination of toxic fractions. These fish were chosen for biological testing since they were quite sensitive to pahutoxin and could be easily collected in local brackish water streams.

B. Isolation of Pahutoxin

1. Methods of detecting toxic fractions

The fish bioassay mentioned previously provided a fast and simple method of tracing pahutoxin. The fractions were taken to dryness under vacuum and a known amount of residue was dissolved in 10 ml of fresh water. Four fish, brackish water mollies of pre-adult size, were placed in solution and the time of death noted. By repeating this test on different concentrations of residue it was possible to get a semiquantitative estimation of the relative amount of toxin in each sample (Table II).

When it was noted that toxic fractions always gave a strongly positive Dragendorff color reaction, the bioassay was replaced with the Dragendorff test.

2. Polyethylene column chromatography

The toxic secretions from six boxfish were heated to denature enzymes, filtered, and tested for toxicity to brackish water mollies (1.45 mg/ml - Death 7 min).

A slurry of 500 g of polyethylene powder and methanol was poured into a large glass column, packed by applying vacuum to the collection flasks (2½" d), and washed exhaustively with water to remove all traces of methanol from the column. The cold aqueous secretions were filtered through the column, followed by 4 l of cold distilled water wash solution. Successive wash solutions of 3 l portions of 25/75, 50/50, 75/25, and 100/0 methanol/water (V/V) were collected and evaporated to dryness in a rotary evaporator under reduced pressure. Only the residue of the 75 per cent methanol/water fraction, a white solid weighing 435.6 mg, proved to be toxic (0.106 mg/ml - Death 8 min). Comparison of the toxicity data of the crude toxic secretions and that of the processed residue demonstrated that polyethylene chromatography effected a 15-fold purification of pahutoxin. Re-examination of a small portion of this fraction on polyethylene using the same elution scheme showed a subsequent loss in toxicity, thus indicating the necessity of performing this step as fast as possible.

3. Polyamide column chromatography

Preliminary studies suggested polyamide (pre-washed with 2-propanol and chloroform to remove impurities) as a useful adsorbent. A chloroform slurry of 100 g polyamide was poured into a suitable glass column and allowed to settle. The adsorbent was packed and washed with chloroform using reduced pressure to increase the flow rate. Half of the previously obtained toxic residue, 217 mg dissolved in chloroform, was applied to the polyamide column. Gradient elution was performed using chloroform and chloroform/2-propanol mixtures. At approximately 75-90 per cent 2-propanol/chloroform toxic fractions appeared (0.026 mg/ml - Death 9 min). A careful analysis of the weights of individual fractions indicated that, although the polyamide was carefully prewashed, a small amount of impurities was washing off the column. Thin-layer chromatographic analysis of different fractions on alumina using a 75 per cent methanol/2-propanol developing solvent showed the following results:

Fractions 1 - 18 showed only UV active material and were non-toxic.

Fractions 26 - 28 showed two Dragendorff-positive spots accompanied by UV active material and were slightly toxic.

Fractions 31 - 39, the most toxic fractions, contained only one UV-active spot and one Dragendorff-positive spot.

4. Cellulose column chromatography

The toxic secretions of ten fish were processed on polyethylene. The 75/25 methanol/water fraction was evaporated until only an aqueous solution remained. Extraction of this solution with butanol gave a strongly toxic butanol residue (0.1 mg/ml - Death 7 min) and a non-toxic aqueous residue. This procedure eliminated the necessity of removing water, a step responsible for hydrolytic decomposition.

Cellulose chromatography of this fraction using a hexane/2-propanol elution scheme proved to be more useful than the previous nylon chromatography. Toxic Dragendorff positive fractions were eluted with 90 per cent 2-propanol/hexane. The semipure toxic residues (0.026 mg/ml - Death 4-6 min), weighing 25 mg, could be precipitated from ethyl acetate. Strong absorption in the 3000 cm^{-1} range of the IR suggested that the toxin was fatty in nature and indicated that possibly greater purification could be accomplished using silicic acid as an adsorbent.

5. Modified isolation techniques and silicic acid chromatography

The fact that pahutoxin was easily extracted from aqueous solutions into butanol suggested that immediate extraction of crude toxic secretions would reduce hydrolytic degradation to a minimum. This would remove the need to heat toxic secretions and perhaps make

polyethylene chromatography, a step known to cause loss in toxicity, unnecessary.

Extraction of the secretions of twenty fish (2 mg/ml - Death 7 min) with butanol gave an organic residue of 831 mg (0.1 mg/ml - Death 7 min). Toxicity tests indicated that butanol extraction of the crude secretion provided more than a 20-fold increase in purification making polyethylene treatment unnecessary.

Thirty-two gram of silicic acid was prepared according to the method described by Wren.¹⁷ A chloroform slurry of the adsorbent was poured into an appropriate column and allowed to settle. After washing with chloroform, the butanol residue (831 mg) was dissolved in chloroform and introduced onto the column. A gradual elution scheme using 2.5 per cent, 5 per cent, 10 per cent, and 12 to 15 per cent methanol/chloroform (V/V) as eluent solutions, accomplished the desired separation. The toxic Dragendorff-positive fraction, eluted with 12 to 15 per cent methanol/chloroform, contained 284 mg of a semi-crystalline substance that could be reprecipitated from hot acetone. However, repeated chromatography and reprecipitation would not yield a crystalline product.

6. Crystalline pahutoxin

Attempts to form a crystalline picrate by the addition of picric acid to a 95 per cent ethanol solution of the toxin failed. If pahutoxin contained a quaternary

nitrogen, then it should be possible to make a picrate using the picrate form of Dowex 1-X4 anion exchange.

Replacement of the chloride form of the anion exchange resin with the picrate anion was attempted. Since picric acid is highly ionized in solution, passing such a solution through the ion exchange should convert the chloride form to the picrate form. The amount of picric acid retained on the column suggested that this was the case. Pahutoxin was then passed through the ion exchange resin and the appearance of a green eluate indicated that the picrate had been formed. However, trituration of the solid residue with anhydrous ether dissolved the yellow-green picric acid impurity, leaving a white residue that recrystallized in long needles from acetone. The NMR spectrum demonstrated the absence of the picrate anion and proved identical to that of the amorphous pahutoxin.

Chloride anion exchange treatment, diethyl ether trituration or addition of small amounts of water to the crystallizing solvent did not render a crystalline toxin. Although the reason was not clear, the picric acid-coated anion exchange treatment was incorporated into the isolation scheme of pahutoxin (Figure 1).

Crude Toxic Secretion of the Boxfish (*Ostracion lentiginosus* Schneider)

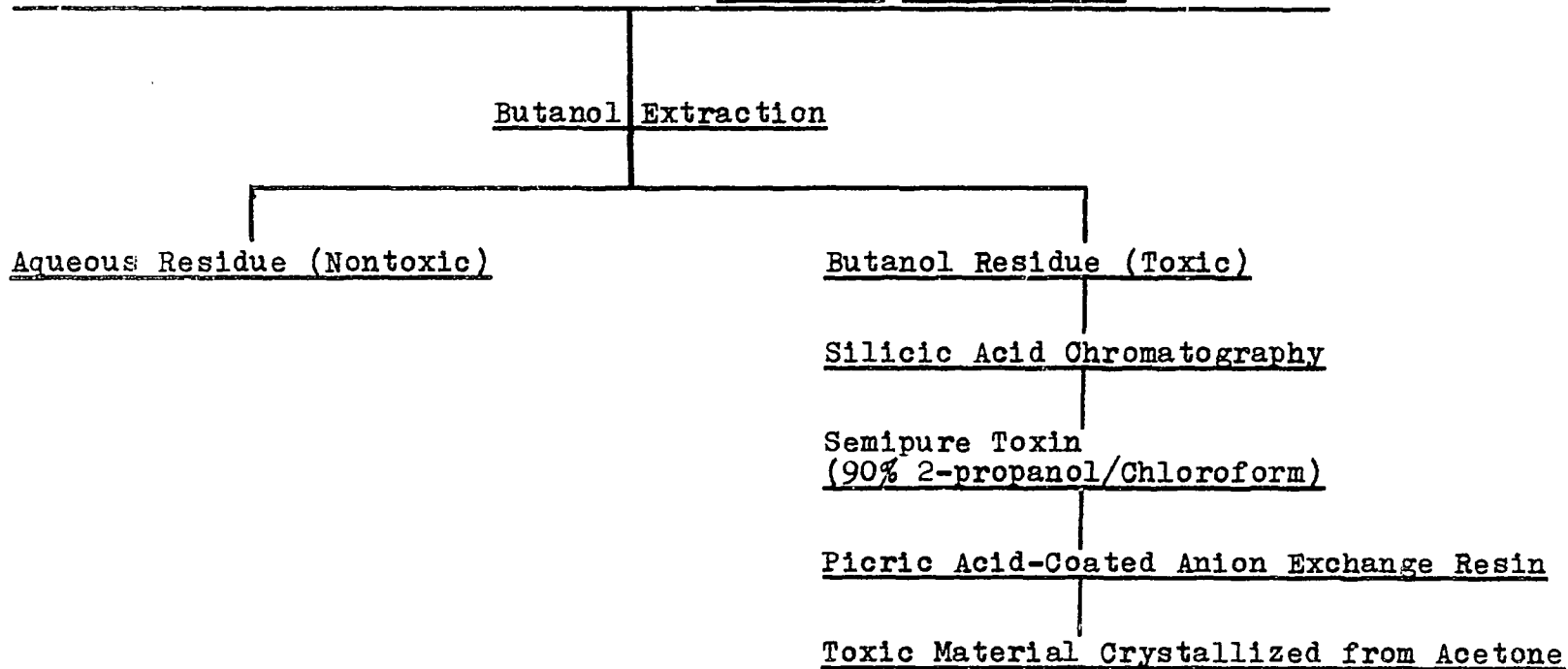


Fig. 1. Scheme Leading to Isolation of Crystalline Pahutoxin

C. Characterization of Pahutoxin

1. Physical and chemical properties

a) Crystallization and solubility

Pahutoxin crystallized in fine white needles from acetone. The crystalline material was very soluble in water, alcohol, chloroform, hot acetone, and hot ethyl acetate, and was slightly soluble in benzene and ether.

b) Halide test

A halide test with silver nitrate in dilute nitric acid gave a white precipitate which dissolved in excess ammonium hydroxide and reappeared with an excess of nitric acid.

c) Sodium fusion

Sodium fusion tests indicated the presence of chlorine and nitrogen.

d) Functional group analyses

A hydroxamic acid test was positive (deep purple). A ninhydrin test was negative.

Pahutoxin gave strongly positive Dragendorff and silicotungstic acid tests indicating tertiary or quaternary nitrogen. Also weakly positive Mayers and picric acid tests were observed.¹⁸

e) Stability to pH changes

Ten-milligram samples of pahutoxin were dissolved in 10 ml solutions of pH 8.9, 7.8, 4.5 and allowed to stand at room temperature for 2½ days. The resulting

solution, when tested with brackish water mollies, indicated the following results.

pH	Standing Period at Room Temperature	Toxicity
8.9	2½ days	Nontoxic
7.8 (sea water)	2½ days	Nontoxic
4.5	2½ days	Toxic

Conductometric and potentiometric titration of pahutoxin was studied in an attempt to detect titratable functional groups. Titration with 0.1 N sodium hydroxide gave no smooth titration curve but did indicate that there was a slow uptake of base.

f) Melting points

Pahutoxin melted between 74-75° C. Repeated recrystallizations from acetone did not change the melting point range.

g) Optical rotation

The readings were taken in 0.1 dm cell with a 0.9 ml capacity. A total of ten reference and ten sample readings were taken. A sample of 0.04593 g of crystalline pahutoxin dissolved in 2 ml of anhydrous methanol gave $\alpha = +0.007$, or $[\alpha]_D^{22} = +3.05$.

h) Elemental analysis

Anal. calcd. for

$C_{22}H_{42}NO_5Cl$: C, 60.55; H, 9.67; N, 3.19.

$C_{22}H_{44}NO_5Cl$: C, 60.27; H, 10.04; N, 3.19.

$C_{22}H_{42}NO_4Cl$: C, 62.85; H, 10.00; N, 3.33.

$C_{22}H_{44}NO_4Cl$: C, 61.33; H, 9.77; N, 3.32.

Found: C, 61.73; H, 9.92; N, 3.24

i) Ultraviolet absorption spectrum

In methanol only strong end absorption was observed.

j) Infrared absorption spectrum

The IR spectrum of 20.3 mg of crystalline pahutoxin in 2 ml of chloroform showed the following major absorption bands (Figure 2): 3320 cm^{-1} (b,m), 2950 (sh,s), 2850 (sh,s), 2450 (b,w), 1730 (s), 1620 (w), 1470 (b,m), 1375 (m), 1250 (b,s), 1220 (w), 1162 (b,w), 1130 (w), 1090 (w), 1045 (w), 1025 (w), 957 (w).

k) Nuclear magnetic resonance spectrum

The NMR spectrum of 38 mg of pahutoxin dissolved in 7 ml of deuteriochloroform showed the following signals (Figure 3): 0.9 δ - triplet (3), 1.35 (24), 2.1 (3), 2.6 - doublet (2), 3.6 -singlet (9), 4.2 (2), 4.6 (2), 5.2 (1).

l) Hydrolytic degradation studies

Basic hydrolysis of Pahutoxin at elevated temperature. To insure complete hydrolysis of the toxin

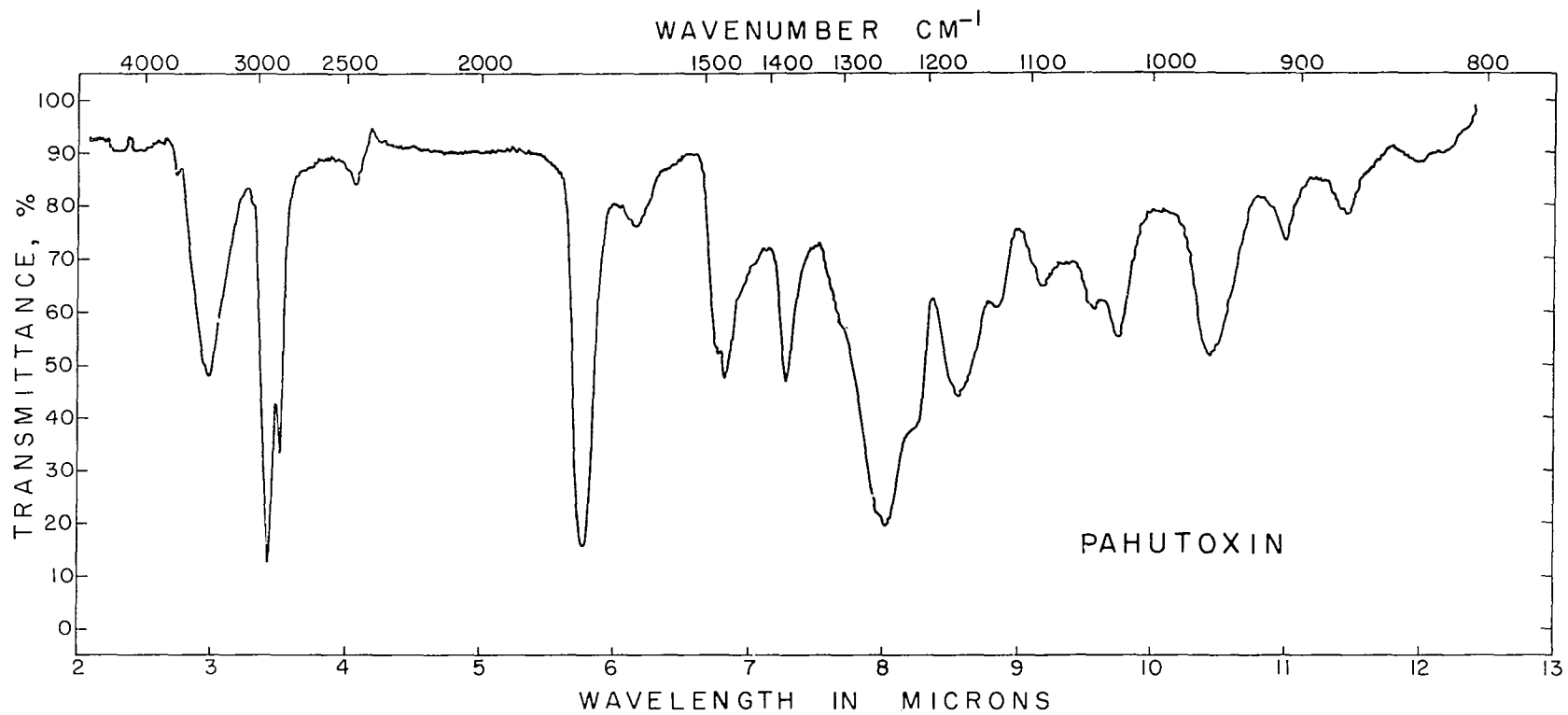


Figure 2. IR Spectrum of Pahutoxin in Chloroform.

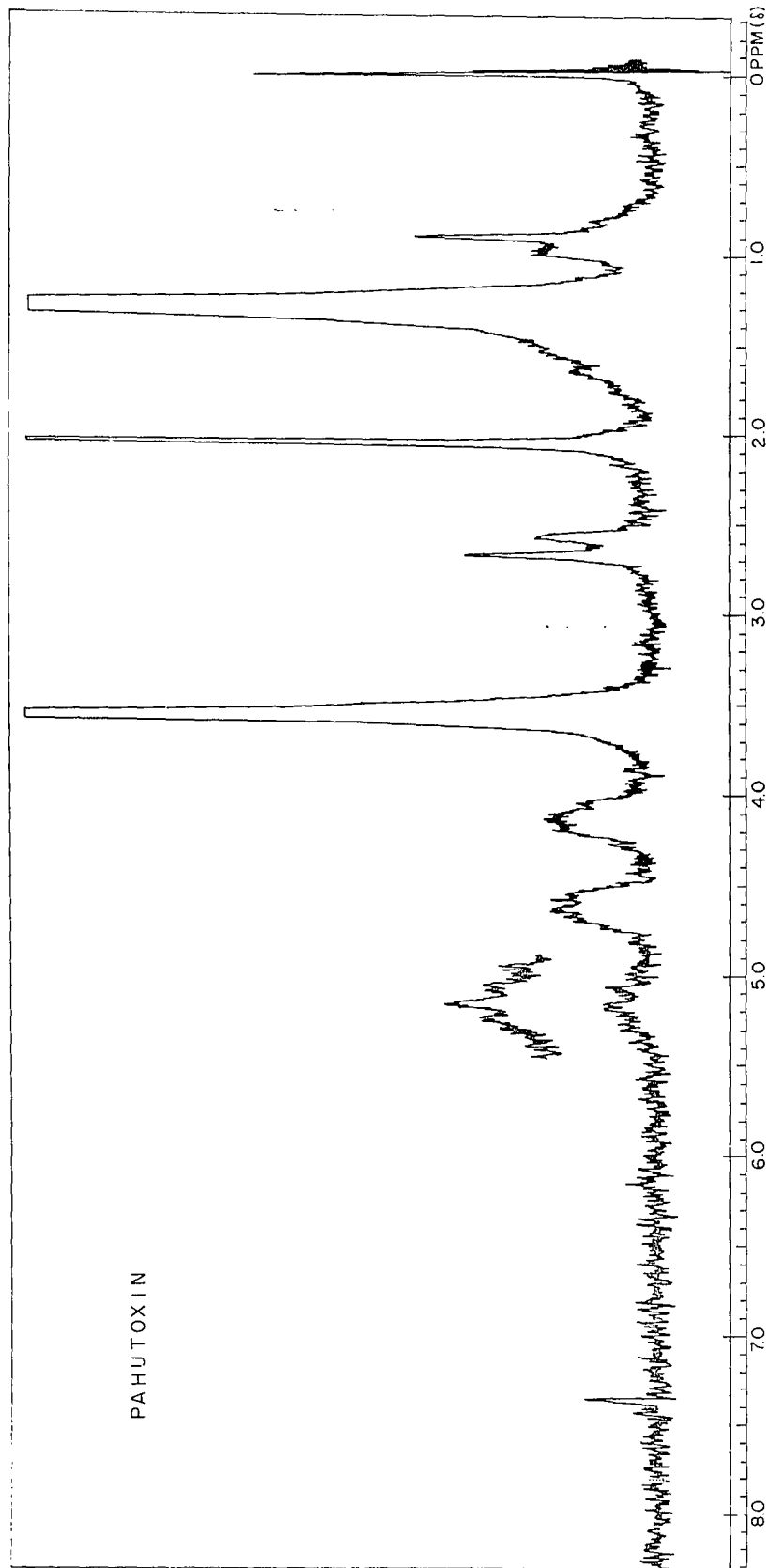


Figure 3. NMR Spectrum of Pahutoxin in Deuteriochloroform.

313 mg was added to an excess of 1 N sodium bicarbonate solution and hydrolyzed at 50° C. for 7 hr. The resulting hydrolysate was no longer toxic to brackish water mollies. The solution was acidified and extracted with butanol. The butanol residue (331.3 mg) was triturated with hexane and separated into hexane-soluble and hexane-insoluble fractions.

(1) Hexane-soluble fraction

The hexane soluble residue consisted of 145 mg of a colorless oil that gave a negative Dragendorff test. The IR spectrum of this hydrolysis product showed the following bands:

3300-3000 cm^{-1} (b,m), 2950 (sh,s), 2850 (sh,s),
2650 (w), 1725 (b,s), 1650 (m), 1475 (m),
1420 (m), 1280 (m), 1230 (w).

The NMR spectrum of this oil showed the following signals (Figure 5):

0.85 δ - triplet (3), 1.25 (22), 2.1 (2), 5.5 -
triplet ($\frac{1}{2}$), 5.8 - triplet ($\frac{1}{2}$), 6.85 - triplet ($\frac{1}{2}$),
7.1-triplet ($\frac{1}{2}$).

Esterification of this compound in 2 N methanolic sulfuric acid resulted in a colorless oil whose IR spectrum is shown in Figure 4. An NMR spectrum of this product in CCl_4 revealed the following signals: 0.85 δ - triplet (3), 1.25 (22), 2.1 (2), 3.6 (3), 5.5 - triplet ($\frac{1}{2}$), 5.8 - triplet ($\frac{1}{2}$), 6.7 - triplet ($\frac{1}{2}$), 7.0 - triplet ($\frac{1}{2}$).

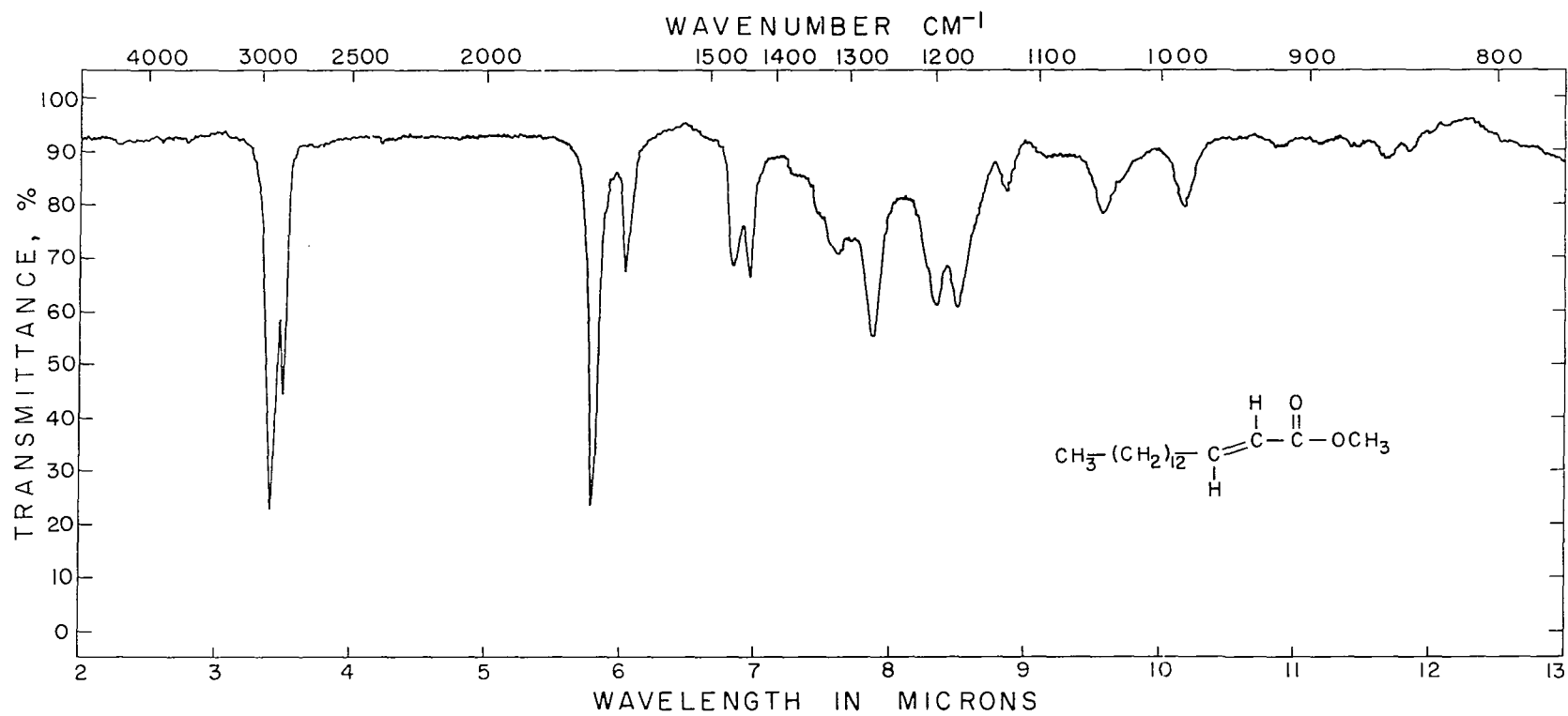


Figure 4. IR Spectrum of Methyl 2-hexadecenoate in Carbon tetrachloride.

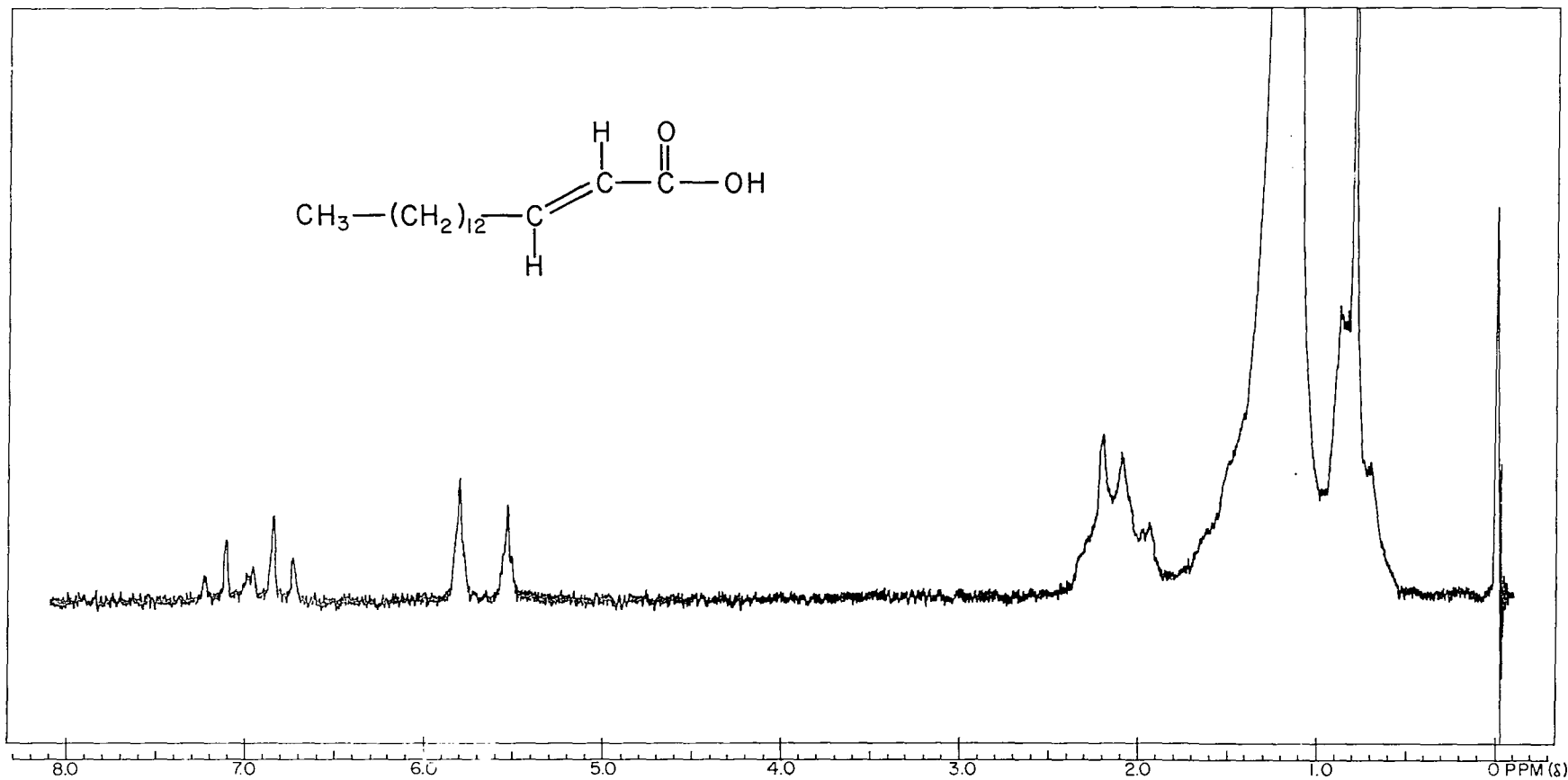


Figure 5. NMR Spectrum of 2-Hexadecenoic acid in Carbon tetrachloride.

Hydrogenation of 50 mg of this ester in 15 ml of ethanol was accomplished using 6 mg of $\text{PtO}_2 \times \text{H}_2\text{O}$ as a catalyst and hydrogen at a pressure slightly above atmospheric pressure. After 2 hr, the reaction mixture was filtered and taken to dryness under vacuum. An NMR spectrum of the colorless oil residue showed the following signals: 0.85 δ - triplet (3), 1.25 (26), 2.15 (2), 3.6 (3).

The NMR spectrum was compared with authentic methylhexadecanoate synthesized by treating hexadecanoic acid (chromatographic standard-Mann Research Laboratories) with 2 N methanolic sulfuric acid.

(2) Hexane insoluble fraction

The hexane insoluble portion proved to be a complex mixture of compounds and had to be chromatographed on silicic acid using an hexane/ether elution scheme.

Although chromatographic separations were not complete, the IR analysis of different fractions provided evidence for the presence of trace amounts of previously unidentified compounds. One compound in particular showed two strong IR absorption bands, one at 1740 cm^{-1} and one at 1710 cm^{-1} . However, lack of material prohibited detailed spectral studies.

Dilute basic hydrolysis of pahutoxin at room temperature. Hydrolysis of 236 mg of crystalline pahutoxin with 20 ml of 0.1 N sodium bicarbonate was performed

at room temperature for 24 hr. The basic hydrolysate was extracted with butanol to give a butanol fraction (B) and an aqueous fraction. The aqueous fraction was acidified and re-extracted with butanol.

(1) Aqueous portion (A)

The aqueous portion (A) was taken to dryness (32 mg) and the residue was triturated with anhydrous 2-propanol. The 2-propanol residue (28.5 mg) gave a strongly positive Dragendorff test, indicating the presence of a polar hydrolysis product containing a tertiary or quaternary nitrogen. Long white needles which crystallized from an acetone-2-propanol solution gave a strongly positive Dragendorff test and were very hygroscopic. An IR absorption spectrum of the sample in a KBr pellet showed the following absorptions: 3400 to 3200 cm^{-1} (b,s), 3020 (sh,w), 2900 (b,w), 1475 (m), 1400 (\bar{m}), 1348 (w), 1135 (w), 1092 (m), 1005 (w), 955 to 950 (b,s), 870 (w).

An NMR absorption spectrum of the sample in D_2O showed the following absorption signals:

3.25 δ - singlet (9), 3.55 (2), 4.1 (2).

The above NMR and IR spectral data were identical with those of an authentic sample of choline chloride (Eastman).

(2) Butanol fraction (B)

The butanol extract (B) was taken to dryness in a vacuum rotary evaporator. The residue, after

being triturated with hexane to remove stopcock grease, weighed 100.7 mg and gave a strongly positive Dragendorff test. However, attempts to crystallize this from numerous solvents failed. The residue was dissolved in 80 per cent ethanol and added to an excess of picric acid. After refrigeration for 2 days a small amount of yellow precipitate formed that could be recrystallized from 75 per cent ethanol. This material with a melting point at $100^{\circ} - 102^{\circ} \text{ C}$ was dried under vacuum for 24 hr at 50° C .

Anal. Calcd. for $\text{C}_{27}\text{H}_{44}\text{N}_4\text{O}_9$:

C, 57.03; H, 7.74; N, 9.84. Found: C, 57.34; H, 7.71; N, 9.86.

The picrate was converted to the chloride, designated (DB-3) by passing its solution through a Dowex 1-X4 chloride anion exchange column. The eluting solvent mixture was acetone-methanol-water (6:2:1). An IR spectrum of DB-3 showed the following absorption pattern: 3360 cm^{-1} (b,m), 2950 (sh,s), 2850 (sh,s), 1730 (s), 1650 (m), 1470 (b,m), 1375 (m), 1240 (b,s), 1177 (b,m), 1130 (w), 1030 (w), 957 (w).

Methanolysis of the chloride in refluxing 2N methanolic sulfuric acid was followed by hexane extraction. The hexane residue, a small amount of colorless oil, gave a negative Dragendorff test. The IR spectrum of this compound in carbon tetrachloride showed the following absorption bands (Figure 4):

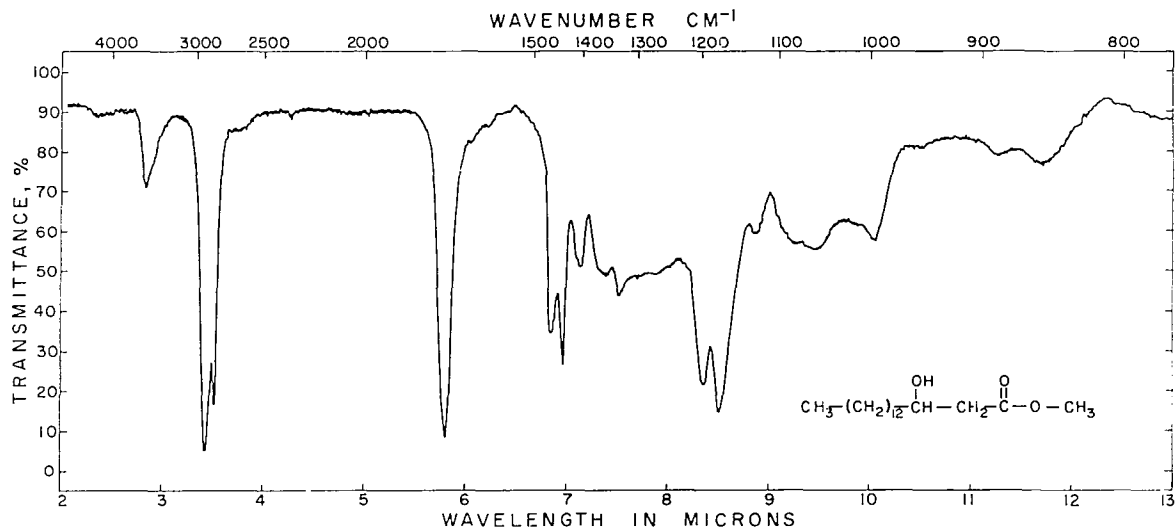
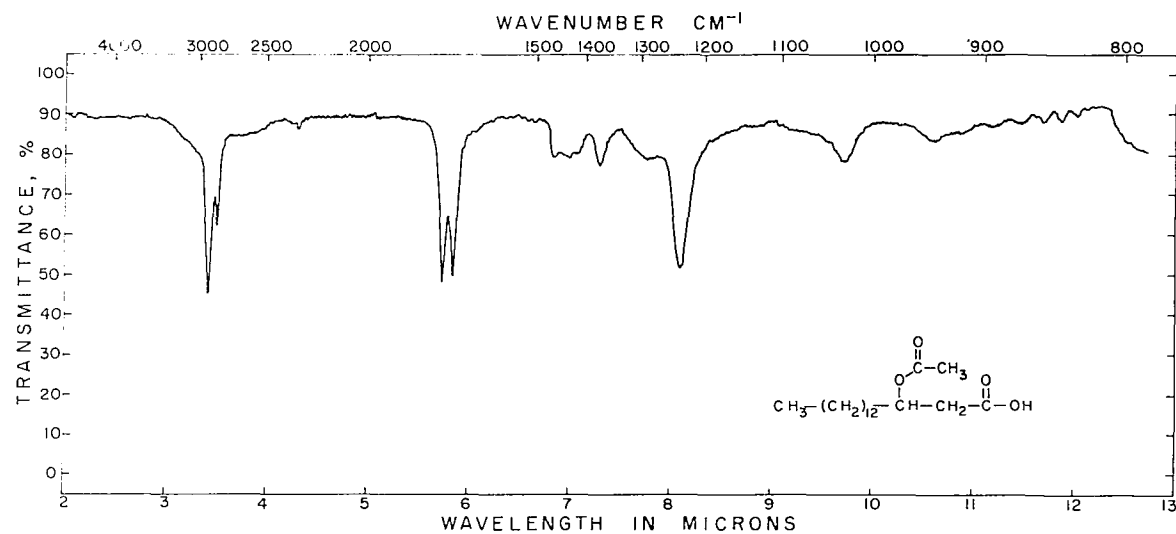


Figure 6. IR Spectra (carbon tetrachloride) of:
A. 3-Acetoxyhexadecanoic acid
B. Methyl 3-hydroxyhexadecanoate.

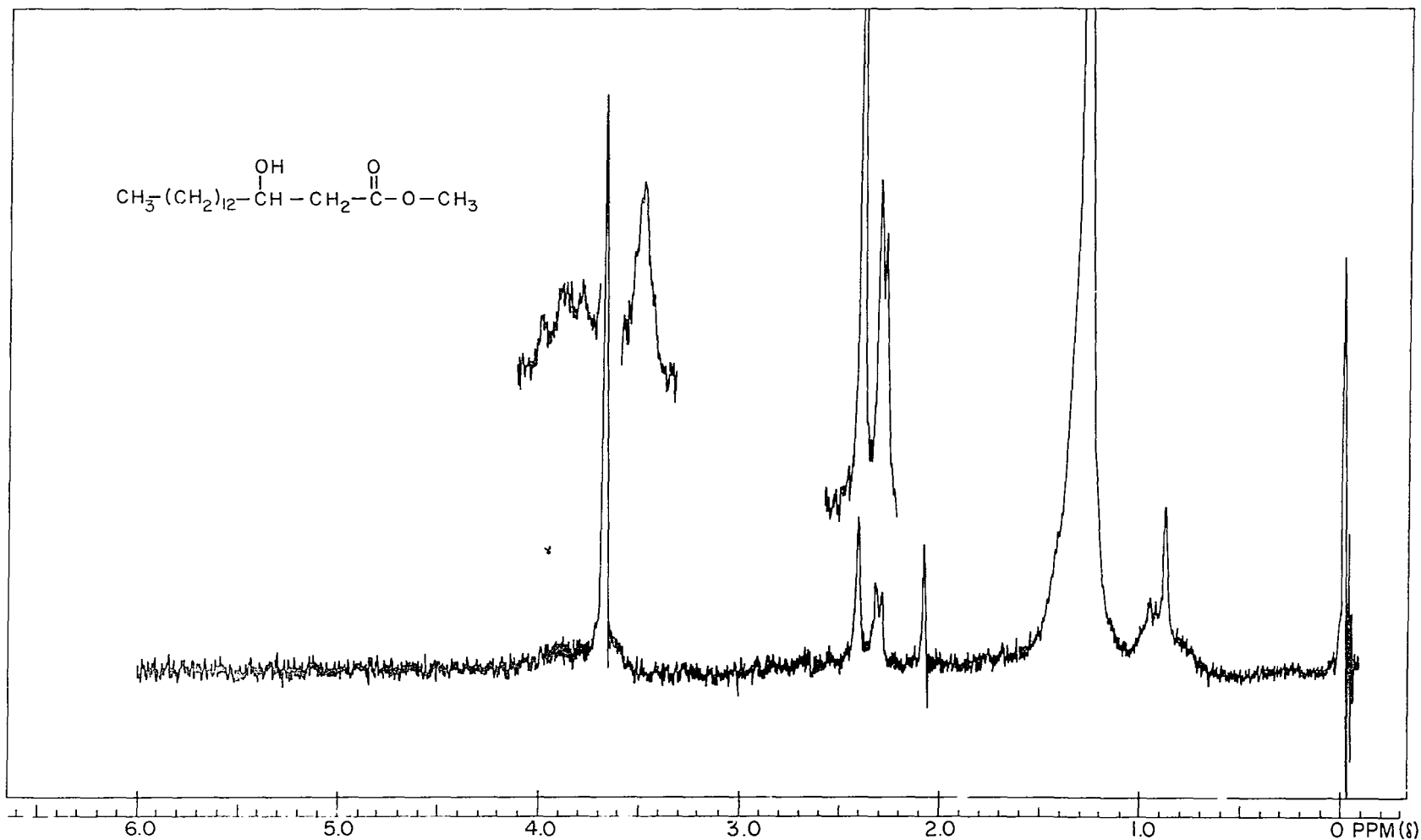


Figure 7. NMR Spectrum of Methyl 3-hydroxyhexadecanoate in Carbon tetrachloride.

2950 cm^{-1} (sh,s), 2850 (sh,m), 1725 (sh,s), 1660 (m),
1470 (m), 1440 (m), 1410 (2), 1320 (b,w), 1270 (m),
1200 (m), 1177 (m), 1130 (w).

Acidic methanolysis of pahutoxin at elevated temperatures. Pahutoxin (100 mg) was placed in 2 N methanolic sulfuric acid for 3 hr at 60° C. The acidic methanol solution was then extracted with hexane. After washing with methanol, the hexane fraction was taken to dryness. The resulting white crystalline solid, m.p. 46-48° C, accounted for over half of the expected product. An IR spectrum of this material in carbon tetrachloride showed the following absorption bands (Figure 6-B):
3550 cm^{-1} (w), 2930 (sh,s), 2850 (sh,s), 1725 (sh,s),
1470 (sh,m), 1440 (sh,m), 1400 (w), 1330 (w), 1200 (sh,s),
1178 (sh,s), 1130 (w), 1000 (w).

The NMR spectrum showed the following signals (Figure 7): 0.98 δ triplet (3), 1.38 (24), 2.3-doublet (2), 3.5 (1), 3.7-singlet (3), 3.8-multiplet (1).

Basic Hydrolysis of pahutoxin at room temperature. A 1N sodium bicarbonate hydrolysis of pahutoxin (400 mg) was conducted overnight at room temperature. The hydrolysate was neutralized with 1N sulfuric acid and extracted with butane. The butanol residue (356 mg) was chromatographed on silicic acid using a hexane/diethyl ether elution scheme. The first fraction (156 mg) eluted with 1 per cent diethyl ether/hexane gave an IR and NMR

identical with that of DB2. At 17 per cent diethyl ethyl/hexane, a colorless oil residue (78 mg) was eluted which partially crystallized on cooling. This residue sublimed at 95° C (pump pressure) to give a white crystalline compound, m.p. 44°-45° C.

Anal. Calcd for C₁₈H₃₄O₄:

C, 68.78; H, 10.86.

Found: C, 68.74; H, 10.75.

The IR spectrum showed the following absorption bands (Figure 7-A): 2930 cm⁻¹ (sh,s), 2850 (sh,s), 1740 (sh,s), 1715 (sh,s), 1460-1400 (w), 1375 (w), 1235 (b,s).

The NMR spectrum gave the following signals (Figure 8): 0.98^δ-triplet (3), 1.3 (24), 2.1-singlet (3), 2.64-doublet (2), 5.25 multiplet (1).

Acid hydrolysis of pahutoxin for detection of volatile products. Pahutoxin (100 mg) was added to a solution of 1 ml of trifluoro-acetic acid in 5 ml of deuterium oxide and heated to 60° C for 12 hr in a moisture-tight distillation system. The volatiles were distilled and analyzed directly by NMR. The only volatile compound detected gave a singlet at 2.18 that grew in intensity when acetic acid was added.

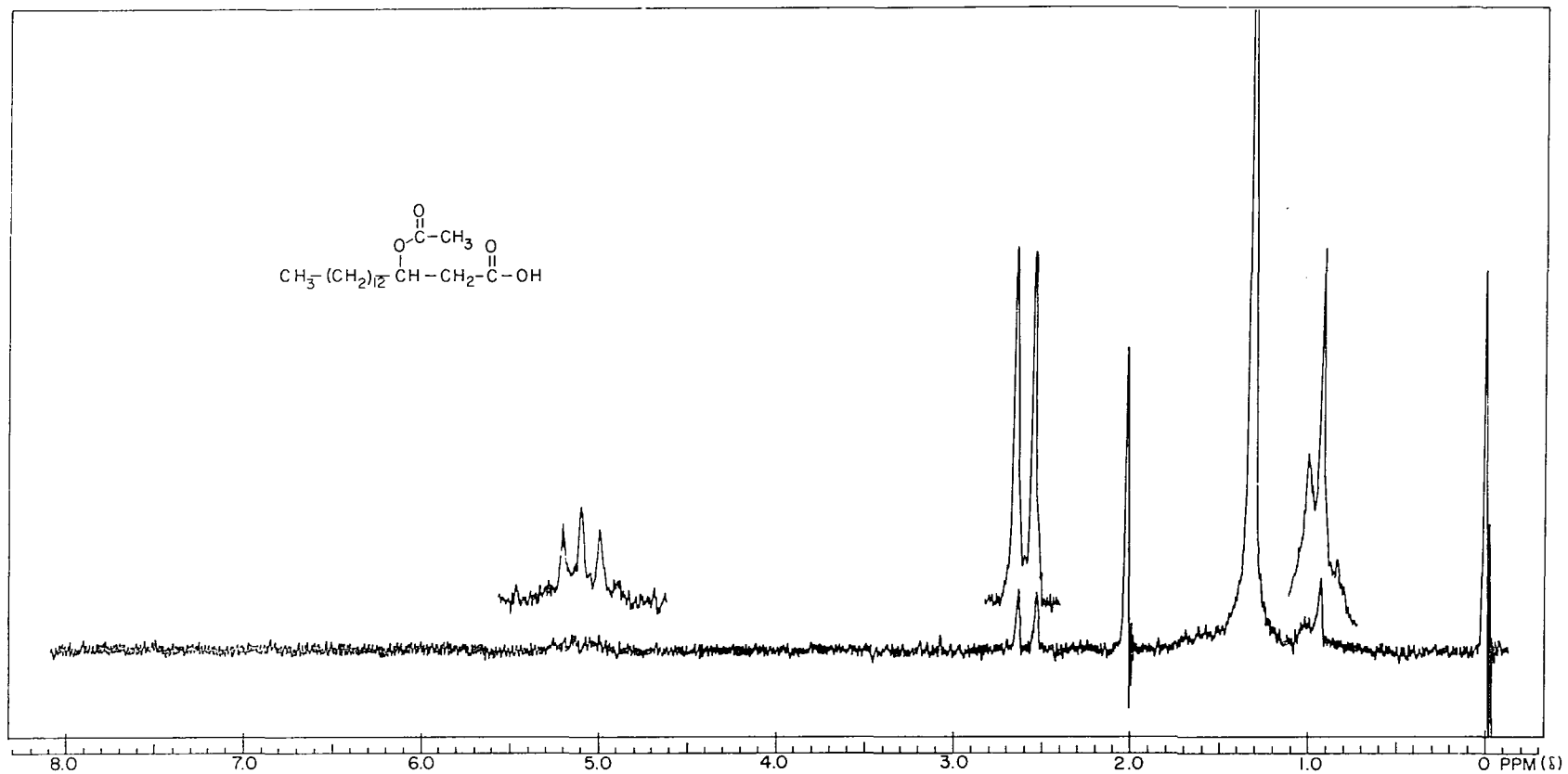


Figure 8. NMR Spectrum of 3-Acetoxyhexadecenoic acid in Carbon tetrachloride.

D. Synthesis of Pahutoxin and Homologues.

Several schemes for the synthesis of pahutoxin were investigated.

1. The first scheme involved conversion of 3-iodohexadecanoic acid to 3-acetoxyhexadecanoic acid. Since this was a preliminary investigation to determine the worth of such a reaction scheme, none of the synthetic products were purified. All reported yields are approximate and have been determined from NMR spectral evidence.

2-Bromohexadecanoic acid. Five gram of Eastman Kodak grade palmitic acid (20 mmoles) and 12 ml carbon tetrachloride were added to 0.5 ml of phosphorus trichloride and heated to reflux. The solution was then allowed to cool and a small amount of red phosphorus was added. Bromine 1.5 ml was introduced slowly and a gentle reflux was maintained for 12 hr.¹⁹ The volatiles were removed under vacuum and the residue was dissolved in butanol, washed with water and concentrated in vacuum. The resulting product, a yellowish solid, was not further purified.

The NMR spectrum of this crude product showed a triplet at 4.2 δ (.6-.7), a strong signal at 1.38 δ (28-29), and a triplet at 0.9 δ (3). This evidence suggested a yield of 2-bromohexadecanoic acid in the vicinity of 70 per cent.

2-Hexadecenoic acid. The crude 2-bromohexadecanoic acid product was added to 150 ml of anhydrous tert.-butyl alcohol containing 16 g (0.16 mmole) sodium tert.-butoxide. The solution was stirred for 3 hr at reflux temperature. After neutralization with 6 N sulfuric acid and removal of tert.-butyl alcohol, the aqueous solution was extracted with 1-butanol. The organic layer was washed with water and concentrated under vacuum. The resulting light yellow semi-solid showed NMR signals at: 0.98 δ , 1.38, 2.28 (3), 5.4 (1/3), 5.58 (1/2), 5.83 (1/2), 6.8 (1/2), 7.1 (1/2). All but the signal at 5.58 were identical with signals shown in Figure 5. Based on NMR evidence the reaction yield was estimated at 85 per cent or 2.8 g (11 mmoles) of 2-hexadecenoic acid.

3-Iodohexadecanoic acid. To 2.5 g of crude 2-hexadecenoic acid (approximately 1.3 g or 6 mmoles of pure 2-hexadecenoic acid) was added 15 ml chloroform and 20 ml acetic acid. The solution was then treated with an excess of hydrogen iodide.^{20,21} The reaction mixture was allowed to stand for 12 hr and was refluxed for 1 hr to remove excess hydrogen iodide. The solvent was removed by vacuum distillation and the remaining residue was poured into cold water. The precipitate, approximately 3.4 g, was a light pink solid and gave the following NMR spectrum: 0.98 δ -triplet; 1.35; 2.2 (2); 3.1-doublet; 4.3 (1) multiplet.

3 Acetoxyhexadecanoic acid. Several methods were attempted for the synthesis of 3-acetoxyhexadecanoic acid.

The first method involved reaction of crude 3-iodohexadecanoic acid (100 mg) with a solution of sodium acetate saturated acetic acid at 60° C for 2 hr. The reaction gave a major product whose NMR spectrum was identical with that shown in Figure 5.

Stirring 300 mg of crude 3-iodohexadecanoic acid with silver acetate in acetic acid for 12 hr at room temperature gave a complex mixture of products that could be partially separated on silicic acid using a hexane/diethylether elution scheme. The major component gave an NMR spectrum identical with that of Figure 5. Other minor components detected were starting material, a compound with an IR absorption at 1820 cm^{-1} , the desired compound with two IR absorptions at 1740 and 1710 cm^{-1} , and several unidentified compounds.

Crude 3-iodohexadecanoic acid (300 mg) stirred with an excess of silver acetate in acetone for 16 hr gave 2 reaction products. The major product showed a strong absorption in the IR at 1820 cm^{-1} ; the minor component was the desired product as indicated by IR spectrum containing strong absorption at 1740 and 1710 cm^{-1} . Reaction of the crude product (124 mg) with 121 mg (1.179 mmoles) of acetic anhydride and one drop of concentrated sulfuric acid at 90° C for 1 hr²² resulted in the

disappearance of the IR 1820 cm^{-1} band in the IR spectrum and enhancement of the 1740 and 1710 cm^{-1} bands of the desired product. Chromatography of the crude product (149.5 mg) on silicic acid using a hexane/ether elution scheme resulted in 40 mg of pure white residue and showed an IR spectrum identical with that shown in Figure 6-A.

2. The most useful method of synthesis was the application of a modified Reformatsky reaction.²⁴

Tetradecanal. Tetradecanol 30 g (0.14 moles) was oxidized by 66.6 g lead tetraacetate (0.15 moles) in 700 ml of anhydrous pyridine to tetradecanal.²³ The initial dark brown solution turned colorless after 12 hr of stirring. The solvent was removed under vacuum. The solid residue was triturated with hot hexane and the hexane solution was cooled in an ice bath. The unreacted tetradecanol crystallized from solution leaving relatively pure tetradecanal dissolved in hexane. Removal of hexane yielded 20 g (0.094 moles) of pure aldehyde. The NMR spectrum of this solid white residue showed the following signals: $0.98\text{ }\delta$ -triplet (3), 1.3 (22), 2.2 (2), 9.2-triplet (1).

3-Hydroxyhexadecanoic acid. Tetradecanal 20 g (0.094 moles) and ethyl bromoacetate (32 ml, 0.284 moles) were dissolved in 100 ml anhydrous benzene and placed in a separatory funnel. Zinc (16 g, 0.415 moles) was placed in a 3-necked round bottom flask equipped with a mechanical

stirrer and a downward condenser. The zinc was covered with anhydrous benzene and the benzene was distilled to remove all traces of water in the system. The round bottom flask was then equipped with a reflux condenser containing a calcium chloride drying tube. A small amount of pre-dried nitrogen was continuously passed through the system to prevent accumulation of moisture through system leaks.

Ten milliliter of the solution of reagents was introduced to the flask and the contents were heated in order to initiate the reaction. The remainder of the reactants was added at such a rate so as to maintain a gentle reflux. After 4 hr of additional refluxing the mixture was cooled and poured into cold 10 per cent sulfuric acid. The product was extracted with ether and washed with 10 per cent sodium bicarbonate. After removal of the solvent, the residue was hydrolyzed with 10 per cent ethanolic potassium hydroxide at 50° C for 3 hr. Acidification of the hydrolysis mixture, extraction with ether and crystallization of the ether residue from carbon tetrachloride gave 5.56 g of white crystalline solid mp 183.5° C.

The NMR spectrum of this product showed the following signals: 0.98 δ -triplet (3), 1.3 (24), 2.35 (2), 4.0 (1), 3.5 (1).

3-Acetoxyhexadecanoic acid. Acetylation of 5.56 g of 3-hydroxyhexadecanoic acid (20.5 mmoles) with 6.8 ml of acetyl chloride (80 mmoles) was accomplished after 24 hr

of stirring at room temperature. The reaction residue was washed with water and dried under vacuum. Chromatography of the reaction products on silicic acid using a hexane/ether elution scheme gave 4.0 g of crystalline white compound m.p. 45° - 46° C. The IR and NMR spectra of this residue were identical with those of the natural hydrolysis product shown in Figures 6-A and 8.

Choline 3-acetoxyhexadecanoate. To 0.72 ml of thionyl chloride (10 mmoles), 1.5 g of 3-acetoxyhexadecanoic acid (4.8 mmoles) in 10 ml of chloroform (passed through woelm alumina) was added slowly with stirring. After 1 hr the excess thionyl chloride was removed under vacuum and the resulting acid chloride was added to 1.4 g (13.4 mmoles) of anhydrous powdered choline. The contents were stirred for 12 hr and the residue was triturated with diethyl ether to remove unreacted 3-acetoxyhexadecanoic acid.

The insoluble residue was triturated with hot acetone and the acetone soluble residue was chromatographed on silicic acid using a chloroform/methanol elution scheme. The choline 3-acetoxyhexadecanoate fraction was detected using the Dragendorff test. The residue, accounting for over a 50 per cent conversion in this reaction, was passed through picric acid coated Dowex 1-X4 chloride anion exchange resin using anhydrous ethanol as a solvent. The product, a yellowish solid, was triturated

with anhydrous ether. The white residue crystallized in long colorless needles from acetone and melted at 60°-61° C.

Crystalline material for analysis was dried under vacuum for 8 hr at 35° C and sealed in a vacuum ampule.

Anal. Calcd. for $C_{23}H_{46}NO_4Cl$: C, 63.30;

H, 10.55; N, 3.21.

$C_{23}H_{48}NO_5Cl$: C, 60.78;

H, 10.58; N, 3.01

Found: C, 60.64, 60.65; H, 10.40, 10.49;

N, 3.13, 3.18.

Crystalline natural pahutoxin was processed under identical conditions and submitted for combustion analysis.

Anal. Calcd. for $C_{23}H_{44}NO_5Cl$: C, 61.33; H, 9.77;

N, 3.11.

Found: C, 61.81, 61.65; H, 9.89, 9.95;

N, 3.29, 3.20.

The NMR and IR spectra of choline 3-acetoxyhexadecanoate were identical with those of natural pahutoxin shown in Figures 2 and 3.

Mass spectral data of choline 3-acetoxyhexadecanoate and natural pahutoxin showed a majority of fragments below m/e 100 with major peaks at m/e 58 and 71. Small but detectable peaks appearing at m/e 237, 281, 325 were not accompanied by resolved metastable peaks. Peaks at m/e 660 and 580 in the natural toxin were absent in the

synthetic product and a peak at 405 m/e in the synthetic product was absent in the natural pahutoxin spectrum.

Synthesis of homologues. Choline esters of 3-acetoxynonanoic, dodecanoic and tetradecanoic acids were synthesized by the same route. Data concerning the intermediates and products synthesized are summarized in Table I.

E. Biological Testing of Pahutoxin and Synthetic Homologues

1. Toxicity to fish

Toxicity studies were conducted using brackish water mollies (Mollienesia latipinna Le Seur) as test fish.¹⁰ This fish was chosen because it is reasonably sensitive to pahutoxin, is readily available in local brackish water streams, and can survive equally well in fresh and salt water. Although the test fish were not weighed or measured prior to testing, the size was kept reasonably constant (pre-adults). Slight variations in size seemed to have less effect on the time of death than did individual fish resistance to the toxin. For this reason a minimum of four fish were tested for each concentration of toxin.

Pure crystalline toxin was dried in a desiccator containing calcium chloride for one week. A sample was weighed accurately and dissolved in a specific volume of fresh water using a volumetric flask. Aliquots were then taken from this sample and diluted with fresh water in

Table I. Synthetic Homologues of Pahutoxin

Reformatsky products: ethyl esters of -	Potassium hydroxide hydrolysis products:	Acetylation products:	Esterification products: choline esters of -
1) 3-hydroxynonanoic acid	3-hydroxynonanoic acid	3-acetoxynonanoic acid	3-acetoxynonanoic acid
a) b.p. 100-102° C (1-2 mm)	crystallized from anhydrous ether/hexane	IR- 2 carbonyl peaks-1740 cm ⁻¹ 1710	a) reaction yield calcd. by NMR - 75%
b) yield calcd. by NMR - 75%			b) product purified on silicic acid; sol. in chloroform; Dragendorff positive; crystallized from anhydrous ether/ acetone.
c) NMR (neat soln.) 4.4-3.9 (4), 2.4-doublet (2), 1.3-9.0 (16)			c) NMR (deuteriochloro- form)-0.9 triplet (3), 1.35 (10) 2.1-singlet (3), 2.6-doublet (2), 3.6 (9), 4.2 (2), 4.6 (2), 5.2 (1).

Table I. (continued)

2) 3-hydroxydodecanoic acid	3-hydroxydodecanoic acid	3-acetoxynonanoic acid	3-acetoxydodecanoic acid
a) b.p. 143 C. (1-2 mm) b) yield calcd. by NMR 78% c) NMR (neat soln.)- 4.4-3.9 (4), 2.4-doublet (2), 1.3-9.0 (22)	crystallized from hexane	IR- 2 carbonyl peaks-1740 cm ⁻¹ 1710	a) product purified by silicic acid: sol. in chloroform; Dragen- dorff positive; crystallized from acetone b) NMR (deuteriochloro- form)- 0.9 -triplet (3), 1.35 (16), 2.1-singlet (3), 2.6-doublet (2), 3.6 (9), 4.2 (2), 4.6 (2), 5.2 (1)
3) 3-hydroxytetra- decanoic acid	3-hydroxytetra- decanoic acid	3-acetoxytetra- decanoic acid	3-acetoxytetradecanoic acid
a) b.p. 163 ^o C (1-2 mm) b) yield calcd. by NMR 66% c) NMR (neat soln.) 4.4-3.9 (4), 2.4-doublet (2), 1.3-0.9 (26)	crystallized from hexane	IR- 2 carbonyl peaks-1740 cm ⁻¹ 1710.	a) product purified by silicic acid chromatography: chloroform soln.; Dragendorff positive; precipitated from acetone. b) NMR (deuteriochloro- form)- 0.9 (3), 1.35 (20), 2.1-singlet (3), 2.6-doublet (2), 3.6 (9), 4.2 (2), 4.6 (2), 5.2 (1).

order to obtain desired concentrations. Brackish water mollies were submerged in 5 ml volumes of various concentrations of pahutoxin and the toxic effects were noted (Table II). The inverse of survival time, or averaged individual death times in minutes, was plotted against the corresponding concentration in mg/ml (Figure 9). In order to make the graph more significant, the range of survival times averaged for each concentration of toxin was indicated by an extension from each point.

Using the above procedure, samples of synthetic pahutoxin and homologues were also tested for toxicity to fish (Table II) and their inverse survival times were plotted against concentration (Figure 9).

2. Hemolytic test²⁵

Red blood cells from big eye tuna blood were washed with a modified Alsevier's buffer solution consisting of glucose (10.5 g), citric acid monohydrate (0.55 g), sodium citrate dihydrate (8.00 g), and sodium chloride (4.20 g) in one liter of water. The cell suspensions were centrifuged and rewashed until the wash solution was colorless. If more than four washings were required, the blood was discarded and a new sample obtained. These washed cells were diluted with Alsevier's buffer until total hemolysis of 1 ml of this suspension diluted to 8 ml with 0.1 N sodium bicarbonate solution gave an optical density reading of 0.68 at the 541 m μ wave

Table II. Toxicity of Pahutoxin and Synthetic Homologues Toward Fish.

Av. Tox. is average death time in minutes of four fish (Mollienesia lalipinna Le Seur).

$\frac{1}{\text{Av. Tox.}}$ is the reciprocal of the average death time.

Natural Pahutoxin	Concentration (mg/ml)	Av. Tox.	$\frac{1}{\text{Av. Tox.}}$
	0.0056	60	0.017
	0.0060	14	0.072
	0.0090	13	0.077
	0.012	11	0.089
	0.015	10	0.096
	0.018	8.5	0.12
	0.048	5.0	0.20
Synthetic Toxin	0.0038	0.0	0.00
	0.0050	41	0.024
Choline 3-acetoxy-hexadecanoate	0.010	12	0.083
	0.021	7.0	0.14
	0.068	5.0	0.20
	0.24	4.3	0.22
Choline 3-acetoxy-tetradecanoate	0.017	24	0.041
	0.024	17	0.058
	0.034	13	0.074
	0.042	12	0.081
	0.051	8.0	0.12
	0.080	5.6	0.17
	0.12	5.0	0.20
	0.25	4.7	0.21
Choline 3-acetoxy-dodecanoate	0.050	0.0	0.013
	0.10	80	0.068
	0.15	14	0.084
	0.20	12	0.11
	0.25	8.0	
Choline 3-acetoxy-nonanoate	0.24	0.0	0.00
	1.2	33	0.030

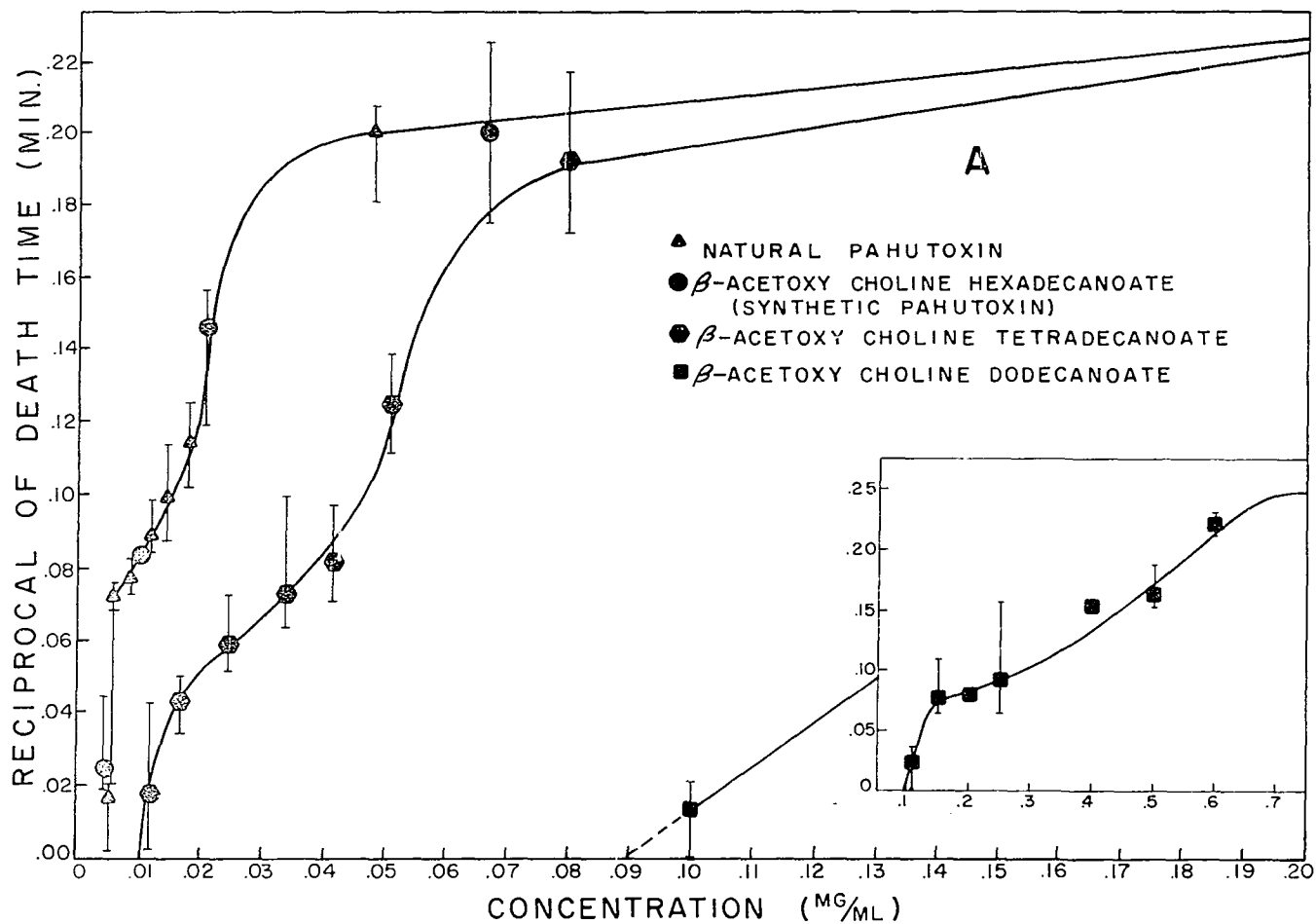


Figure 9. Reciprocal of Death Time (Min^{-1}) Versus Concentration (Mg/Ml)

Each point corresponds to the reciprocal of the average death time in minutes of four or more fish submerged in a specific concentration of toxin. The concentration range in the inset is expanded to include significant toxicity values for 3-acetoxycholine dodecanoate. The reciprocal of death time is plotted to allow a direct comparison of the toxicity versus concentration curve (A) with hemolysis versus concentration curve (B), Figure 10.

length of a Beckman DB double beam spectrophotometer. This standard suspension was placed in ice water and used as soon as possible.

Samples of pahutoxin and synthetic homologues were weighed accurately and diluted with Alsevier's buffer to a known volume. Aliquots of these samples were diluted to specific volumes in order to obtain accurate concentrations. To each 7 ml sample 1 ml of standard red cell suspension was added, and the resulting suspension was allowed to stand for 10 min, followed by centrifuging for 3 min at 2,000 rpm. Allowing more than 10 min for the reaction or more than 3 min for centrifuging resulted in peptization of the ghost cell walls causing useless colloidal suspensions. All optical densities were measured with a double beam Beckman DB Spectrophotometer at the 541 m wave length, using the centrifuged solution from an identical portion of unhemolyzed blood cells as the reference solution. The observed optical density over the optical density of a totally hemolyzed identical blood sample was reported as the per cent hemolysis or the per cent lysis (Table III). These hemolytic values were plotted against concentration in mg/ml (Figure 10).

Table III. Hemolytic Ability of Pahutoxin and Synthetic Homologues Toward Big Eye Tuna Red Blood Cells

% Lysis is a measure of the ability of the toxin to hemolyze or rupture red blood cells.

Natural Pahutoxin	Concentration (mg/ml)	% Lysis
	0.0030	5.0
	0.0050	8.0
	0.0060	10
	0.0080	28
	0.0090	40
	0.010	46
	0.011	65
	0.015	72
	0.020	82
	0.022	96
	0.025	97
	0.036	100
	0.048	100
	0.15	100
<hr/>		
Synthetic Toxin		
Choline 3-acetoxyhexa- decanoate	0.004	6.0
	0.006	10
	0.009	29
	0.010	35
	0.013	55
	0.016	72
	0.020	88
	0.025	97
	0.035	100
	0.040	100
	0.180	100

Table III (continued)

Choline 3-acetoxytetra- decanoate	0.015	1.0
	0.020	3.0
	0.025	5.0
	0.030	5.0
	0.040	8.0
	0.055	15
	0.062	42, 46
	0.071	61
	0.075	90
	0.080	97
	0.090	99
	0.100	100
0.120	100	
<hr/>		
Choline 3-acetoxydo- decanoate	0.100	0.0
	0.150	1.0
	0.250	3.0
	0.300	3.0
	0.350	3.0
	0.450	6.0
	0.500	10
	0.600	17
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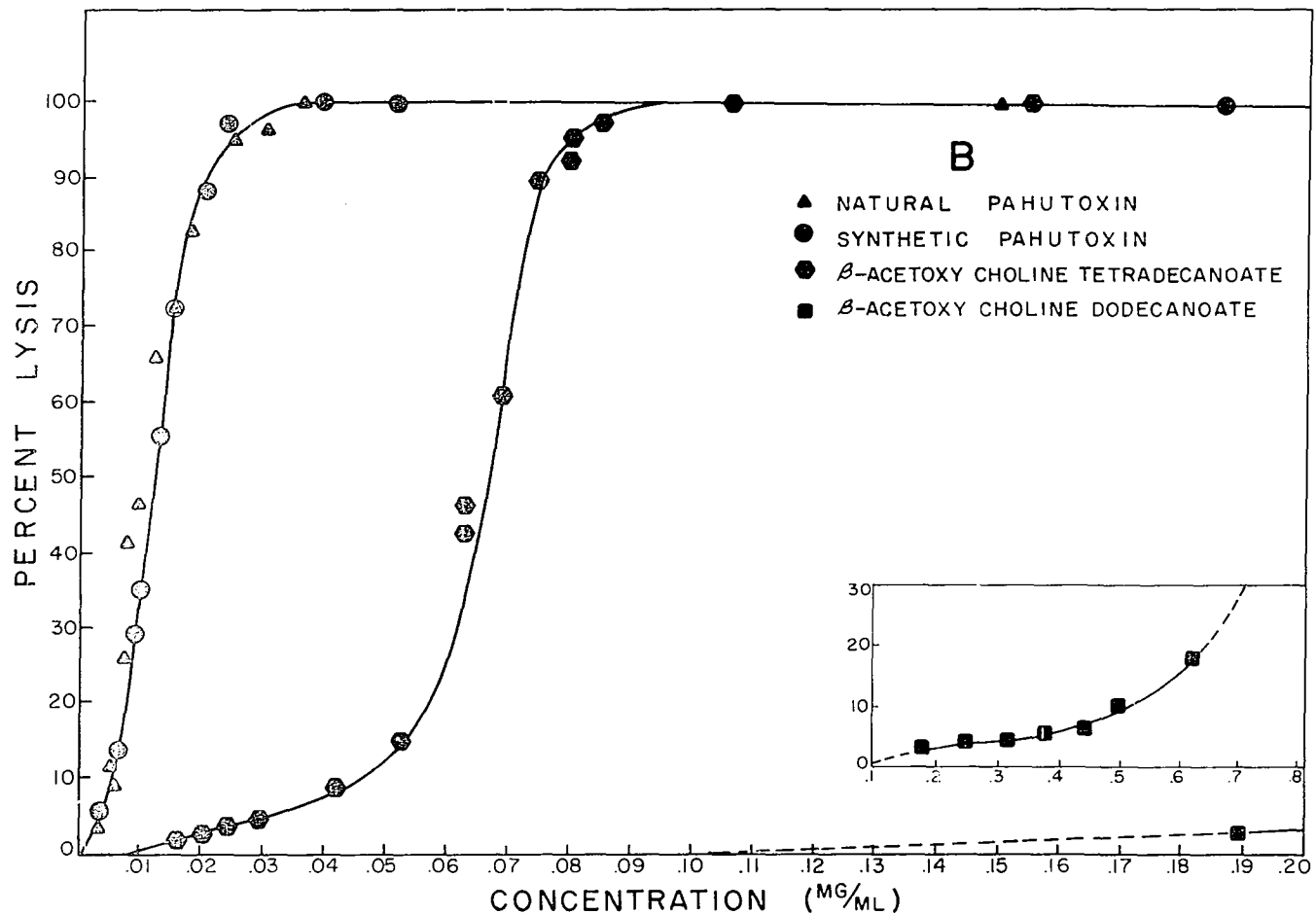


Figure 10. Percent Lysis Versus Concentration (Mg/ML).

Percent lysis is a measure of the ability of the toxin at a certain concentration (mg/ml) to hemolyze or rupture big eye tuna red blood cells. The concentration range in the inset is expanded to include significant hemolysis values for 3-acetoxy choline dodecanoate. The toxicity versus concentration curve (A), (Figure 9) can be compared directly with % lysis versus concentration, curve (B).

F. Pharmacology of Pahutoxin

The effect of pahutoxin on the anesthetized rat was determined using the Grass Polygraph Model 5D. Intravenous administration of boxfish toxin through the jugular vein caused blood pressure and respiration responses as shown in Figure 11.

Pahutoxin (200 $\mu\text{g}/\text{gm}$ mouse weight) was injected intraperitoneally and the symptoms were recorded.

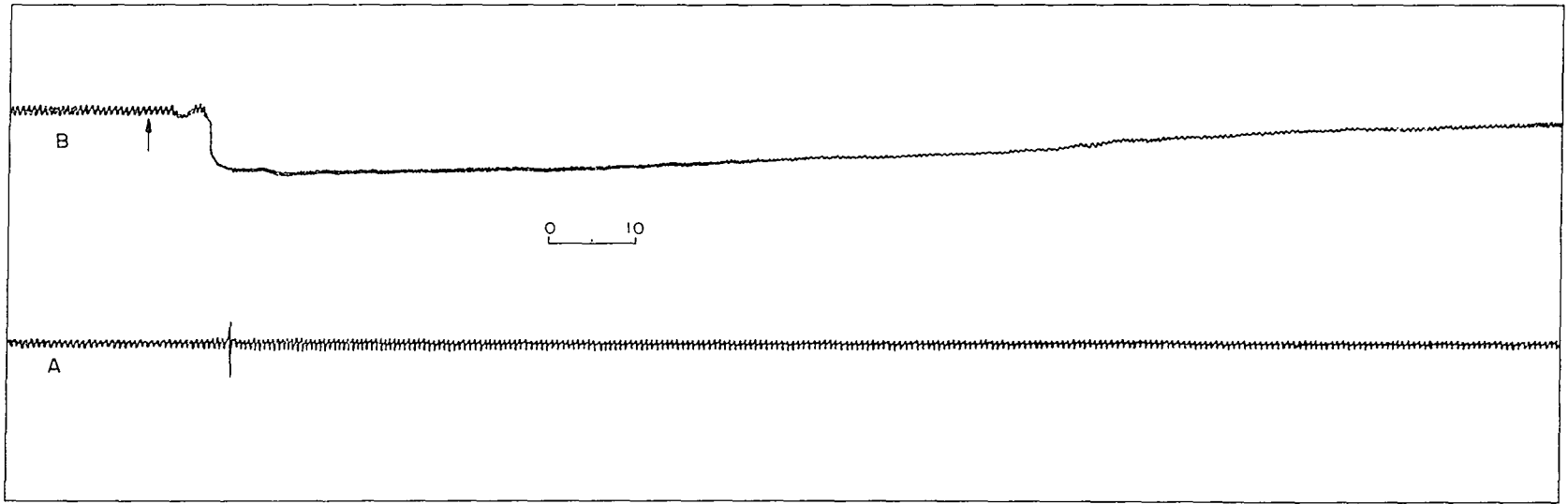


Figure 11. Effect of Pahutoxin on an Anesthetized Rat:

- A. Respiration Response
- B. Blood Pressure Response

Pahutoxin was injected intravenously through the jugular vein and signs were recorded by the Grass Polygraph Model 5 D. Scale indicates seconds.

III. DISCUSSION OF RESULTS

A. Isolation and Purification

During the search for suitable isolation methods it became evident that pahutoxin was relatively unstable. A useful isolation scheme, therefore, needed to be gentle, efficient, and fast. The initial isolation scheme did not meet these requirements and had to be modified drastically.

The final extraction and purification process required only one day for conversion of the crude toxic aqueous secretions to the final crystalline product. Loss of toxicity, caused by enzymatic or chemical hydrolysis of crude aqueous extracts, was reduced to a minimum by immediate butanol extraction. The butanol residue was chromatographed on silicic acid to yield a semipure toxic principle which could be precipitated from hot acetone. A pass of this toxin through a picric acid-coated anion exchange resin yielded a yellow solid which, after being washed with ether, crystallized in long colorless needles from hot acetone. From IR and NMR spectra it was evident that this crystalline material was identical with the previously obtained amorphous pahutoxin. This picric acid coated anion exchange treatment can not be rationalized, since passing the toxin through uncoated anion exchange resin, addition of picric acid to pahutoxin, addition of

small amounts of water, and other possible modifications did not provide crystalline material. However, since this method provided a means of obtaining crystalline toxin it was incorporated into the isolation scheme (Figure 1). By this procedure it was possible to isolate as much as 60 mg of crystalline toxin from one adult boxfish.

B. Pahutoxin

Pahutoxin gave a strongly positive Dragendorff test which indicated the presence of a tertiary or quaternary nitrogen. A positive silver nitrate test for ionic halides further suggested that the nitrogenous portion of the molecule was quaternary in nature.

Elemental analysis of pahutoxin, recrystallized to a constant melting point and constant optical rotation, gave results from which no unequivocal empirical formula could be derived. The analytical data seemed to fit best an approximate composition of $C_{23}H_{44}NO_5Cl$. However, this formula did not agree with structural information from other sources.

The NMR spectrum of pahutoxin (Figure 3) proved to be quite useful. The strong signal at 3.6δ (9) and the two signals at 4.2δ (2) and 4.6δ (2) suggested the presence of a choline moiety. A strong signal at 1.35δ (24) and a triplet at 0.9δ (3) indicated the presence of a normal aliphatic chain of thirteen carbons. The other signals provided no initial clues to the structure.

The IR absorption spectrum (Figure 4) further confirmed our conclusions. Absorption at 3320 cm^{-1} band suggested a large saturated aliphatic portion, and the 1730 cm^{-1} and 1250 cm^{-1} bands showed that one or more carbonyl groups were incorporated in the molecule as ester functions.

Conductometric and pH titration studies indicated that hydrolysis of pahutoxin occurred under weakly basic conditions and suggested that hydrolytic studies would be useful.

The first hydrolysis scheme which was investigated involved a stringent basic hydrolysis since such a process should yield simple, easily identifiable hydrolysis fragments. Hydrolysis of pahutoxin in 1 N sodium bicarbonate was carried out at 50°C for 3 hr. The butanol extract of the acidified hydrolysate gave a hexane-soluble oil residue designated as DB-2 and a hexane-insoluble residue. The hexane-soluble portion accounted for more than half of the total hydrolysate. The IR spectrum of DB-2 showed strong absorption in the 2900 cm^{-1} region which suggested a large aliphatic portion and a broad carbonyl absorption at 1725 cm^{-1} which when coupled with the absence of a strong absorption in the 1200 cm^{-1} region indicated a carboxylic acid group. The presence of an absorption at 1650 cm^{-1} suggested a carbon-carbon double bond.

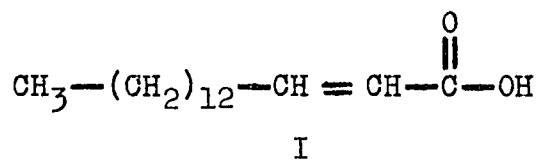
The NMR spectrum of DB-2 (Figure 5) further supported the IR assignments. A strong $1.35\text{ } \delta$ (22) signal and a

triplet at 0.85δ (3) indicated a normal twelve-carbon chain. Four triplets at 5.5, 5.8, 6.85 and 7.1δ each integrating for (1/2 H) suggested a double bond conjugated to a carbonyl function. Resonance between the carbonyl group and the double bond would cause the carbon atom beta to the carbonyl group to become sufficiently electro-positive to move the signal of a hydrogen attached to it down-field from a normal olefinic position. Comparison of the NMR spectrum with that of crotonic acid²⁶ confirmed this assignment.

Esterification of this compound in (DB-2) 2 N methanolic sulfuric acid resulted in a colorless oil whose IR spectrum is shown in Figure 4. The presence of a strong absorption in the 1200 cm^{-1} region and the sharpness of the 1730 cm^{-1} carbonyl band indicated that the methyl ester had formed. The signal at 3.6δ (3) in the NMR provided further evidence for the methyl ester.

Hydrogenation of 50 mg of this ester gave a colorless oil whose NMR showed signals at 0.85δ (3) and 1.3δ (26) indicating a normal fourteen-carbon chain, at 3.6δ (3) indicating a methyl ester, and at 2.5δ (2) indicating the lack of substitution on the carbon alpha to the carbonyl. This spectrum was identical with that of methyl hexadecanoate which was synthesized from hexadecanoic acid.

The structure of DB-2, therefore, was 2-hexadecenoic acid (I).



Investigation of the hexane-insoluble products of this basic hydrolysis reaction provided IR evidence for other compounds in trace amounts. Of particular interest was a compound containing two carbonyl absorptions, one at 1740 cm^{-1} and the other at 1710 cm^{-1} . However, the small quantities present prohibited a detailed spectral study.

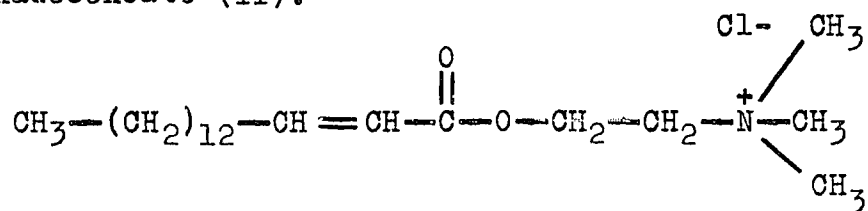
A milder hydrolytic scheme was suggested since it was desirable to learn more about the ester linkages in pahutoxin. When pahutoxin was placed in a solution of 0.1 N sodium bicarbonate and allowed to stand at room temperature for 24 hours, the resulting hydrolysate could be separated into two fractions, an aqueous portion (A) and a butanol extract (B).

A Dragendorff-positive, non-toxic substance was isolated from fraction (A) and was crystallized from 2-propanol. The IR and NMR spectra were identical with those of an authentic sample of choline chloride.

Fraction B also gave a positive Dragendorff test. However, attempts to crystallize this compound from numerous solvents failed. A yellow picrate precipitated in small yield from 80 per cent ethanol and was recrystallized from 75 per cent ethanol m.p. $100-102^{\circ}\text{C}$.

Elemental analysis suggested a formula of $C_{27}H_{44}N_4O_9$ which corresponds to a parent compound of composition $C_{21}H_{42}NO_2Cl$.

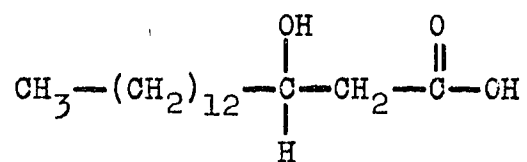
A corresponding chloride, designated as DB-3, was produced by passing the picrate through a Dowex 1-X4 chloride anion exchange resin; it had an IR absorption spectrum much like that of pahutoxin (Figure 2). However, the appearance of a sharp peak at 1650 cm^{-1} and loss of the 1220 cm^{-1} shoulder suggested a slight modification in structure. Further hydrolysis of DB-3 in refluxing 2 N methanolic sulfuric acid was followed by hexane extraction of the hydrolysate. The hexane residue, a colorless oil, gave an IR spectrum identical with that of the methyl ester of DB-2 (Figure 4). This hydrolysis product was therefore assigned the structure of methyl 2-hexadecenoate. Since choline was the only other logical hydrolysis product of this reaction, DB-2 was assigned the structure choline 2-hexadecenoate (II).



II

Infrared spectral analysis and combustion data of DB-2 supported the assigned structure.

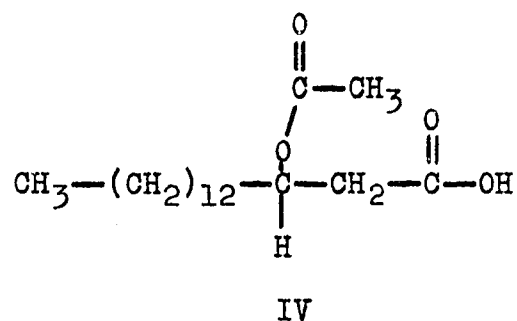
It became evident that basic hydrolysis of pahutoxin generally resulted in an unsaturated hydrolysis product. Therefore, acidic hydrolysis was investigated with the hope of isolating products that would provide more information concerning the base labile portion of pahutoxin. The methanolysis of pahutoxin in 2 N methanolic sulfuric acid for 3 hr followed by hexane extraction gave a hexane residue. This residue, a white crystalline compound melting at 46-48° C, accounted for a large portion of the expected hydrolysate. The IR spectrum (Figure 6) showed hydroxyl absorption at 3550 cm^{-1} , and absorption bands at 1730 and 1200 cm^{-1} indicating the presence of a methyl ester. The NMR spectrum (Figure 7) showed a singlet at 3.6 δ (3) indicating a methyl ester, a multiplet at approximately 3.5 δ assigned to the carbon bearing the hydroxyl group and the hydroxyl hydrogen, and a doublet at 2.3 δ (2) indicating the carbon alpha to the carbonyl group contained two hydrogens split by only one hydrogen on the beta carbon. From this evidence it was concluded that the hydroxy substituent had to be positioned on the beta carbon atom. The aliphatic signals at 0.9 δ (3) and 1.3 δ (24) indicated the presence of a normal thirteen-carbon chain. The only structure which fitted this information was 3-hydroxyhexadecanoic acid (III). This structure was proven through synthesis.



III

In an earlier hydrolysis experiment (1N sodium bicarbonate at elevated temperature) a compound with two carbonyl functions was detected in trace amounts. Since it was desirable to isolate more of this compound a milder hydrolytic scheme was attempted. Pahutoxin was hydrolyzed in 1 N sodium bicarbonate at room temperature for 24 hr. After neutralization and extraction with butanol, the butanol residue was chromatographed on silicic acid using a hexane/diethyl ether elution scheme. The residue of the 11 per cent diethyl ether/hexane eluate amounted to over 35 per cent of the butanol residue and its IR and NMR spectra were identical with those of 2-hexadecenoic acid. The 17 per cent diethyl ether/hexane eluate residue amounted to about 22 per cent of the butanol residue and appeared as a colorless oil. Sublimation of this oil gave a white crystalline compound (m.p. 44-45°) whose elemental analysis agreed with an empirical formula of $\text{C}_{18}\text{H}_{34}\text{O}_4$. The IR spectrum (Figure 8) demonstrated two carbonyl peaks at 1740 and 1720 cm^{-1} . The absorption at 1235 cm^{-1} suggested that at least one of these carbonyls was present as an ester. If this product was a precursor of the previously identified fragments,

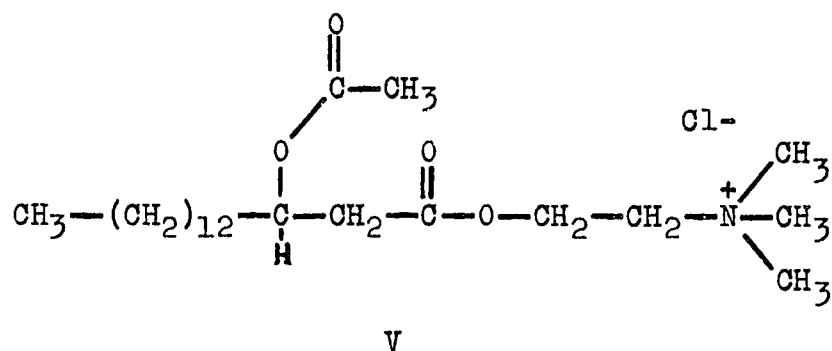
then it must have a two-carbon, carbonyl-containing functional group in a position that is easily eliminated in base to give 2-hexadecenoic acid or hydrolyzed in acid to give 2-hydroxyhexadecanoic acid. The logical compound which has these requirements would be 3-acetoxyhexadecanoic acid (IV).



Comparison of the NMR spectrum of this hydrolysis product (Figure 9) with that of methyl 3-acetoxybutyrate²⁷ strongly supported this structural assignment.

Further evidence supporting this structure (IV) was obtained with a hydrolysis scheme which was devised to enable detection of volatile hydrolysis products. Pahu-toxin was hydrolyzed in a strongly acidic deuteriated trifluoroacetic, deuterium oxide medium. The NMR of the distillate showed only one peak at 2.1 δ which increased in intensity as acetic acid was added. This provided conclusive evidence that acetic acid was the only volatile hydrolysis product.

The above degradative evidence indicated that pahu-toxin was choline 3-acetoxyhexadecanoate (V).



But, since the postulated structure (V) suggested a molecular formula of $\text{C}_{23}\text{H}_{46}\text{NO}_4\text{Cl}$ and combustion data suggested a formula of $\text{C}_{23}\text{H}_{44}\text{NO}_5\text{Cl}$,* positive identification was sought through synthesis.

C. Synthesis and Structure of Pahutoxin

The first synthetic scheme which was investigated concerned the conversion of 3-iodohexadecanoic acid to 3-acetoxylhexadecanoic acid. Since this was a preliminary investigation to determine the worth of such a reaction, the crude products were not purified and, therefore, only approximate yields could be estimated by NMR spectral evidence.

Synthesis of 2-bromohexadecanoic acid was accomplished using hexadecanoic acid, bromine, and phosphorus trichloride. An NMR spectrum of the yellow-brown product showed a triplet at 0.9δ (3) indicating a terminal aliphatic methyl group, strong absorption at 1.38δ (26) indicating

*Although these formula differ by H_2O --while pahutoxin is hygroscopic--we have no evidence for a single molecule of water.

an aliphatic thirteen-carbon chain and a triplet at 4.2δ ($.6-.7$) indicating that the carbon alpha to the carbonyl group contained bromine and hydrogen. The ratio of the 4.2δ integrated signal, accounting for one hydrogen in the 2-bromohexadecanoic acid, to the 0.98δ integrated signal, accounting for three terminal methyl hydrogens on both the product and the unreacted hexadecanoic acid, was 0.7 to 3.0. This indicated a ratio of 7 molecules of 2-bromohexadecanoic acid to 10 molecules of reaction product and unreacted material indicating an approximate reaction yield of 70 per cent.

Dehydrohalogenation of this crude 2-bromohexadecanoic acid with sodium tert.-butoxide in tert.-butyl alcohol resulted in a yellow semi-solid whose NMR spectrum, except for a signal at 5.4δ , was identical with that shown in Figure 5 and was assigned the structure 2-hexadecenoic acid. The 5.4δ signal was assigned to the olefinic hydrogens on a double bond not conjugated with the carbonyl group (probably the hexadecenoic acid). The ratio of the integrated signals at 5.58δ (1), 5.83 (1), 6.8 (1), and 7.1 (1) to the integrated signal at 5.4δ ($2/3$) was 6 to 1 indicating a reaction yield of 2-hexadecenoic acid of approximately 85 per cent or an overall yield of about 60 per cent.

Addition of hydrogen iodide to crude 2-hexadecenoic acid gave complete conversion to a saturated product. The

NMR spectrum of this product showed no olefinic hydrogen signals but did show a multiplet at 4.38δ (1) that was assigned to one hydrogen on a carbon bearing iodine and a doublet at 3.68δ (2) that could only be explained by two hydrogens on carbon alpha to the carbonyl group with an iodo-methylene group in the beta position.

Conversion of 2-iodohexadecanoic acid to 2-acetoxy-hexadecanoic acid proved to be the most difficult reaction of this synthetic sequence. Since the hydrogen on the carbon next to the carbonyl group was acidic, a method had to be devised which minimized beta elimination. For this reason various reaction conditions were investigated.

The first method concerned an attempted displacement of the iodo group with sodium acetate in acetic acid at 60°C . The major product was shown by NMR to be 2-hexadecenoic acid (Figure 5).

The reaction conditions were changed so as to favor more an SN_1 reaction. 3-Iodohexadecanoic acid was stirred in a silver acetate-acetic acid solution at room temperature for 12 hours. Chromatography of the reaction residue on silicic acid using a hexane/diethyl ether elution scheme effected partial separation of several products. The major product had an NMR spectrum identical with that of 2-hexadecenoic acid (Figure 5). Other minor components detected were starting material, a compound with an IR carbonyl absorption at 1820 cm^{-1} , tentatively assigned to

the beta-lactone structure of 3-hydroxyhexadecanoic acid, and a very small amount of a double carbonyl compound with strong absorption at 1740 cm^{-1} and 1719 suggesting that the desired 3-acetoxyhexadecanoic acid had formed.

The solvent was changed from acetic acid to acetone for it was thought that a less polar reaction medium would suppress beta elimination. Silver acetate, and 3-iodohexadecanoic acid were stirred in acetone for 12 hours. Infrared analysis of the reaction product indicated that both the 3-acetoxyhexadecanoic acid and the beta-lactone had formed in good yield. Although the beta-lactone was identified as the desired major product, it could easily be converted to the 3-acetoxyhexadecanoic acid by reaction with acetic anhydride in the presence of a sulfuric acid catalyst.

Since this reaction scheme was tedious and since the products were difficult to purify, other methods of synthesis of 3-acetoxyhexadecanoic acid were investigated.

A modified Reformatsky reaction was the key to a simple synthesis of 3-acetoxyhexadecanoic acid. All of the reactants could be obtained commercially or synthesized easily. If the apparatus was scrupulously dried, yields in excess of 70 per cent could be realized. Tetradecanal, synthesized from lead tetra-acetate oxidation of tetradecanol in pyridine, reacted with ethyl bromoacetate in the presence of zinc to give a white compound which upon

hydrolysis with 10 per cent ethanolic potassium hydroxide, could be crystallized from carbon tetrachloride, m.p. 183.5° C. The NMR spectrum of this compound showed signals at 0.9δ (3) and a 1.3δ (24) indicating a normal thirteen-carbon aliphatic chain, and a doublet at 2.35δ indicating that two hydrogens on the carbon alpha to the carbonyl group were split by one hydrogen on the beta carbon atom. The multiplet at 4.0δ can then be explained by a hydroxyl group occupying the beta position. These data indicated that the desired 3-hydroxyhexadecanoic acid had formed. Furthermore, the NMR spectrum of the methyl ester of this compound, formed by reacting the acid with 2 N methanolic sulfuric acid, was identical with that of the acidic methanolysis product of natural pahu-toxin (Figure 7).

Acetylation of 3-hydroxyhexadecanoic acid with acetyl chloride gave a quantitative conversion to 3-acetoxyhexadecanoic acid. This was ascertained when the IR and NMR spectra were found to be identical with those of the natural degradation product (Figures 6-A and 8).

Formation of the acid chloride of 3-acetoxyhexadecanoic acid followed by esterification with anhydrous choline chloride gave a Dragendorff-positive compound which could be precipitated from hot acetone. Silicic acid chromatography followed by passing an anhydrous ethanol solution of the

Dragendorff positive fraction through a picric acid coated Dowex chloride anion exchange resin gave a colorless residue which crystallized from anhydrous acetone m.p. 60-61° C. Combustion analysis of this crystalline compound, dried in vacuum at 35° for 8 hours, most nearly fitted an empirical formula of $C_{23}H_{48}NO_5Cl$. If the elements corresponding to a molecule of water are subtracted from this formula, the resulting $C_{23}H_{46}NO_4Cl$ formula is that of choline 3-acetoxyhexadecanoate.

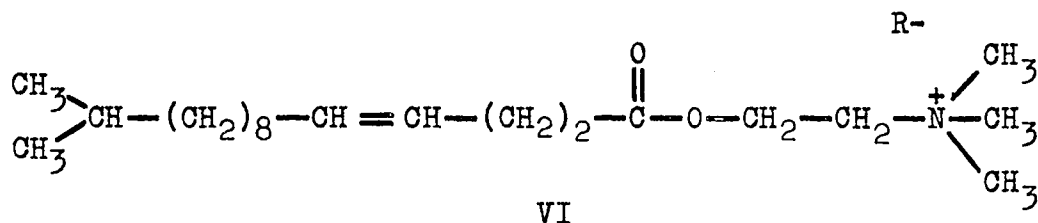
Crystalline natural pahutoxin was prepared for analysis in exactly the same manner. A calculated empirical formula of $C_{23}H_{44}NO_5Cl$ best fitted the analytical data, while the known structure of pahutoxin required a composition of $C_{23}H_{46}NO_4Cl$. This was consistent with earlier combustion data. The low hydrogen and high oxygen values in this formula cannot be rationalized since the IR and NMR spectra of pahutoxin are identical with those of choline 3-acetoxyhexadecanoate. Since the synthetic product was a racemate, the optical rotation and melting points could not be compared.

Mass spectral data did, however, suggest a possible answer to the problem. Both pahutoxin and choline 3-acetoxyhexadecanoate were submitted for mass spectral determination. The majority of the fragments observed were below an m/e of 100 with major peaks at m/e 58 assigned to the fragment $(CH_2 = \overset{+}{N} \begin{matrix} \text{CH}_3 \\ \text{CH}_3 \end{matrix})$, and m/e 71

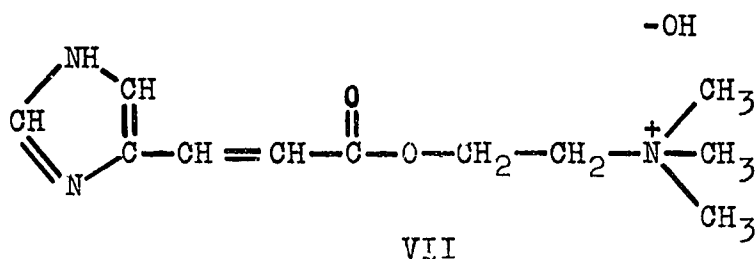
assigned to $(\cdot\text{CH}_2-\text{CH}_2-\overset{+}{\text{N}}(\text{CH}_3)_2)$. Small but detectable fragments appeared at m/e 237, 281, 325 in both spectra, however, these could not be assigned since their corresponding metastable peaks were not resolved. The presence of peaks as high as m/e 660 and 580 in the spectrum of natural toxin, accompanied by the absence of such peaks in the spectrum of synthetic toxin suggested the presence of subtle impurities even though natural pahutoxin was crystalline. The appearance of a 405 mass peak found only in the spectrum of the synthetic toxin can be rationalized by the presence of a subtle impurity or an unlikely recombination peak. Over all, however, a majority of peaks were present in both spectra. No molecular ion was observed in either case. This was not surprising since pahutoxin is a quaternary nitrogen cation and must eliminate methyl chloride or a similar fragment before it becomes volatile enough to enter the vacuum chamber of the mass spectrometer. The largest expected molecular weight fragment--provided no recombination fragmentation pattern occurs--would be m/e 385 if this excited fragment were stable enough to show a mass peak.

The structure of pahutoxin is quite unique among known fish toxins. It bears some resemblance to the structure of a glycolipid isolated from the soft tissues of a Japanese oyster. The fatty acid moiety of this glycolipid

was choline 14 methyl-4-pentadecenoate (VI).³⁶



Pahutoxin is also distantly related structurally to urocanylcholine or murexine (VII) which was isolated from the hypobranchial body of the mollusk Murex trunculus and other related species.³⁷ However, there is a marked difference between biological activity of these compounds and of pahutoxin.



D. Biological Activity of Pahutoxin and of Synthetic Homologues

It had been shown previously that crude pahutoxin was extremely toxic to fish and possessed strong hemolytic ability toward big eye tuna blood.¹⁰ A major objective of this investigation was to establish a correlation between structural modification of the length of the aliphatic chain and biological activity. For this reason synthetic pahutoxin or choline 3-acetoxyhexadecanoate and the choline

esters of 3-acetoxynonanoic, dodecanoic, and tetradecanoic acids were synthesized and their biological activities compared with those of natural pahutoxin.

Toxicity studies using brackish water mollies (Mollienesia latipinna Le Seur) as test fish showed interesting results. The data are summarized in Table II. Reciprocals of time--to death (measured in minutes) were plotted against concentration of pahutoxin and synthetic homologues (Figure 11).

Inspection of Figure 11 shows conclusively that natural pahutoxin and synthetic pahutoxin or choline 3-acetoxyhexadecanoate exhibited the same toxic activity. This provides additional evidence that the two compounds are identical. The minimum lethal concentration of pahutoxin which is required to kill a representative population in approximately one hour using 50 ml test solutions was 1.76×10^{-4} mg/ml or 0.176 ppm.

The biological data also showed that a decrease in chain length from C₁₆ to C₁₂, all other structural features remaining constant, was accompanied by a marked decrease in toxic activity. Choline 3-acetoxynonanoate was found to be non-toxic at concentrations as high as 1 mg/ml.

Similar studies on synthetic alkylbenzene sulfonates indicated that toxicity to fish increased with an increase in aliphatic chain length and leveled off at an alkyl chain of fourteen carbons.²⁸

Since toxicity data reported in the literature are measured under a variety of conditions, no attempt was made to compare reported values with those of pahutoxin.

Hemolytic results using big eye tuna blood cell suspensions were reported as per cent lysis. The data are summarized in Table III and are plotted against concentrations of pahutoxin and synthetic homologues (Figure 10).

From this graph it is evident that pahutoxin, natural and synthetic, exhibits the same hemolytic behavior.

A decrease in the chain length from C₁₆ to C₁₂ resulted in a marked decrease in hemolytic activity. Choline 3-acetoxynonanoate was found to be non-hemolytic in concentrations greater than 1 mg/ml.

Analogous studies with a variety of soaps have shown that sodium salts of C₁₀ to C₂₀ fatty acids are hemolytic with maximum hemolytic ability occurring between the C₁₄ and C₁₈ homologues.²⁹ Among a series of choline esters of fatty acids studied, palmitic and stearic esters were found to be most hemolytic with properties slightly inferior to those of lysocithin.³⁰ Because of the varied experimental conditions reported in the literature, no direct comparison of hemolytic ability could be made among known hemolytic biotoxins such as holothurin A, digitonin, quillaria saponin,³¹ starfish saponin,³² and pahutoxin.

A surprising correlation seemed to exist between the graph showing inverse toxicity versus concentration

(Figure 9) and the graph showing per cent lysis versus concentration (Figure 10). Not only do toxic activity and hemolytic ability decrease proportionally as the chain length decreases, but the two effects occur at approximately the same concentrations for each homologue. The striking resemblance of the two curves suggests that either hemolysis is the cause of toxicity to fish or is closely related to it.

Many theories have been proposed concerning the mechanism of action of fish poisons.³³ This experiment was not designed to provide enough information to enable one to postulate a reaction mechanism. However, it did provide the basic structure-activity relationship that will be useful for future studies in this direction.

It is also felt that information gained through the biological examination provided additional proof that the structure of pahutoxin is choline 3-acetoxylhexadecanoate.

E. Pharmacology of Pahutoxin

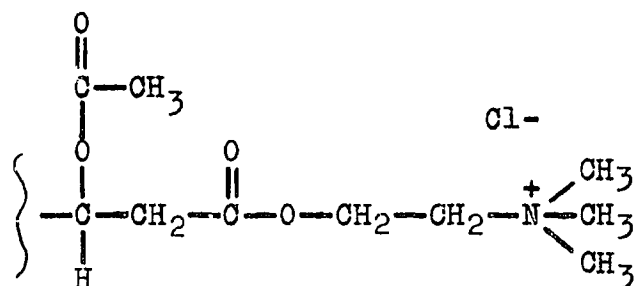
Pahutoxin administered intravenously¹³ to a rat (Figure 13) caused a prompt drop in blood pressure with rapid recovery within two minutes. A slight increase in respiration amplitude and frequency was also observed. Repeated doses caused similar effects; however, the blood pressure remained subnormal indicating only partial recovery. Death occurred after approximately 15M g toxin per gram rat had been injected.

Intraperitoneal injection of 200 μ g of toxin per gram mouse caused miosis (constriction of the pupils), prostration, and death in three hours.

These data clearly demonstrate that pahutoxin causes no immediate nicotinic action as is observed in anticholinesterase toxins, e.g., ciguatera toxin. Inhibition studies further showed that pahutoxin did not inhibit bovine erythrocyte cholinesterase.

The observed results are attributed to a muscarinic type activity.

The pharmacology of choline 3-acetoxyhexadecanoate is an interesting subject for speculation since the polar portion of the molecule



would be expected to act as a stimulant similar to acetylcholine or possibly even exhibit curare activity such as dicholinesuccinate,³⁴ where the lipid portion of the molecule would be expected to exhibit symptoms much the same as choline palmitate.³⁵

The experiment performed was clearly of a preliminary nature and only after detailed pharmacological tests can the exact mode of action of pahutoxin be determined.

IV. SUMMARY AND CONCLUSION

A simple bioassay, developed by Thomson, using brackish water mollies as test fish proved useful in devising an efficient isolation scheme. The following procedure was developed to limit the loss of toxin and turned out to be a simple process requiring only one day for isolation of pure toxin. The crude aqueous secretion was immediately extracted with butanol in order to precipitate proteins and deactivate harmful enzymes. The butanol extract, after being washed with distilled water, was concentrated to dryness and applied to a silicic acid column. Gradient elution using chloroform and chloroform/methanol, yielded a residue which after being passed through a picric acid-coated chloride exchange resin could be recrystallized from anhydrous acetone. Since it gave a positive test with Dragendorff's alkaloid reagent, toxic column fractions could be detected easily.

Sodium fusion tests demonstrated the presence of nitrogen and chlorine and the hydroxamic acid test gave positive results indicating the presence of an ester group. Conductometric and pH titrations indicated that a hydrolysis was taking place and suggested the use of mild hydrolytic conditions for structural degradation studies.

The nuclear magnetic resonance spectrum of pahutoxin using deuteriochloroform as a solvent and tetramethylsilane

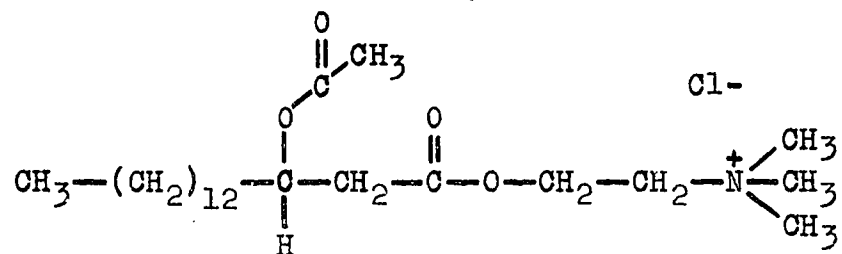
as an internal reference gave the most valuable information about the structure. A broad singlet at 3.6δ (9) was attributed to a trimethyl quaternary nitrogen group and broad absorption at 4.2δ and 4.6δ each integrating for two hydrogens raised suspicions that a choline moiety was present. A prominent peak at 1.35δ (24) and a triplet at 1.0δ (3) suggested a simple C_{13} -alkyl chain. Other signals included a singlet at 2.18δ (3) assigned to an acetate group, a doublet at 2.6δ (2) indicating the presence of 2 hydrogens on the carbon alpha to a carbonyl group, and a multiplet at 5.2δ (1) that was attributed to the hydrogen on an acetoxy-methylidene group beta to the carbonyl.

Infrared bands at 3320 cm^{-1} , 3000, 1730 (broad) indicated a trimethyl quaternary nitrogen group, a large CH_2 moiety and more than one carbonyl group; a 1250 cm^{-1} band was assigned to an ester group. The ultra-violet spectrum showed only strong end absorption beginning at 220 m μ .

Several of the most useful degradation schemes involved hydrolysis using aqueous sodium bicarbonate at room temperature, and methanolic sulfuric acid at 50° C . Mild basic hydrolysis yielded several products which were separated by solvent extraction and by silicic acid chromatography and the structures were determined by spectral methods. Choline, 2-hexadecenoate, isolated as a picrate and confirmed by elemental analysis, and

2-hexadecenoic acid were isolated. Methanolysis of pahutoxin in methanolic sulfuric acid gave methyl 3-hydroxyhexadecenoate as a major product. This was ascertained by NMR spectral evidence and comparison with an authentic synthetic sample. Deuteriated trifluoroacetic acid hydrolysis was used to detect any volatile hydrolytic products since the distillate could be used directly for NMR analysis. This procedure revealed acetic acid as the only volatile component. Sublimation of a sodium bicarbonate hydrolysis residue gave an additional crystalline fragment which was identified as 3-acetoxypalmitic acid.

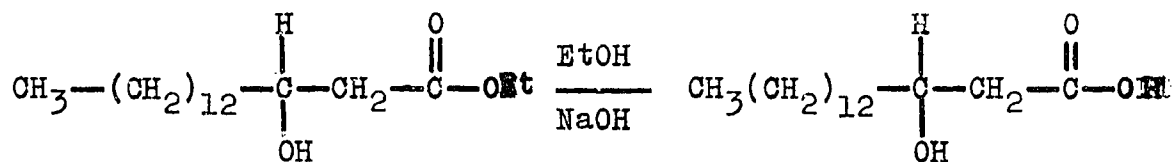
The total structure postulated for pahutoxin is choline 3-acetoxypalmitate (I).



I

Positive proof of the above structure rested in the synthesis of racemic pahutoxin. Several synthetic schemes were attempted, but a modified Reformatsky reaction gave the best results.





Acetylation of 3-hydroxyhexadecanoic acid followed by formation of the acid chloride with thionyl chloride and esterification with choline gave a racemic choline ester whose IR and NMR spectra were identical with those of natural pahutoxin.

The synthetic product gave rise to a combustion analysis which best fitted the formula $\text{C}_{23}\text{H}_{48}\text{NO}_5\text{Cl}$ which differs by a molecule of water from the formula $\text{C}_{23}\text{H}_{46}\text{NO}_4\text{Cl}$ which matches the composition of choline 2-acetoxylhexadecanoate. However, natural pahutoxin, prepared for analysis under identical conditions gave rise to an analysis that best fitted the formula $\text{C}_{23}\text{H}_{44}\text{NO}_5\text{Cl}$.

This discrepancy in combustion analysis was best explained by mass spectral data. The mass spectra of the synthetic and natural pahutoxin exhibited fragmentation peaks at 58, 71, 281, and 325 with a small inconsistent population of peaks above 400 mass units. The appearance of peaks at 660 and 580 mass units in the natural pahutoxin spectrum that were absent in the spectrum of the synthetic analogue suggested the presence of subtle impurities even though natural pahutoxin was a crystalline compound.

Since the synthetic procedure was not complicated and the starting materials could be secured easily, we decided to synthesize a number of homologues in order to compare their biological activities with those of pahutoxin. The choline esters of 3-acetoxynonanoic (C_9), -dodecanoic acid (C_{12}), -tetradecanoic (C_{14}), and -hexadecanoic (C_{16}) acids were synthesized in good quantities and tested for toxicity to fish and hemolytic ability. Results showed that the natural and synthetic toxin possessed the same toxicity and hemolytic values despite the fact that the synthetic toxin is a racemate and pahutoxin an optically active compound. These tests also pointed out that the toxicity and hemolytic values vary markedly with alkyl chain length from the most active C_{16} acid derivative to the non-active C_9 derivative. Surprisingly, these results revealed that toxicity concentration curves correlated exactly with hemolysis concentration curves. This indicated that hemolysis was the direct cause of toxicity to fish or was related closely to the actual cause.

Pahutoxin was also found to cause a typical muscarinic type activity when injected intravenously into rats and caused death at a dose of 15 $\mu\text{g/g}$.

In conclusion, it should be pointed out that pahutoxin is the first fatty choline ester isolated from fish secretions and can only be compared with a glycolipid

isolated from a Japanese oyster whose lipid portion is the choline ester of 14-methyl-4-pentadecenoic acid. It is distantly related choline β -4-imidazolyl acrylate isolated from a Mediterranean welk.

Evidence of an exotoxin, similar to pahutoxin, has been detected in the cowfish, Lactoria fornasini (Linnaeus), but not in the closely related puffer fish Sphaeroides rubrypes (Temminick and Schlegel). The actual function of pahutoxin is presumed to be defensive since the inability of the boxfish to maneuver makes him an easy prey of predators.

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