Palytoxin: A New Marine Toxin from a Coelenterate

Abstract. *Palytoxin has been isolated from the zoanthids* "limu-make-o-Hana" *(Tentatively identified as* Palythoa *sp.) as a noncrystalline, chromatographically pure entity. Apart from polypeptide and protein toxins, it is the most highly toxic substance known, with a lethal dose (LD_{59}) in mice of 0.15 microgram per kilogram by intravenous injection. Unlike the potent toxins batrachotoxin, saxitoxin, and tetrodotoxin which have molecular weights of 500 or less, palytoxin has an estimated molecular weight of 3300 and contains no repetitive amino acid or sugar units.*

Limu make o Hana (deadly seaweed of Hana) is the Hawaiian phrase for a toxic organism which Malo (*1*) described as follows: ". . . In Muolea, in the district of Hana, grew a poisonous moss in a certain pool or pond close to the ocean. It was used to smear on the spear points to make them fatal. . . . The moss is said to be of a reddish color and is still to be found. It grows nowhere else than at that one spot." According to Hawaiian legend (*2*) there lived in the Hana district a man who always seemed to be busy planting and harvesting. Whenever the people in the neighborhood went fishing, upon their return one of the group was missing. This went on for some time without anyone having an explanation for the mysterious disappearances. At last the fishermen became suspicious of the man who tended his taro patch. They grabbed him, tore off his clothes, and discovered on his back the mouth of a shark. They killed and burned him and threw the ashes into the sea. At the spot where this happened, so goes the legend, the *limu* became toxic. The tidepool containing the toxic *limu* subsequently became *kapu* (taboo) to the Hawaiians. They would cover the *limu* with stones (*1*) and were very secretive about its location. They firmly believed that disaster would strike if anyone were to attempt to gather the toxic *limu*.

Our long interest in marine toxins and specifically in the biological origin of ciguateric fishes prompted us to try and locate the toxic *limu*. Through the efforts of Professor A. H. Banner and Dr. P. Helfrich of the Hawaii Institute of Marine Biology and by an elaborate chain of informers the tidepool was located at the end of a lava flow at Muolea (Kanewai), south of Hana, Maui, and a small collection of the toxic *limu* was made on 31 December 1961. Local residents reminded the members of the field party of the ancient *kapu* and warned of impending misfortune. Coincidentally, on that same afternoon a fire of unknown origin destroyed the main building of the Hawaii Marine Laboratory at Coconut Island, Oahu.

The collection was preserved in ethanol and returned to the laboratory. To check gross toxicity a portion of the supernatant solvent was evaporated and the residue was tested by intraperitoneal injection in mice. The alcohol extract had apparently become highly toxic, since even very dilute samples caused death in mice.

Taxonomic examination of the toxic organism showed that it was not a *limu*, but an animal of the phylum Coelenterata, order Zoantharia, family Zoanthidae, possibly a new species tentatively assigned to the genus *Palythoa* (*3*). Halstead (*4*) does not list the family Zoanthidae among toxic coelenterates; this may well indicate that our discovery was the first demonstration of toxicity among the Zoanthidae. Since then Attaway (*5*) has reported on toxic *Palythoa caribaeorum* and *P. mammilosa* from Jamaica and the Bahamas, and Hashimoto *et al.* (*6*) found toxic *P. tuberculosa* at Ishigaki Island in the Ryukyus.

These toxic zoanthids (Fig. 1) were again collected from the tidepool at Muolea in May 1963 and in January 1964. Each polyp was severed at the base of the stalk to free it from the lava substrate. A slimy mucus was immediately exuded into the water. The polyps were removed from the pool with a small net, drained, and placed in an airtight container with ethanol, and brought back to the laboratory. Each collection yielded about 250 g of wet polyps. The zoanthid population in the tidepool appeared to be almost entirely restored within 6 months.

The collected samples were kept for 1 to 2 days, and then the dark orange-colored toxic extract was removed by decantation. The polyps were then soaked twice in 70 percent aqueous ethanol, just enough to cover the animals, for 2 to 3 hours; this extract was then separated by decantation. The zoanthids were then ground in a Waring Blendor with 70 percent aqueous ethanol, the extract was drawn off by suction, and the ground polyps were washed twice with 70 percent aqueous ethanol. The combined extracts and washings (3.8 liters) were evaporated under reduced pressure at 50°C to remove ethanol, and the concentrate (about 0.5 liter) was extracted three times with benzene and twice with 25-ml portions of 1-butanol saturated with water. The 1-butanol extract was backwashed three times with water saturated with 1-butanol.

The combined aqueous portion, after removal of dissolved 1-butanol under reduced pressure at 50°C, was passed

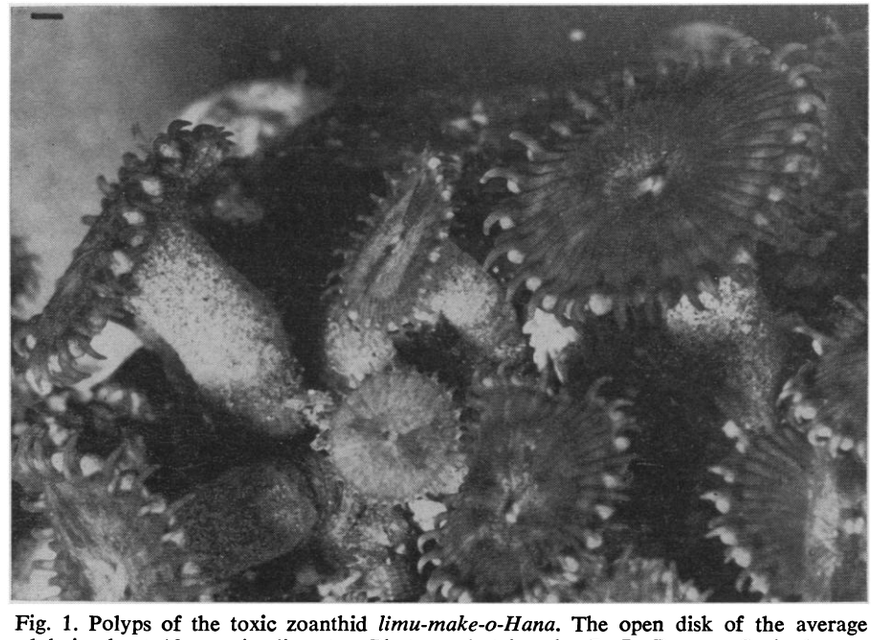

Fig. 1. Polyps of the toxic zoanthid *limu-make-o-Hana*. The open disk of the average adult is about 10 mm in diameter. Photograph taken by R. L. Bowers. Scale 1 mm.

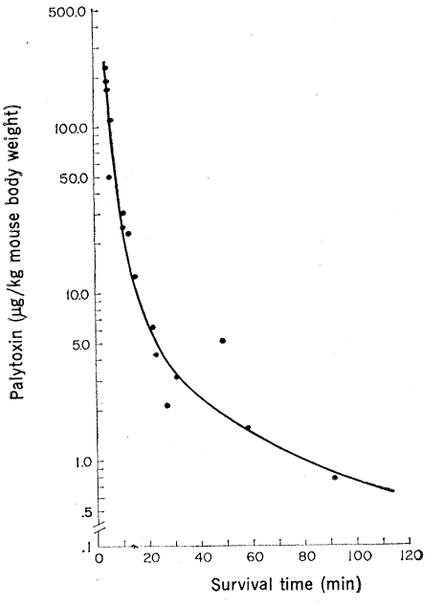

Fig. 2. Dose-response curve of palytoxin in 0.9 percent saline injected intraperitoneally into mice. Five mice (SPF strain) having an average weight of 20 g were used to determine each point.

with suction through a column (21 by 24 cm) of 200-mesh polyethylene (*7*). The column was washed thoroughly with water to remove inorganic salts and very polar organic material, and the toxin was eluted with 500 ml of a mixture of 50 percent ethanol and water. The toxic fraction was evaporated at reduced pressure at 30°C to give about 0.4 g of a brown glass.

The toxic material, dissolved in 0.5 to 1 ml of 0.02*M* sodium phosphate buffer of *p*H 7.0, was now introduced onto a column (33 by 2 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 that had been equilibrated with 0.02*M* sodium phosphate buffer of *p*H 7.0. Elution was continued with the same buffer and 5-ml fractions were collected. Fractions 8 to 10, which contained most of the toxin and showed pronounced ultraviolet absorption at 263 nm, were combined and desalted on a column (19 by 3 cm) of polyethylene as described above. Fractions 11 to 15, which contained small amounts of toxin were combined, desalted, and recycled on DEAE-Sephadex. The total amount of crude toxin from the desalted fractions 8 to 10 was 200 mg.

This 200 mg of crude toxin was dissolved in 0.5 to 1 ml of 0.02*M* sodium dihydrogen phosphate, placed on a column (33.5 by 2 cm) of carboxymethyl (CM)-Sephadex C-25 that had been equilibrated with 0.02*M* sodium dihydrogen phosphate, and eluted with 0.02*M* sodium dihydrogen phosphate; 10-ml fractions were collected. Fractions 18 to 28 were combined and desalted on a column (19 by 3 cm) of polyethylene as described above. The yield of palytoxin from the desalted fractions 18 to 28 was 68 mg (0.027 percent).

Progress in the purification of palytoxin was monitored by intraperitoneal or intravenous injections of portions of a given extract into mice.

A portion (0.1 ml) was taken from each fraction; the solvent (if organic) was evaporated at reduced pressure; the residue was dissolved in 10 ml of 0.9 percent saline; 0.5-ml portions of this solution were injected intraperitoneally into three to five mice (SPF strain). The fractions that killed all mice were retained.

To ascertain whether toxicity was lost during the isolation, varying amounts of toxin at each purification stage were dissolved in 0.5 ml of 0.9 percent saline and injected intraperitoneally into mice at lethal and sublethal dosage. The lethal dose (LD_{50}), in mice, of 27 g of extract of the zoanthids collected from Hana, Maui, in January 1964 was estimated to be 40 μg/kg. After the lipids and pigments from 27 g of this extract were removed with benzene and 1-butanol, the remaining material was chromatographed on polyethylene powder and DEAE-Sephadex and 200 mg of crude toxin with an LD_{50} of 0.4 μg/kg was obtained. Recovery of toxicity to this point was therefore 74 percent. After chromatography on CM-Sephadex the yield of palytoxin was 68 mg with an LD_{50} of only 0.4 μg/kg by intraperitoneal injection, but an LD_{50} of 0.15 μg/kg by intravenous injection; thus by the latter route 92 percent of the toxicity was in the 68 mg. While no appreciable differences in toxicity were noted when the toxin was administered either intraperitoneally or intravenously before purification on CM-Sephadex, the purified palytoxin was consistently 2.7 times less toxic in mice when administered intraperitoneally than when administered intravenously.

To determine the concentration of palytoxin in an unknown solution that had been purified on CM-Sephadex, a portion was diluted with 0.9 percent saline and injected intraperitoneally into at least five mice, and the time

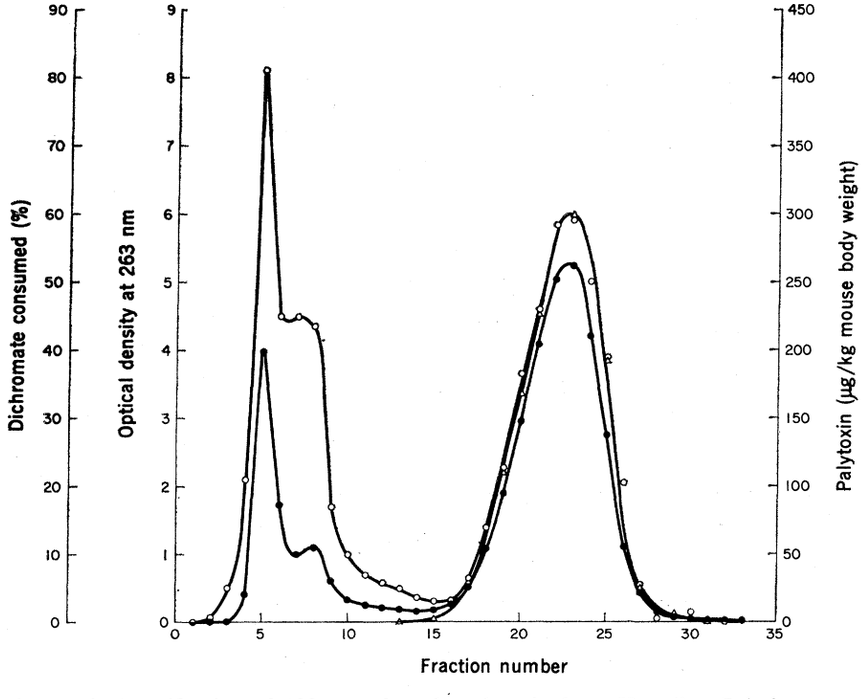

Fig. 3. Final purification of 100 mg of crude palytoxin from Hawaiian *Palythoa* on a column of CM-Sephadex C-25 (2 by 33.5 cm) with 0.02*M* sodium dihydrogen phosphate. Ten-milliliter fractions were collected. (Curve 1, ●–●) Absorbance at 263 nm. (Curve 2, ○–○) Assay of total organic material [method of Märki and Witkop (*8*)]. (Curve 3, △–△) Assay of activity. A 0.10-ml portion of each fraction was diluted to 5.00 ml with 0.9 percent saline, five 20-g mice were injected with 0.5-ml doses, and an average death time was determined. The dosage in micrograms of palytoxin per kilogram of body weight (mouse) was then obtained from the dose-response curve (Fig. 2).

of death was measured. Because the response is rather variable, this number was needed to obtain a significant average. The dose was then determined from Fig. 2, and the amount of palytoxin in the unknown solution was calculated. To determine the amount of palytoxin in a crude extract or at any stage prior to chromatography on CM-Sephadex, the same method was used, except that the dose obtained from Fig. 2 must be divided by 2.7 if the value is to be compared with the intravenous response to pure toxin.

Symptoms in mice given lethal doses are decreased locomotion, lowering of the anterior trunk, extension of forelimbs, paralysis in hind limbs, diarrhea, severe convulsions, dyspnea, and finally death from respiratory collapse.

The material obtained from CM-Sephadex chromatography is considered to be pure palytoxin. Assays (Fig. 3) for ultraviolet absorption at 263 nm, total organic material (*8*), and toxicity indicate that a homogeneous substance is present in fractions 18 to 28. The ultraviolet absorption spectra of all of these fractions are identical; for example, the ratio of the absorbances at 233 and 263 nm is 1.71 in all spectra. Palytoxin is probably a single compound; however, it is possible that palytoxin may be a mixture of very closely related compounds or homologs.

The behavior of our purest samples of palytoxin in a countercurrent distribution experiment between 1-butanol and water also indicates homogeneity. The partition coefficient for the distribution of palytoxin between 1-butanol and water was 0.21 at 25°C based on comparison of the absorbance at 263 nm for the two layers.

Palytoxin is a white, amorphous, hygroscopic solid which has not yet been crystallized. It is insoluble in chloroform, ether, and acetone, sparingly soluble in methanol and ethanol, and soluble in pyridine, dimethyl sulfoxide, and water. The toxin shows no definite melting point and only chars when heated to 300°C. It is optically active, having a specific rotation of $+26° \pm 2°$ in water. The optical rotatory dispersion curve of palytoxin (*9*) exhibits a positive Cotton effect with $[\alpha]_{250}$ being $+700°$ and $[\alpha]_{215}$ being $+600°$.

The behavior of palytoxin on CM-Sephadex indicates the presence of a basic (cationic) group in the molecule. Negative tests, however, were obtained with ninhydrin and Dragendorff reagents. After a solution of palytoxin was refluxed for 6 hours in 6*N* hydrochloric acid a faint ninhydrin reaction was obtained with the hydrolysate. Examination of the acid hydrolysate by thin-layer chromatography on cellulose indicated the presence of an amine giving a very faint violet spot with ninhydrin. The test with Nessler's reagent was definitely positive. The R_F of this amine, 0.70 in isopropyl alcohol–water–concentrated ammonium hydroxide (7 : 2 : 1) and its low sensitivity to ninhydrin differ from those of any of the common amino acids. Most of the nitrogenous material in the acid hydrolysate is probably ammonia as shown by a strong positive test with Nessler's reagent.

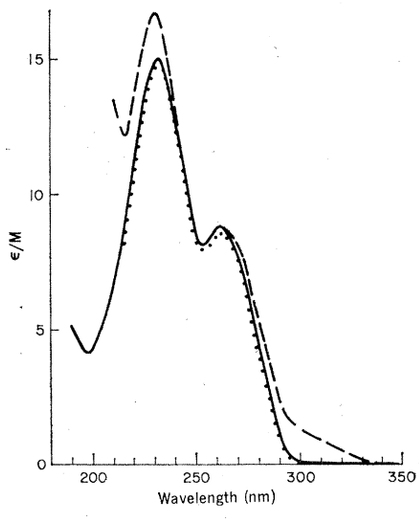

Fig. 4. The ultraviolet spectrum of palytoxin in water (——), aqueous 0.05*N* HCl (. . . . .), and aqueous 0.05*N* NaOH (– – –). The spectrum in acid or base was determined within 2 minutes after mixing. Neutralization within 2 minutes regenerated palytoxin with no apparent loss of toxicity.

In aqueous solution palytoxin behaves like a steroidal saponin, producing a foam on agitation. Unlike a steroidal saponin, the toxin cannot be cleaved by acid hydrolysis into lipophilic and hydrophilic moieties; the possibility that the hydrophilic part might be composed of simple sugar units is eliminated by a negative Molisch test. The toxin reacts with sodium metaperiodate, the lipophilic portion precipitating from the medium as a gummy mass as the oxidation proceeds.

Attempts to obtain a molecular weight and formula by mass spectrometry have failed. The toxin is nonvolatile and decomposes, as does the trimethylsilyl derivative, in the heated inlet system. From the nuclear magnetic resonance (NMR) spectrum in D_2O, however, the number of protons in the molecule can be estimated. If the broad doublet at δ 8.1 represents a single proton, integration of areas of the band at δ 8.1 and of the remainder of the spectrum gives an average proton ratio of 1 : 263. Combustion analysis (*10*) showed 52.6 percent C, 8.0 percent H, and 1.7 percent N. Sulfur and phosphorus (as phosphate ester) were absent. Oxygen was the only other element present. On the basis of the NMR and combustion data, we estimate that the molecular weight is about 3300, and that the formula is $C_{145}H_{264}N_4O_{78}$. The error is probably no more than 10 percent.

The ultraviolet spectrum of palytoxin is shown in Fig. 4. Bands at 233 nm (ε 49,800) and 263 nm (ε 29,000) (*11*) suggested at first that palytoxin might be related to the Colombian arrow poison, batrachotoxin (*12*); however, the lability to both acid and base coupled with a negative Ehrlich reaction shows that a 2,4-dialkylpyrrole-3-carboxylate chromophore is not present in palytoxin. In methanolic 0.05*N* HCl and aqueous 0.05*N* NaOH, the band at 263 nm disappears with a half-life of 85 and 55 minutes, respectively. Loss of toxicity is concomitant with the destruction of the chromophore absorbing at 263 nm. One must conclude that palytoxin has at least two chromophores. If the molar extinction coefficient (ε) of the band at 263 nm is approximately the same (27,000) as for sorbamide (*13*) and that 90 percent of the absorption at 263 nm is due to this chromophore, then the molecular weight of palytoxin would be about 3400.

The infrared spectrum of palytoxin shows a band at 1670 cm^{-1} which is tentatively assigned to an α,β-unsaturated amide carbonyl group.

At least four molar equivalents of hydrogen are absorbed catalytically (platinum) in ethanol under ordinary conditions. Equivalent weights of 630 and 822 were obtained from two ultramicrohydrogenation experiments, the latter probably being a more reliable value as it was obtained with a much larger sample. The data indicate the presence of four or five double bonds that can be catalytically hydrogenated. The ultraviolet spectrum of hydrogenated palytoxin shows only end absorption.

RICHARD E. MOORE
PAUL J. SCHEUER
Department of Chemistry,
University of Hawaii,
Honolulu 96822

References and Notes

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3. Originally, the species was thought to be *Palythoa vestitus* Verrill, but reinvestigation of the Hawaiian zoanthids by G. E. Walsh and R. L. Bowers (in preparation) makes the binomial uncertain at present.
4. B. W. Halstead, *Poisonous and Venomous Marine Animals of the World* (Government Printing Office, Washington, D.C., 1965), vol. 1, pp. 297–307.
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6. Y. Hashimoto, N. Fusetani, S. Kimura, *Bull. Japan Soc. Sci. Fish.* **35**, 1086 (1969).
7. We thank the Dow Chemical Co. through the efforts of Dr. K. Terada for the powdered polyethylene, designated as experimental resin QX-2187 (density 0.960, melt. index 1), which we found to be highly satisfactory for our work.
8. F. Märki and B. Witkop, *Experientia* **19**, 329 (1963). We thank Dr. Witkop for providing us with details of the micro determination: A 0.100-ml portion of each fraction was mixed with 1.00 ml of dichromate reagent (1.00 g sodium dichromate dihydrate in 20 ml of water, diluted to 1 liter with concentrated sulfuric acid) and oxidized by heating in a boiling water bath for 20 minutes. The solution was then diluted to 4.00 ml with water, and the optical density at 350 nm was determined.
9. We thank Professor C. Djerassi for determining the optical rotatory dispersion curve.
10. Analyses by Berkeley Analytical Laboratory, Berkeley, Calif.
11. Extinction coefficients are based on an assumed molecular weight of 3300 and can therefore be expressed as 15.1 M or 8.8 M, where M is the molecular weight (Fig. 4).
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Norepinephrine Biosynthesis Inhibition: Effects on Memory in Mice

Abstract. *Diethyldithiocarbamate, a dopamine beta hydroxylase inhibitor, decreases biosynthesis of norepinephrine in the brain. The effects of this inhibitor coincide with alterations in memory as demonstrated in single-trial passive avoidance in C57BL/6J mice.*

Recent evidence suggests that norepinephrine may play an important role in memory. Roberts *et al.* (*1*) have demonstrated that amnesia resulting from administration of puromycin can be reversed by a variety of drugs that compete for adrenergic receptor sites. Seiden and Peterson (*2*) have shown that reserpine and α-methyl-*p*-tyrosine, both of which decrease the concentration of amines in the brain, cause a temporary failure to perform a well-learned conditioned avoidance response. Our study demonstrates that inhibition of norepinephrine synthesis in the brain at the dopamine β-hydroxylase stage is associated with early enhancement and later impairment of memory for a one-trial passive avoidance response in the C57BL/6J strain of mice.

Diethyldithiocarbamate (DDC), a dopamine β-hydroxylase inhibitor, decreases the synthesis and the brain concentration of norepinephrine, while the concentration of dopamine remains unchanged or slightly increased (*3*). Inhibition of the biosynthesis of norepinephrine in the brain was measured by two separate methods. In the first procedure, the conversion of [^{14}C]dopa to [^{14}C]dopamine and [^{14}C]norepinephrine was analyzed in the brains of control mice and those treated with DDC (*4*). The second determination assayed endogenous norepinephrine concentrations fluorometrically in both groups (*5*).

Adult male mice (70 to 100 days of age) of the C57BL/6J strain were placed in the start compartment of a two-chamber apparatus previously described by Quartermain and McEwen (*6*). After 15 seconds, the door to an adjacent larger compartment was opened with simultaneous activation of a timer and a flashing white light at the far end of the chamber. When the mouse entered the second compartment, the door was shut, and the timer stopped. For the last 2 seconds of a 20-second period after entry, a scrambled foot shock (0.16 ma for 2 seconds) was automatically delivered through the floor bars. Retention of the avoidance response was tested by replacing the mice in the start compartment and recording the latency to reenter the second compartment. The light flashed continuously during the test period. Animals failing to enter within 3 minutes were removed and given a score of 180.0 seconds.

In our first experiment, different groups of mice were injected subcutaneously with DDC [250 mg per kilogram of body weight (0.3 ml); $N = 10$] or with saline (0.3 ml; $N = 10$) 30 minutes before the foot-shock training trial and tested for retention at 1 minute, 5 minutes, 1 hour, 6 hours, or 24 hours after the training trial. Although this dose of DDC reduces spontaneous activity by approximately 30 percent (*7*), latencies to enter the compartment on the training trial were consistently shorter for the mice injected with DDC than for the controls injected with saline. Mean initial latency for all DDC injected mice was 5.6 seconds and for all saline injected mice 7.4 seconds, indicating no impairment, due to the drug, of motor ability to enter the second chamber.

The results of the retention tests are illustrated in Fig. 1. The saline injected control groups of mice show an increase in latency to reenter the compartment in which they had received foot shock after the 1-minute and 5-minute tests. The relatively poor short-term memory in controls of this strain

Table 1. The effect of DDC on [^{14}C]norepinephrine (NE) biosynthesis from [^{14}C]dopa and on endogenous NE concentrations in the brain of C57BL/6J mice. The animals were treated subcutaneously with 250 mg of DDC per kilogram, and 1 hour after administration of [^{14}C]dopa (specific activity, 3.18 mc/mole; 5 μc per mouse, given intravenously) the animals were killed. In separate groups of animals the concentrations of endogenous NE (last column) were determined. The results are the means from three experiments ± the standard error of the means. N.D., not detectable (below 200 count/min).

Time interval after administration of DDC	[^{14}C]NE (count/min per brain)	NE (nanograms per gram of tissue)
Controls (saline)	2800 ± 150	385.0 ± 11.5*
30 minutes		260.0 ± 8.5
90 minutes	N.D.	205.0 ± 6.0
4½ hours	N.D.	202.5 ± 7.0
8½ hours	1100 ± 100	215.0 ± 6.5

* There was no difference in brain NE between control mice tested 1 minute after training and those either injected with saline 30 minutes before training or those killed immediately after removal from their home cages.

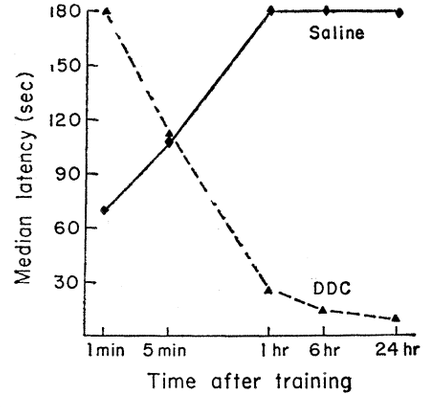

Fig. 1. Median latency to enter the large compartment on the retention test as a function of the time interval between the training trial and the retention test. Injections of DDC and saline were given 30 minutes before the training trial in all groups.