Isolation and Partial Purification of Hemolytic Toxin from Sea Anemone, *Stoichactis helianthus*

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Abstract □ The isolation of a toxic principle(s) from the sea anemone, *Stoichactis helianthus*, was effected by subjecting the anemones to a freeze–thaw cycle followed by immersion in deionized water. The crude toxic material was removed from the aqueous phase by precipitation with acetone and was partially purified by gel filtration. The partially purified toxin has a molecular weight greater than 5000 and an LD_{50} of 0.25 mg/kg in mice, and it causes complete hemolysis of a 1% suspension of sheep red blood cells at 0.5 μg/ml. The toxicity in mice is destroyed upon interaction of the partially purified toxin with concanavalin A.

Keyphrases □ *Stoichactis helianthus* (sea anemone)—isolation and partial purification of toxin with direct hemolytic activity □ Sea anemone—isolation and partial purification of toxin with direct hemolytic activity □ Hemolytic toxin, direct—isolation and partial purification from *Stoichactis helianthus* (sea anemone)

The pharmacological and chemical nature of coelenterate toxins has received much attention (1–5). While the properties of whole animal or whole tentacle homogenates employed in earlier studies are difficult to interpret, the more recent use of toxin preparations obtained from the rupture of isolated nematocysts (6–10) or the controlled discharge of attached nematocysts (11) has permitted a more definitive assessment of the mode of action of these substances and their chemical composition.

Various methods have been employed to effect rupture of the isolated nematocysts (6, 12). Of these methods the freeze–thaw cycle and immersion in distilled water, when effective, are perhaps the most gentle and the least destructive.

In this investigation, the isolation of a highly toxic and hemolytic principle from the sea anemone, *Stoichactis helianthus*, was most conveniently effected by applying the rupture techniques mentioned to the whole animals with neither homogenization nor nematocyst isolation. Partial purification was effected by the removal of low molecular weight constituents by gel filtration.

There is no direct proof that this substance is a constituent of the nematocyst apparatus; however, such a localization is implied by the high toxicity, the mode of preparation, and the fact that further extraction following homogenization failed to yield toxic material.

EXPERIMENTAL

The anemones (average 10 cm in diameter) were collected on the fringe reef off the St. James coast of Barbados, frozen (−20°), and shipped to these laboratories by air freight.

Crude Toxin Extraction—After storage (−20°) for 10 days the anemones (about 8 liters in volume) were defrosted, and the viscous liquid that separated was decanted (200 ml, Extract A). They were then gently mixed with deionized water (4 liters) and kept at room temperature for 4 hr with intermittent mixing. Care was taken in these operations to avoid mechanical tissue damage. The aqueous portion (5 liters) was decanted, filtered, and divided into two equal parts (Extracts B and C).

Table I—Isolation of Crude Toxin

Preparation	Weight, g	LD_{50}, mg/kg	Extract	Volume, ml	LD_{50}[a], ml/kg
A	3.6	4.5	A	200	0.25
B	25	3.3	B	2500	0.33
C	82	11.3	C	2500	0.34

[a] Extrapolated values (see text).

Table II—Gel Filtration of Water-Soluble Portion of Preparation B

Sample	Fraction	V_e/V_0[a] Range	Weight[b], mg	LD_{50}, mg/kg
1[c]	1–6	0.92–1.08	356	0.25
2	7–20	1.08–1.43	82	>10
3	21–35	1.43–1.80	205	>10
4	36–58	1.80–2.39	180	>10

[a] V_e = effluent volume, and V_0 = void volume. [b] Combined fractions were freeze dried. [c] Elemental analysis: C, 41.2%; H, 6.8%; N, 8.3%; P, nil; S, nil.

Crude Toxin Preparations—Extract A was diluted to 700 ml with acetone and kept at 0° for 4 hr. The precipitated material was collected, washed with acetone, and dried to constant weight under reduced pressure at room temperature. The resulting beige solid (9.6 g) was stirred with deionized water (100 ml) for 10 min, and the fine suspension that formed was centrifuged. The supernate was collected and freeze dried to provide Preparation A as a beige solid (3.6 g).

Extract B (2.5 liters) was diluted to 6 liters with acetone and kept at 0° for 24 hr. The precipitated material was collected, washed with acetone, and dried at room temperature under reduced pressure to provide Preparation B as a yellow-brown solid (25 g).

Extract C was freeze dried to provide Preparation C as a beige solid (82 g).

Gel Filtration of Water-Soluble Portion of Preparation B—The dextran gel[1] (500 g) was swollen in deionized water (saturated with chloroform) at 25° for 4 hr prior to use. The column (5 × 100 cm) had a void volume[2] (V_0) of 795 ml and was operated at a flow rate of 120 ml/hr (6 ml/cm²/hr). Fractions (20 ml each) were monitored by the biuret reaction according to the technique of Gornall *et al.* (13).

LD_{50} Determinations—The acute toxicity was determined in mice (female, ICR strain, approximately 2 months old) and based on a 6-day observation period. Intraperitoneal injections were given in a volume of 0.5 ml/20 g of mouse. Ten mice were used per dosage point, and the estimated LD_{50} was calculated by the method of Litchfield and Wilcoxon (14).

Hemolytic Studies—The hemolytic effect was determined by the addition of increasing toxin concentrations in isotonic saline (0.5 ml) to 4.5 ml of a 1.1% suspension of washed sheep red blood cells in isotonic saline. After 20 min, the suspensions were centri-

[1] Sephadex G-25 medium, Pharmacia, Montreal, Quebec, Canada.
[2] As determined with Dextran 2000, Pharmacia.

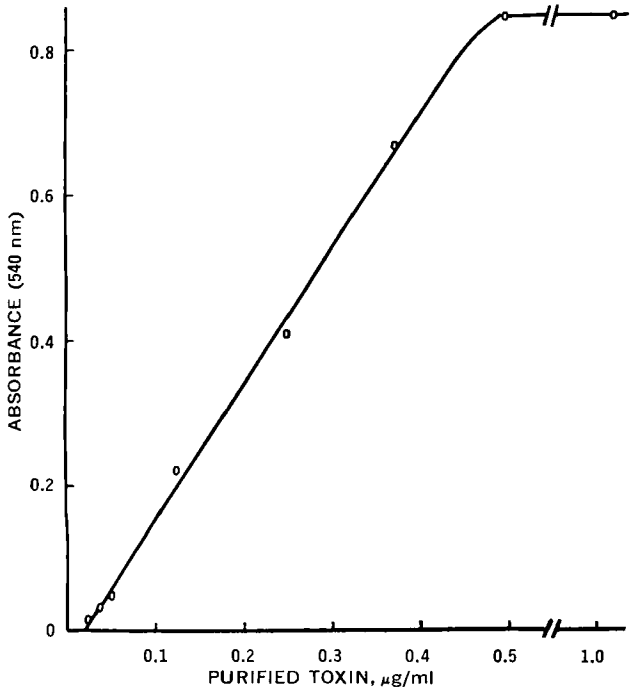

Figure 1—*Hemolysis of a 1% suspension of sheep red blood cells with the partially purified toxin in saline.*

fuged and the optical density of each supernate was measured[3] at 540 nm.

Interaction with Concanavalin A—The interaction of the purified toxin with concanavalin A[4] was determined by the development of turbidity upon the addition of a solution (3 ml) of concanavalin A to increasing toxin concentrations in a total volume of 4 ml. The medium employed was 15% aqueous sodium chloride, and the turbidity was measured[3] at 420 nm after 20 min at 25°. Corrections were made with respect to the absorption of the toxin solutions in the absence of concanavalin A.

RESULTS AND DISCUSSION

Isolation of Crude Toxin—The toxicities of Preparations A, B, and C are presented in Table I. Extrapolations of the toxicities of Preparations B and C back to the original extracts gave LD_{50} values of 0.33 and 0.34 ml/kg, respectively. Preparations B and C had a common origin and differed only in the mode of concentration, *i.e.*, acetone precipitation and lyophilization, respectively. Therefore, the toxic principle was insoluble in 50% aqueous acetone and apparently unaffected by this method of isolation.

Partial Purification—Preparation B (20 g) was stirred at room temperature with deionized water (300 ml) and then kept, without stirring, for 5 min. The milky aqueous portion was decanted. The insoluble material was treated twice more with deionized water (2 × 150 ml) in the same manner. The aqueous portions were combined and centrifuged (850×*g* for 1 hr at 5°). The supernate was decanted and the precipitate was mixed with water (200 ml) and again centrifuged (850×*g* for 1 hr at 5°). The latter supernate was combined with the former (total volume 800 ml), freeze dried to about 100 ml, and again centrifuged (850×*g* for 1 hr at 5°). The resulting clear pale-yellow solution was freeze dried. The beige solid (2.95 g, LD_{50} 0.6 mg/kg) obtained in this manner retained 90% of the toxic principle from Preparation B.

A portion (1 g) of this preparation (LD_{50} 0.6 mg/kg) was dissolved in deionized water (10 ml) and purified by gel filtration (see *Experimental*). The biuret-positive material was primarily in the first six fractions immediately following the void volume. The toxic effects were exclusively restricted to this narrow band (Table II) and represented an 86% recovery of the toxic principle. This behavior indicates that the molecular weight of the toxic principle(s) is greater than 5000.

Stability and Composition of Partially Purified Toxin—The toxin at this level of purity (Sample 1, Table II; hereafter referred to as toxin) was soluble in deionized water (>10%) and in 20% (w/v) sodium chloride solution (1%). At a concentration of 1 mg/ml, it was destroyed (LD_{50} >10 mg/kg) in acidic (0.05 *N* HCl at 25° for 1 day) and in basic (0.05 *N* NH_4OH at 25° for 1 day) media but was very stable (LD_{50} 0.25 mg/kg) in solution (25° for 2 days) in deionized water.

The toxin gave a positive biuret (protein) and a positive Molisch (carbohydrate) reaction.

Hemolytic Effects—The toxin caused complete hemolysis of washed red blood cells (sheep) at 0.5 μg/ml (Fig. 1). This effect was fully retained when a saline solution of the toxin at this concentration was kept at room temperature for 2 weeks. Since serum or lecithin addition was not required for this activity, the toxin can be classified as a direct hemolysin (15). The same level of activity was observed with whole blood (cat).

Direct hemolytic activity has been found in toxins of the sea wasp, *Chironex fleckeri* (16, 17), and the sea anemone, *Stoichactis kenti* (18), but not in that of the Portuguese man-of-war, *Physalia physalis* (7).

Interaction with Concanavalin A—Concanavalin A, a hemagglutinating protein from jack bean, *Canavalia ensiformis*, forms insoluble complexes with many polysaccharides (19) and glycoproteins (20). Therefore, the interaction of the toxin with concanavalin A was investigated (Fig. 2). Precipitation was complete at the ratio of 6 mg toxin to 1 mg concanavalin A. With this effect in mind, the toxin (60 mg) was treated with concanavalin A (15 mg) in 15% aqueous sodium chloride (15 ml). After 25 min (25°), the suspension was centrifuged and the supernate was decanted and diluted to 250 ml with deionized water. The resulting solution (equivalent of 0.25 mg toxin/ml saline) was found to be nontoxic at 50 ml/kg.

This result implies a close relationship of the polysaccharide moiety of the partially purified toxin with the toxic effect. The significance of the protein portion, however, remains unknown.

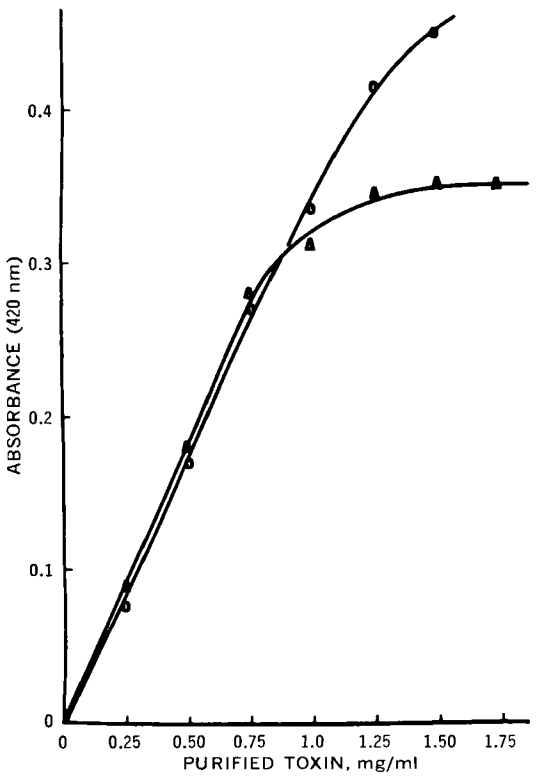

Figure 2—*Development of turbidity upon the interaction of the partially purified toxin with concanavalin A at concentrations of 0.250* (△) *and 0.375* (○) *mg/ml in 15% aqueous sodium chloride.*

[3] Spectronic 20, Bausch and Lomb Co.
[4] Pharmacia.

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Phosphorus–Nitrogen Compounds XVI: Phosphoramantadine Derivatives

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Abstract □ Nine derivatives containing adamantyl moieties were synthesized and screened for antileukemic properties. Five 1-*N*-adamantylphosphoramidate esters, phenyl *N*-1-adamantylphosphoramidochloridate, phenyl P-1-aziridinyl-*N*-1-adamantylphosphonamidate, and *N,N'*-diadamantyl-*N''*-bis(2-chloroethyl)phosphoric triamide were inactive. P,P-Bis(1-aziridinyl)-*N*-1-adamantylphosphinic amide, however, showed good activity when tested against L-1210 lymphoid and P-388 lymphocytic leukemia, giving %T/C values of 225 (20 mg/kg) and 244 (10 mg/kg), respectively.

Keyphrases □ Phosphoramantadine derivatives—synthesized and screened for antileukemic properties □ Phosphorus–nitrogen compounds—synthesis and screening of phosphoramantadine derivatives for antileukemic properties □ Amantadines, phosphorylated—synthesis and screening for antileukemic properties □ Anticancer agents, potential—synthesis and screening of phosphoramantadine derivatives □ Antileukemic agents, potential—synthesis and screening of phosphoramantadine derivatives

Amantadine (1-aminoadamantane, I) has been shown to inhibit the penetration of Rous Sarcoma virus in chick embryo cells (1) and to decrease mitosis and DNA synthesis in sea urchin ova (2), chick embryo fibroblasts, and Hela cell cultures (3). It also displayed activity *in vitro* against angiosarcoma and pancreatic sarcoma (4). The *N*-methyl, *N*-acetyl, and *N*-formyl derivatives of amantadine also gave an effect in the latter tumor system (4). Other compounds containing the adamantyl[1] moiety which have shown antineoplastic activity include those classified as thiosemicarbazones (5) and pyrimidines (6). Also, the insertion of an adamantyl grouping into cytarabine resulted in an immunosuppressant effect twice that of the parent molecule (7).

This paper reports the synthesis (Schemes I and II and Table I) and anticancer screening results (Table II) of nine phosphorylated amantadines. New ester derivatives (II–V) were synthesized by the phosphonate–carbon tetrachloride–amine method, while VII and XI were prepared by amidation of VI and IX, respectively. Compound VIII was synthesized by the reaction of I and bis(2-chloroethyl)phosphoramidic dichloride.

EXPERIMENTAL

Phosphoramantadine Diesters (II–V)—These compounds were synthesized by the reaction of I, carbon tetrachloride, and the appropriate phosphonate as previously reported for the diphenyl ester (8).

Di-*o*-chlorophenyl *N*-1-Adamantylphosphoramidate (X)—This derivative was prepared from IX, triethylamine, and *o*-chlorophenol according to a previously described procedure (8).

Phenyl *N*-1-Adamantylphosphoramidochloridate (VI)—To a stirred solution of 7.5 g (0.05 mole) of I and 5.0 g (0.05 mole) of triethylamine in 50 ml of anhydrous ether was added 10.5 g (0.05 mole) of phenyl phosphorodichloridate at 30° over 45 min. After remaining overnight, the reaction mixture was filtered and the residue was washed free of chloride with water. Recrystallization from ethanol gave the pure product.

Phenyl P-1-Aziridinyl-*N*-1-adamantylphosphonamidate (VII)—A solution of 3.3 g (0.01 mole) of VI and 1.3 g (0.03 mole) of aziridine in 50 ml of dioxane was refluxed for 4 hr and then left

[1] This name for the radical, rather than the Chemical Abstracts nomenclature of tricyclo(3,3,1,1^{3,7})dec-1-yl, is used in this paper.