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STUDIES ON THE ORIGIN AND DISTRIBUTION OF PALYTOXIN IN A CARIBBEAN CORAL REEF

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S. Gleibs, D. Mebs and B. Werding. Studies on the origin and distribution of palytoxin in a Caribbean coral reef. *Toxicon* **33**, 1531–1537, 1995.—In coral reefs of the Caribbean Sea (Colombia) palytoxin (PTX) has been detected in zoanthid species of the genera *Palythoa* and *Zoanthus* by assaying the delayed haemolysis in human erythrocytes produced by aqueous extracts, which is inhibited by ouabain pretreatment, and by HPLC. The toxin content of the polyps and colonies is highly variable and is not correlated with their reproductive cycle or with the amount of symbiotic algae. Sequestration of PTX has been observed in crustaceans (*Platypodiella* sp.) living in close association with *Palythoa* colonies and in polychaete worms (*Hermodice carunculata*) feeding on the zoanthids. Resistance of marine animals to the toxin may enable it to enter food chains.

INTRODUCTION

Palytoxin (PTX) is considered to be one of the most potent compounds, exhibiting extreme toxicity in mammals (i.v. LD_{50} 10–100 ng/kg; Vick and Wiles, 1975), surpassed only by bacterial toxins. It has been primarily isolated from the marine zoanthids *Palythoa* (Moore and Scheuer, 1971; Attaway and Ciereszko, 1974; Beress *et al.*, 1983). PTX produces a broad range of effects *in vivo* as well as *in vitro* (Habermann, 1989). The toxin acts through the Na^+, K^+-ATPase of cell membranes, inducing channel or pore formation by the enzyme protein. Despite its high lethality in terrestrial animals the toxin occurs also in crabs (Yasumoto *et al.*, 1986), in a sea anemone (Mahnir and Kozlovskaya, 1992) and in fish (Hashimoto *et al.*, 1969; Fukui *et al.*, 1987; Kodama *et al.*, 1989) without causing deleterious effects.

The present study was performed to investigate the origin and sequestration of PTX in a coral reef ecosystem.

MATERIALS AND METHODS

Marine specimens

Palythoa, *Zoanthus* and other marine specimens were collected in the reefs along the coast of Santa Marta, Colombia (Caribbean Sea), by snorkling or SCUBA diving. The samples were frozen and kept at −20°C until use. PTX isolated from *Palythoa caribaeorum* was a generous gift from Prof. L. Beress (Kiel, F.R.G.).

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PTX assay

Haemolysis. In vitro PTX activity was assayed by testing the delayed haemolysis of human erythrocytes (Habermann *et al.*, 1981). One gram of the sample (parts of zoanthid colony, crabs or worms) was homogenized in 1 ml distilled water using a mortar and centrifuged at 900 × *g*. Fifty microlitres of the supernatant (extract) or its dilutions (with saline) was added to 1 ml of 0.5% red cell suspension (human erythrocytes washed three times) in saline containing 0.5 mM boric acid and 1 mM $CaCl_2$, and incubated for 4 hr at 37°C, the optimum duration of incubation for haemolysis (Ahnert-Hilger *et al.*, 1982). After centrifugation the absorbency of the supernatant was measured at 405 nm. Total haemolysis was achieved by adding 50 μl of 1% saponin solution to the red cell suspension. Haemolytic activity was expressed in units. One haemolytic unit (HU) is defined as the amount of material to produce 50% haemolysis within 4 hr of incubation. In a control experiment 100 μM ouabain (Sigma, St Louis, U.S.A.), an inhibitor of PTX-induced haemolysis (Habermann and Chhatwal, 1982), was added to the red cell suspension and incubated at 37°C for 30 min before the haemolysis test. Only those samples in which haemolysis was completely inhibited by ouabain were considered to contain PTX.

HPLC analysis. Before HPLC analysis of PTX the samples were purified by gel filtration on Sephadex G-75 and chromatography on SP-Sephadex C-25 according to the method of Beress *et al.* (1983). Fractions which produced delayed haemolysis were lyophilized, dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to qualitative HPLC analysis using a reversed-phase column (Lichrospher 300, RP-8, Merck, F.R.G.). Elution was performed by a linear gradient of 0.1% TFA, to which 80% acetonitrile was added and mixed.

Fertility assay. Twenty randomly selected *Palythoa* colonies were cut longitudinally under a dissecting microscope and the number of polyps (about 20 each) was counted, which contained eggs, ovaries, testes or both (ovaries and testes in hermaphroditic specimens) or which were entirely sterile. The ratio of fertility was expressed in per cent for each colony. The polyps examined were also tested for their PTX content.

Chlorophyll a *assay. Palythoa* samples (1 g) were extracted with hot (75°C) 90% ethanol. Chlorophyll *a* content was assayed spectrophotometrically according to the procedure of Smith and Benitez (1955).

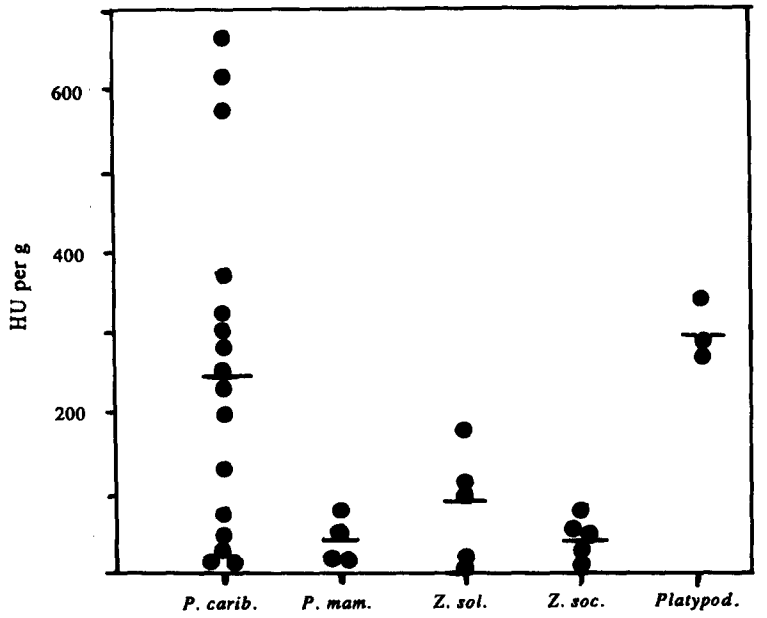

Fig. 1. Delayed haemolysis assay of palytoxin (PTX) in samples of *Palythoa caribaeorum* (*P. carib.*, 20 specimens), *P. mammillosa* (*P. mam.*), *Zoanthus solanderi* (*Z. sol.*), *Z. sociatus* (*Z. soc.*, 5 specimens each) and the crab *Platypodiella spectabilis* (*Platypod*, 3 specimens). The procedure is described under Materials and Methods. Horizontal bar: mean value.

RESULTS

PTX content in Palythoa *and other marine organisms*

Two species of *Palythoa* occur in the reefs along the coast of Santa Marta, Colombia, predominantly at a depth between 2 and 4 m: *Palythoa caribaeorum* and *P. mammillosa*. Both species contain PTX, as demonstrated by delayed haemolysis of human red cells caused by the extracts, which is effectively inhibited by ouabain, but potentiated by borate, two criteria considered to indicate the presence of PTX (Habermann, 1989). Samples from different colonies varied considerably in their PTX content (expressed in haemolytic units); there were extracts with very low haemolytic activity, but also those containing up to 700 HU (Fig. 1).

Two other zoanthid species, *Zoanthus solanderi* and *Z. sociatus*, are the major competitors of *Palythoa* for space in the reef. They grow in colonies close to those of *Palythoa* and contain also variable concentrations of PTX (Fig. 1). The toxin was also identified by HPLC in samples of both *Palythoa* and *Zoanthus* (Fig. 2).

Some crustaceans such as *Platypodiella spectabilis* (Brachyura: Xanthidae) live in crevices or holes under the crust of *Palythoa* colonies (Den Hartog and Türkay, 1991). Three specimens which were available for testing exhibited considerable PTX concentrations comparable to those of the associated colony. The polychaete worms *Hermodice carunculata*

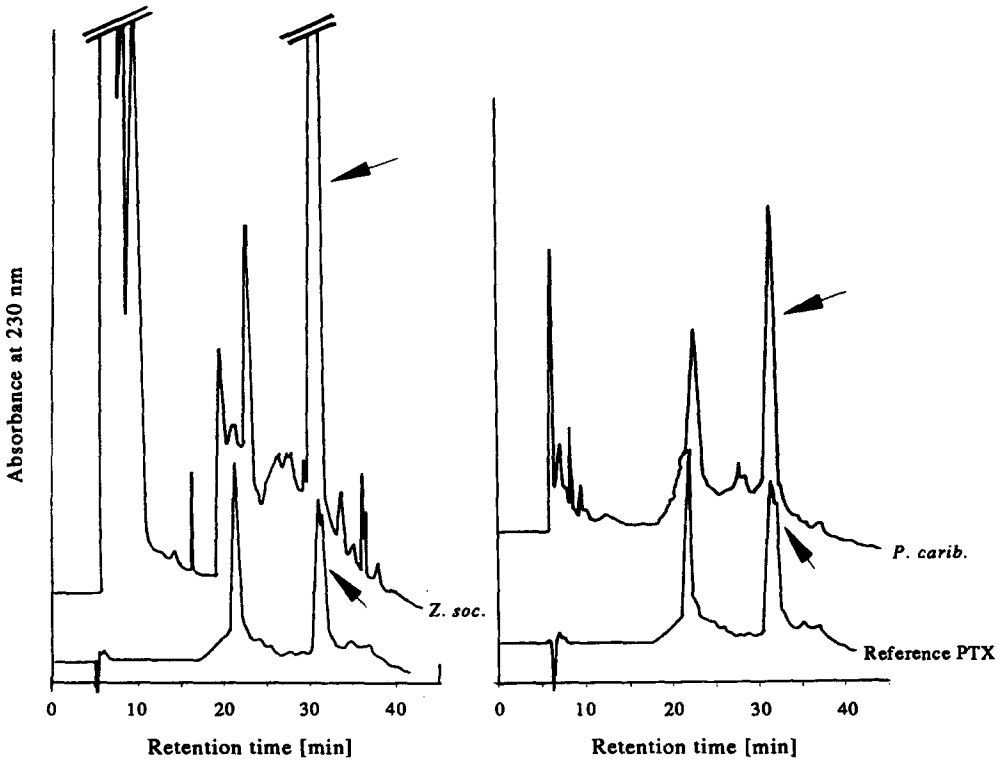

Fig. 2. PTX assay in purified extracts from *Zoanthus sociatus* (left) and *Palythoa caribaeorum* (right) by HPLC.
Lower tracing reference PTX sample (10 μg). The arrow indicates PTX fractions, which produced delayed haemolysis.

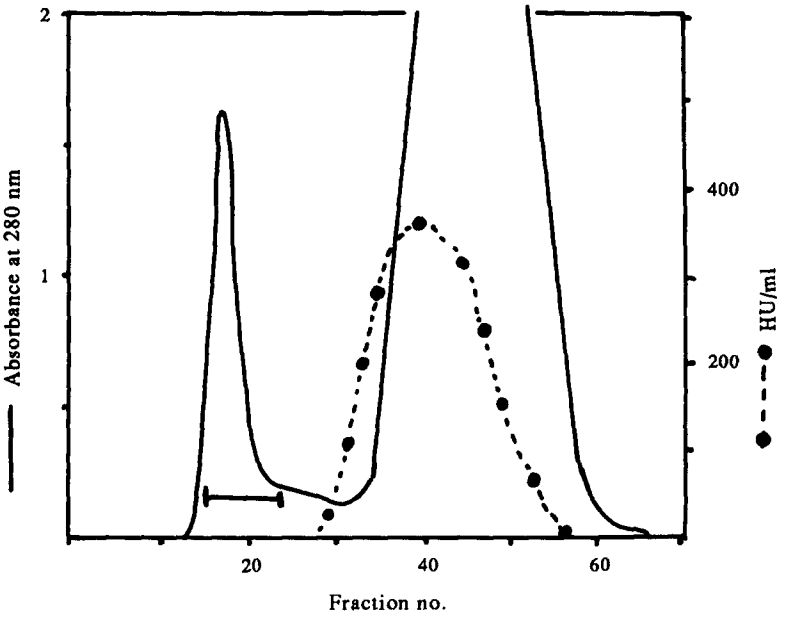

Fig. 3. Gel filtration of extract from the polychaete worm *Hermodice carunculata* on Sephadex G-75.
Column size: 85 × 1.5 cm, elution was performed using 0.1 M ammonium acetate pH 6.8 at a flow rate of 30 ml per hr, fractions of 5 ml were collected. The first fraction (horizontal bar) contained direct haemolytic activity, delayed haemolysis due to PTX (dotted line) was detected in the second peak.

and *Eunice* sp. were observed predating *Palythoa* and feeding on the polyps. This contradicts observations of Sebens (1982), who did not find *Hermodice* feeding on *Palythoa* on the Caribbean coast of Panama. Incubation of extracts from *Hermodice carunculata* specimens with human red cells caused almost instant haemolysis, which was not influenced by ouabain pretreatment of the erythrocytes. However, two haemolytic activities could be separated by gel filtration of the extracts (Fig. 3): one fraction eluted with the void volume, where haemolysis occurred almost instantly but was not inhibited by ouabain, and a second fraction eluted with the low mol. wt part of the extract, where haemolysis was found to occur after 3–4 hr only and was completely inhibited by ouabain pretreatment. This indicates that the polychaete worm contains PTX, which it obtained by feeding on the colonies, and a direct haemolysin of high mol. wt.

PTX content and fertility of Palythoa polyps

As described by Kimura *et al.* (1972), there are usually four types of *Palythoa* polyps to be found: female polyps containing numerous eggs in their mesenteries, males with testes, hermaphroditic polyps possessing both eggs and testes (protogynous hermaphroditism) and sterile, non-reproductive polyps. All four stages appeared simultaneously in the colonies investigated during the period June–October 1993. The number of fertile (female, male, hermaphroditic) polyps varied from colony to colony; sterile polyps were also observed among reproductive specimens.

In 20 randomly collected colonies no correlation was found between fertility such as egg

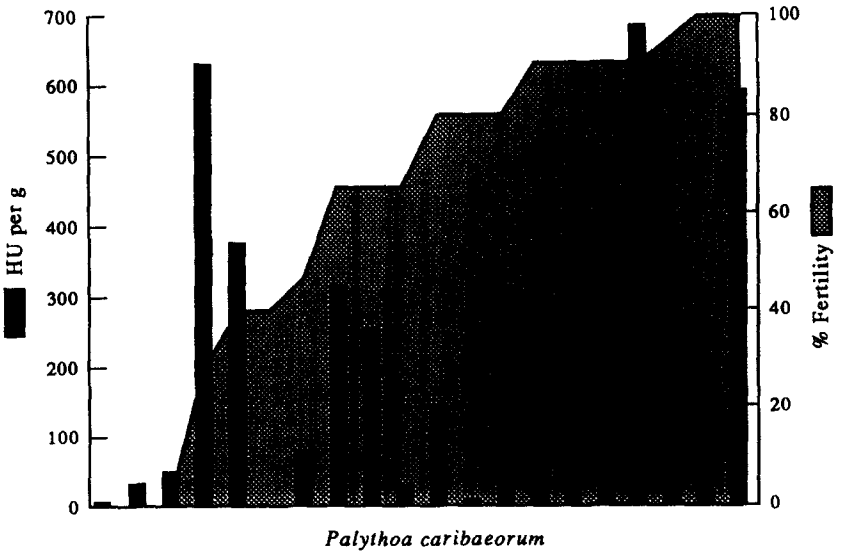

Fig. 4. Percentage fertility and PTX content (delayed haemolysis) of 20 *Palythoa caribaeorum* colonies.

production of the polyps and PTX content (Fig. 4). Besides fertile colonies with low PTX levels, colonies were found exhibiting a low percentage of fertility, but a high PTX content.

Chlorophyll a *and PTX content*

Since zoanthids contain considerable amounts of symbiotic algae (zooxanthellae) in their mesogloea, both chlorophyll *a* and PTX concentrations were assayed in 32 *Palythoa caribaeorum* colonies. As shown in Fig. 5 there exists no correlation between the chlorophyll *a* content as a measure of the amount of algae in the zoanthid and the respective PTX concentration.

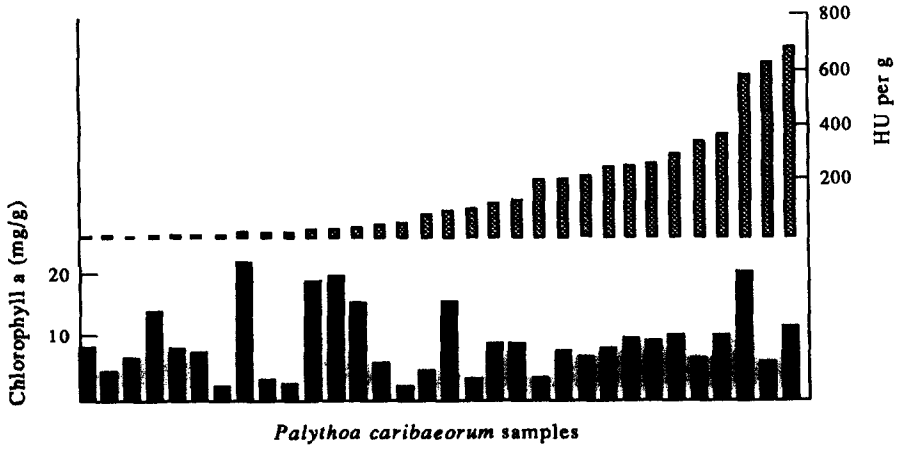

Fig. 5. Chlorophyll *a* content of 32 *Palythoa caribaeorum* samples and their PTX content (delayed haemolysis).

DISCUSSION

The results of this study support the assumption that PTX is more widespread in marine ecosystems than anticipated. As well as in *Palythoa* species the toxin was found in Zoantharia of the genus *Zoanthus*, space competitors of *Palythoa* in the coral reef. Moreover, animals such as crustaceans (*Platypodiella* sp.) living in close association with *Palythoa* colonies or polychaete worms (*Hermodice carunculata*) feeding on *Palythoa* sequester PTX. Since high concentrations of the toxin have been detected in the body of these animals, they must have developed considerable resistance to the toxin. This also applies to other marine animals where PTX has been detected, such as fish (Hashimoto *et al.*, 1969; Fukui *et al.*, 1987; Kodama *et al.*, 1989), crabs (Yasumoto *et al.*, 1986) and a sea anemone (Mahir and Kozlovskaya, 1992). Whether this involves their Na^+, K^+-ATPase, the primary target of PTX (Habermann, 1989), undergoing molecular changes preventing interaction with the toxin, or other mechanisms such as membrane resistance to pore formation, has still to be elucidated. However, resistance to the toxin renders its role as a deterrent for potential predators questionable.

The origin of PTX is still a matter of speculation. Moore *et al.* (1982) suggested a bacterial origin, which has never been corroborated experimentally. Other potential producers such as symbiotic algae, which are able to synthesize secondary products similar to PTX (Nakamura *et al.*, 1993) and which live in large masses in the mesogloea of the Zoantharia, may also be considered. But the lack of correlation between algae (i.e. chlorophyll *a* content) and PTX content appears to contradict their involvement in toxin synthesis. However, direct proof that the algae are toxin producers or are not involved in PTX synthesis may be achieved by culturing the isolated zooxanthellae.

Kimura *et al.* (1972) reported a correlation between the reproductive cycle of *Palythoa* polyps (from the Pacific) and their PTX content. This could not be confirmed in the *Palythoa* species from the Caribbean Sea. Considerable concentrations of PTX were measured even in sterile polyps, but some egg-bearing polyps were entirely free from PTX.

Kodama *et al.* (1989) identified PTX in a mackerel (*Decapterus macrosoma*), a fish often involved in ciguatera poisoning. Resistance of marine animals to the toxin enables its sequestration and accumulation in the food chain. Therefore, symptoms of ciguatera differing from those regularly observed may be due to PTX-like toxins other than ciguatoxin and its congeners (Swift and Swift, 1993).

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