DNA values during lactation are significantly higher than observed at day 1 of lactation.

E was found to augment involution during the initial period after weaning. DNA was decreased 71% from day 1 of lactation, and 50% below the normal controls. By day 10, growth effects of E were evident with a 79% increase above control levels by day 20.

E + P did not prevent the initial decrease of DNA. Involution progressed at same rate as under normal conditions. P in combination with E appears to prevent more rapid decrease in DNA as found when E was injected alone. P may have a "masking" effect upon E, since it has been reported to retard involution at 1 mg day for 6 days(8). E + P growth effects were evident by day 10, with an 82% increase over normal values by day 20.

Ovarian hormones may not retard involution to any significant degree, because DNA values determined at day 5 of involution were as low or lower than observed during normal period. Higher values observed with hormones during later stages of present experiment may be due entirely to growth promoting activity of these hormones upon the mammary gland.

Summary. With DNA as a measure of mammary gland involution, a 42% decrease in DNA between day 1 of lactation and day 5 of involution was noted. By day 10, DNA decreased 85% and remained essentially constant throughout the remainder of the period. E alone appeared to augment retrogression of DNA during initial days of involution, but with E + P this effect was not apparent. With both E and E + P, mammary gland DNA was increased during the later days of experimental period.

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Biologically Active Peptides in *Physalia* Toxin.* (26723)

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Several previous communications from this laboratory have detailed the activity of various kinds of crude *Physalia* toxin preparations(1,2,3). Recently whole toxin has been fractionated chromatographically into a series of peptides. Even after this rigorous treatment, some biological activity persists. This communication presents the results of tests for biological activity of crude toxin on various microorganisms and protozoa, and describes the distribution of toxicity among the component peptides of the crude toxin of *Physalia*.

Materials and methods. Surviving nematocysts were separated from tentacular tissue by controlled autolysis, and were homogenized to liberate their toxic contents as described by Lane and Dodge(1). Nematocysts were homogenized for 20 to 30 minutes in an all-glass homogenizer. The residue was diluted with 2 to 3 ml of distilled water and centrifuged for 20 minutes at 2°C and 10,000 rpm. The residue was resuspended in a

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minimum of water and recentrifuged as before.

The supernatant solution was transferred quantitatively to tared 5-ml drying bulbs and lyophilized at 100-150 μ residual pressure. The drying bulbs were sealed without loss of glass while still evacuated. Six tared empty bulbs were evacuated to 125 μ residual pressure, sealed, cooled and re-weighed. The average weight loss (18.90 mg) provided a correction for calculation of total dry weight of toxin contained in each of the sealed drying bulbs. Lyophilized toxin was stored in evacuated drying bulbs at –10°C.

The yeasts and bacteria used for the auxanographic tests were isolated from Biscayne Bay. *Paramecium caudatum* and *Tetrahymena gellii* were obtained from laboratory cultures and *Uca pugilator* were collected on tidal flats near the Institute of Marine Science.

Fourteen different species of yeasts and 10 species of marine bacteria were exposed in duplicate to 4 different concentrations of *Physalia toxin*. Actively growing cultures of microorganisms were concentrated by centrifuging. The residue was adjusted with sterile distilled water to 20% transmission at 450 mμ. This "constant" suspension was diluted 1 to 20 with agar nutrient medium and poured into Petri dishes. Five sterile, antibiotic testing discs were arranged in a standard pattern on each plate. The center disc received 0.04 ml of sterile, distilled water. The 4 remaining discs each received 0.04 ml of 1.0, 10.0, 100.0, and 1000.0 p.p.m. of toxin solution. The plates were incubated at 25°C for 48 hours, then examined for the presence of zones of inhibition around each of the discs.

The effects of *Physalia* toxin on ciliate protozoa were assayed as follows: 100 living organisms of either *Paramecium caudatum* or *Tetrahymena gellii* were isolated from active cultures into 100 ml of growth medium. 1.0 ml of solution was added to each group of organisms containing the following toxin concentrations: 0.0, 10, 100, and 1000 p.p.m. Each concentration of toxin was replicated 4 times. Cultures were incubated at room temperature for 48 hours. They were then shaken well, and a 1 ml aliquot was removed from each. This was diluted with 5 ml of 10% formalin and brought to 50 ml with distilled water. All the organisms in 0.5 ml of this diluted fixed culture were counted. The 4 replicates of each toxin concentration were counted twice each by 3 individuals, and the results averaged and multiplied by 100 to determine number of organisms per culture. Preliminary to auxanographic tests or ciliate assays the activity of each toxin preparation was established by bio-assay on *Uca pugilator,* as previously described.

For chromatography of toxin fractions lyophilized toxin was diluted to 10,000 p.p.m. with distilled water and this diluted material was added as a stripe 5 inches long on Whatman #1 paper, 7½ by 18 inches in dimension. Chromatograms were run for 18 hours at room temperature in 80% aqueous n-propanol. The unstriped inch and a quarter at each edge of the papers received a single 10 μl spot of the same toxin dilution. The finished papers were dried, these edge strips were cut off and developed in 0.2% ninhydrin dissolved in acetone to reveal the location of the peptides on the rest of the paper. The papers were then cut so as physically to separate each peptide fraction. The fractions were separately eluted at room temperature with 2 to 5 ml distilled water. The chromatographic eluates were collected in tared 30 ml serum bottles and lyophilized directly. After elution of the individual peptides each paper was shown to be ninhydrin negative. Individual peptides separated chromatographically as described have been eluted, lyophilized, hydrolyzed with 6 N HCl in evacuated sealed capsules at 100° for 12 hours. After removal of hydrochloric acid the hydrolysate was rechromatographed. Each peptide was shown to contain more than 2 amino acids. Further details of the amino acid composition of each of these peptides will be described later.

After lyophilization the fractions were weighed and then accurately redissolved in sea water in proportion to their original concentration in the whole toxin. Each fraction was assayed on a group of 10 *Uca pugilator* evenly divided as to sex and of approximately

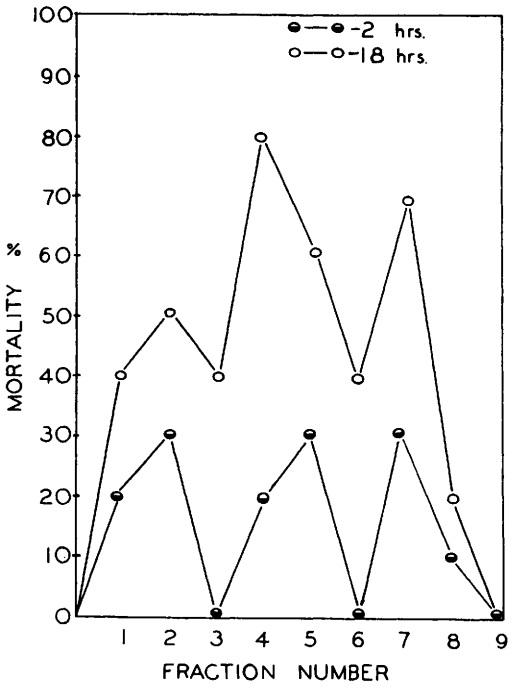

FIG. 1. Toxicity of chromatographic fractions of crude *Physalia* toxin. Ten *Uca pugilator* were inj. with each fraction and mortality recorded at 2 hr and again at 18 hr.

the same weight. 0.10 ml was injected through the arthrodial membrane at the base of the third walking leg. The results of the injection were evaluated after 2 hours and 18 hours.

Results. No toxin preparation exhibited activity in the auxanographic tests with a variety of marine microorganisms. Control colonies of *Tetrahymena gellii* and of *Paramecium caudatum* increased in number from 100 to 7800 ± 230 and 247 ± 26 respectively in 24 hours under the conditions of these tests. No significant change in growth rate occurred with 3 different concentrations of toxin. Fig. 1 shows that all component peptides of the crude toxin possess some toxicity for *Uca*, but peptides numbered 2, 4, 5, and 7, sequentially from the point of origin exhibited the highest biological activity.

Discussion. As has been previously reported from this laboratory, the toxin of *Physalia* is labile and it is easily destroyed by prolonged exposure to the effects of organic solvents. However, under the conditions of these experiments sufficient activity persisted in the chromatographic fractions to permit evaluation of one fraction against another. It is quite clear that the activity of the component peptides of the crude toxin of *Physalia* vary in biological activity as they have been shown to vary in chemical composition.

Each of these peptides is presently being studied in this laboratory and details of their chemical composition, component amino acids, and pharmacologic activity will be reported later.

Summary and conclusions. The crude toxin of *Physalia* nematocysts withstands lyophilization without significant loss of toxicity. *Physalia* toxin may be separated into component peptides by one-dimensional chromatography in 80% aqueous n-propanol. Each of the resultant peptides retains considerable toxicity for the fiddler crab, *Uca pugilator*. Crude toxin of *Physalia* nematocysts has no effect on growth of marine yeasts or marine bacteria. Toxin preparations have been shown to be without significant effect on growth or reproduction of the ciliate protozoans, *Paramecium caudatum* and *Tetrahymena gellii*.

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