Further Studies on the Purification and Physiological Actions of Sea Nettle Toxin[1] (35983)

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The sea nettle (*Chrysaora quinquecirrha*), a venomous jellyfish, occurs in great abundance in the Chesapeake Bay and other coastal waters. This coelenterate inflicts injury to swimmers by injecting a toxin-coated thread into the skin. Recent investigations have demonstrated that soluble toxin released from nettle nematocysts is neurotoxic, myotoxic, and cardiotoxic, as well as capable of producing dermonecrosis and hemolysis (1–3). It has also been shown that *Chrysaora* toxin increased the sodium permeability of active membranes independent of ATPase or cyclic AMP (4). In order to understand the toxin's physiological action at the cellular level, its effect on rat liver mitochondria and lysosome preparations was studied.

Passage of the nematocyst fluid through Sephadex gels resulted in partial purification and separation of the lethal activity into at least 2 fractions (5). In the present investigation, isoelectric focusing was applied to further separate and define the active components.

Materials and Methods. Nematocyst suspensions (NS) were prepared from fresh *Chrysaora* tentacles according to previously described techniques (5). Sonic treatment of NS with 3 A for 90 sec ruptured the nematocysts and released the soluble toxins. This suspension was then centrifuged at 50,000*g* for 1 hr before the resultant supernatant (SU) was inoculated into a Sephadex G-200 gel column (30 cm height × 2 cm diam). Four milliliter fractions were collected and assayed for protein according to the method of Waddell (6). The ascending portion of the major peak in the protein curve of the eluate (FAS) was freeze-dried, resuspended in distilled water and inoculated into the isoelectric focusing apparatus (Model No. 8101, LKB Produkter AB, the Netherlands). Two to three watts were supplied to the focusing column for 2.5 days at 4°. At the conclusion of this run, serial 4 ml fractions of the sample within the column were collected. The pH of each fraction was measured before they were dialyzed and concentrated against weak phosphate buffer (pH 7.0; 0.001 *M*) at 4° in a collodian bag (Schleicher and Schuell, Inc., Keene, NH). A few specimens were treated by dialysis against buffer, freeze-drying and subsequent resuspension in buffer.

All resuspended fractions were analyzed by immunodiffusion and assayed for intravenous mouse lethality, dermonecrosis, and protein content according to previously described techniques (3, 7, 8). In these experiments we observed that the ampholines interfered with the absorption of light, whose wave lengths fell between 215 and 280 mμ. For this reason, it was necessary to measure the protein concentration of the isoelectric column sample by the method of Lowry (8).

The hyperimmune sera used in immunodiffusion was obtained from rabbits inoculated against either NS or SU. The sera of these animals was capable of neutralizing 200 iv mouse LD_{50} units/ml (3).

Disc electrophoresis was performed according to the methods of Davis (9). A power of 2.5 μA per gel was applied for 90 min; a time sufficient to allow the bromophenol

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blue tracer dye to pass to the bottom of each of the gels.

The lysosomal and mitochondrial suspensions were prepared at 4° from rat liver according to previously described techniques (10, 11). The lysosomal suspensions were treated with Triton X or toxic nematocyst preparations before assaying these suspensions for free acid phosphatase and β-*N*-acetyl hexosaminidase (12, 13). The effect of toxin on the mitochondrial respiratory control index was measured by a polarograph chamber (Gilson Medical Electronics Co., Middleton, WI) equipped with a Clark-type membrane-coated oxygen electrode (11, 14).

Results. At least two separate lethal and dermonecrotic toxins were separated from FAS by isoelectric focusing. The p*K* values of these toxins were 4.0 and 6.3, respectively. Approximately 60% of the lethal and dermonecrotic activities inoculated into the isoelectric focusing apparatus were recovered in these two fractions. Greater recovery occurred if the isoelectric focusing was done in ampholytes possessing a pH range of 3–6 units and if rapid concentration was done in collodian bags. The toxin with a p*K* of 6.3 (p*K* 6) contained more total lethal and dermonecrotic activities than that with a p*K* of 4 (p*K* 4). The p*K* 6 was lethal (LD_{50}) if inoculated iv into mice at a dose of 0.4 μg of N_2/g of mouse; whereas p*K* 4 was lethal at a dose of 0.6 μg of N_2/g.

Multiple bands were observed on the acrylamide disc electrophoresis gels inoculated with p*K* 4 and p*K* 6 toxins. Usually 4 or 5 dark bands could be observed ½ to ⅔ the way down the gel after 90 min run (Fig. 1).

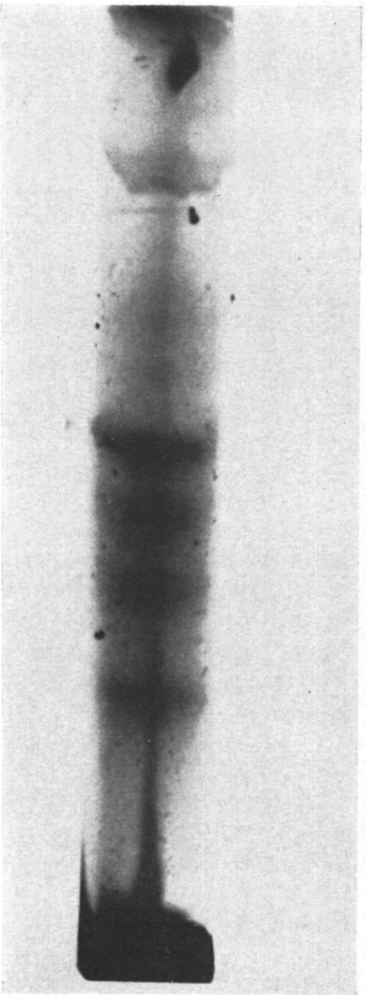

FIG. 1. Disc electrophoresis of p*K* 4 sample: The wavy line near the top is the intersection of the sample and stacking gels. Four distinct bands are visible.

Immunodiffusion and immunoelectrophoresis have been used as techniques to follow the progress of protein purification. The latter technique was not very effective in the present instance since only faint bands were visible when FAS was used as the antigen (Fig. 2). No bands were visible if p*K* 4 or p*K* 6 toxins were inoculated onto the immunoelectrophoresis slides. Contrariwise immunodiffusion could be used as an assay technique if sera from rabbits sensitized to SU were employed in the antibody wells. Three bands were seen on those plates when crude NS was the antigen. SU and FAS antigen wells were surrounded by 2 and 1 lines, respectively (Fig. 2). Wells inoculated with p*K* 4 or p*K* 6 toxins were usually surrounded only by a single band which formed lines of identity with each other. Two lines surrounded p*K* 4 or p*K* 6 toxin wells in 33 and 15% of the tests, respectively.

Triton X (0.15% final concentration), whole tentacle preparations, SU, FAS, p*K* 4, and p*K* 6 released free hexosaminidase from lysosome suspensions (Table I). Heated SU (56°, 30 min) and neutralized SU (SU

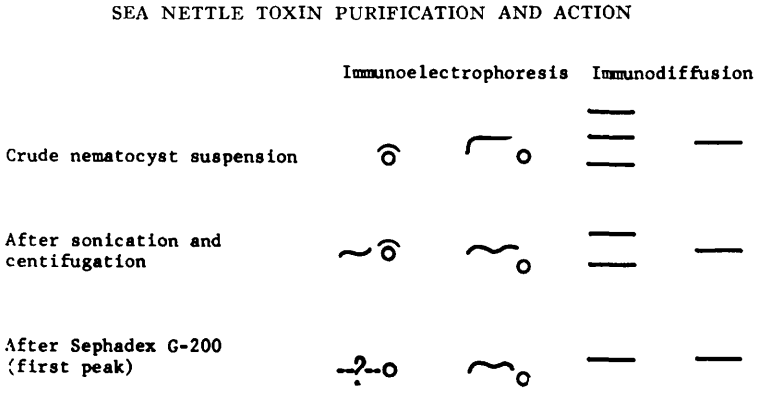

FIG. 2. Immunoelectrophoresis and immunodiffusion of crude and partially purified sea nettle toxins: The sera used in columns 1 and 3 were from an animal immunized with centrifuged preparations; whereas, those in columns 2 and 4 were from an animal immunized with crude nematocysts.

treated 16 hr at 4° with hyperimmune rabbit sera) did not increase free enzyme concentrations. Treatment of the lysosomal preparations with bovine serum albumin (1 mg of albumin/1 mg of lysosomal protein) or methylprednisolone sodium succinate (0.2 mg/ml) did not protect them from the action of SU. Similar results were obtained in experiments in which free acid phosphatase was assayed.

None of the sea nettle toxin preparations affected the respiratory control index of rat liver mitochondria. The maximum dose of toxin inoculated into the chamber contained 1.25 mg of protein and 25 mouse iv LD_{50} doses.

Discussion. The soluble nettle nematocyst fluid contains multiple toxins. Two active fractions were separated by Sephadex gel diffusion (5). The larger of these fractions (FAS) had a molecular weight of 100,000–400,000 whereas the smaller was 5000–70,000. Both these fractions had lethal and dermonecrotic activity but a demonstrable hemolysin was only recovered in FAS.

FAS could be separated by isoelectric focusing into two components possessing lethal and dermonecrotic factors. Some degradation was produced by the procedure since the average total recovery was 60%. The remaining lethal factors had a lower iv LD_{50} (0.4–0.6 μg of N_2/g of mouse) than FAS (iv LD_{50} = 0.93 μg of N_2/g of mouse) (5). The loss or alteration of toxin could have resulted from the electrical current applied during the focusing, the pH, the 4° temperature, or the dialysis procedure. Alteration of the toxin protein structure would also explain the immunodiffusion experiments in which FAS produced a single precipitin band; whereas multiple bands sometimes appeared near the p*K* 4 and p*K* 6 antigen wells. Similar phenomena had been observed with oyster hemolymph treated by different physical techniques (15).

It is interesting that the recovery of lethal activity of p*K* 4 and p*K*6 toxins appeared to be greater if the isoelectric focusing were

TABLE I. The Release of Lysosomal Enzymes by Sea Nettle Toxin.

Treatment applied to lysosomes	Protein inoculated (μg)	Lethality of inocula (mouse iv LD_{50} doses)	4-Methylumbelliferyl-*N*-acetyl-β-D-glucose (n*M* released/min/mg of lysosomal protein)
Triton			30,000
FAS	41	0.17	33,000
p*K* 6	700	0.25	31,500
p*K* 4	350	0.08	7800

performed using ampholines designed to separate proteins whose pK values were between 3 and 6 pH units and if the procedure was shortened by using collodian bags. This observation suggests that the pH of the ampholine gradient and the duration of the separation process might be important in preserving the activity of the lethal factors.

Disc electrophoresis analysis of the pK 4 and pK 6 components revealed that fewer bands were present than were seen with FAS indicating that some purification had been achieved. Although 4–5 bands were seen by electrophoresis, immunodiffusion of the same components revealed only 1 or 2 precipitin bands. This observation suggests that only one or two of the proteins found by disc electrophoresis are antigenically active or that several of the protein bands travel at the same speed through the immunodiffusion gel.

Analysis of the pK 4 and pK 6 fractions by immunoelectrophoresis has not been profitable. No distinct bands have appeared in the gels, presumably because the antigen is diluted by electrophoresis.

Previous experience had shown that the dermonecrotic activities of FAS were heat labile (5). In the present experiments dermonecrotic activity was recovered from pK 4 and pK 6 samples which had been subjected to 4° temperatures for several days during the isoelectric focusing and dialysis procedures. The reason for the stability of the dermonecrotic action in the more purified specimens is not yet known.

Sea nettle toxins appear to rupture lysosomes but do not affect oxidative phosphorylation in mitochondria. pK 6, the more lethal of the 2 fractions isolated after isoelectric focusing, is also more injurious to lysosomes than pK 4. Other animal toxins have been found to release enzymes from lysosomes (16). It is possible that this action might be responsible for the cutaneous cellular injury and pain which accompanies the nettle sting in humans.

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