Isolation of the major toxic protein from the skin venom of the crested newt, *Triturus cristatus*

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Summary. A new protein, of molecular weight 160,000, was isolated from the skin venom of the crested newt and partially characterized by bioassays and biochemical methods. This protein shows the same functional properties as the crude venom, which produces convulsions in mice and is cytotoxic.

The crested newt *Triturus cristatus* extrudes a foamy whitish secretion from granulary skin glands when threatened by predators[2] or in the presence of pathogenic microorganisms (personal communication, G. Habermehl). This secretion deters other animals from attack[2] and is characterized by its cytotoxic activities[3], e.g. hemolysis. Previous studies suggest that the hemolytic activity arises from one or several different proteins with a mol. wt of 45,000[4]. This paper reports the isolation of the major toxin by gel chromatography and ion exchange chromatography. It is an oligomeric protein with an apparent mol. wt of approximately 160,000, composed of subunits of 40,000 to 50,000 mol. wt.

Materials and methods. The crude venom was obtained by sucking up the foamy secretion from the skin of CO_2-anesthetized newts. This suspension was lyophilized and stored at -20 °C. For solubilisation the venom was ground with washed quartz sand in 0.1 M Tris-Cl/0.4 M NaCl, pH 8.5. After centrifugation the clear supernatant was chromatographed on a Sephadex G-200 column and eluted with 0.1 M Tris-Cl, pH 8.5 at 4 °C. The fractions were concentrated by vacuum dialysis. Aliquots were injected s.c. into white mice. The fractions which produced local loss of hair or killed the animals were pooled and applied to a DEAE-Sephadex A50 column. Elution was carried out with a linear saline gradient (0.1-0.4 M NaCl) in 0.1 M Tris-Cl, pH 8.5 at 4 °C.

The purified toxin was submitted to 2 dimensional electrophoresis[5] (native-SDS). For the 1st dimension, 7.5% acrylamide pH 8.9, and for the 2nd, 7.5% acrylamide, 0.1% SDS, pH 7.5 was used. The 1st dimension gel was incubated in SDS buffer[6] on a boiling waterbath for 10 min. In a second 2 dimensional electrophoresis[5] (native-native) the gel system for both dimensions was 7.5% acrylamide, pH 8.9.

Unstained gels were incubated for 60 min at 37 °C on agar slabs containing human blood (for further details see[7]).

Results and discussion. Within 2 months, and adult newt (10 g) can produce its total amount of venom (approximately 250 mg, dry weight) containing 135 mg of unknown proteins and 45 mg of toxic protein. After its isolation (figure 1), this toxin shows the same properties as the crude venom; apparently it corresponds to its main toxic component. It induces cell damage and produces convulsions when injected s.c. into white mice (table). It is completely inactivated by proteolytic treatment (trypsin) or by heat. If administered perorally with a teflon tubing into the stomach, it has no toxic effects. These properties provide strong evidence for the protein nature of the toxin. During its purification, a significant increase of the toxicity for white mice is observed: crude venom LD_{50} 4.5 mg kg^{-1}, Sephadex G-200 purified material LD_{50} 3.8 mg kg^{-1}, DEAE-Sephadex purified material LD_{50} 2.5 mg kg^{-1}. The relatively small increase in toxicity is probably caused by the lability of the protein.

The mol. wt of the native protein is approximately 160,000 as determined by gel filtration on Sephadex G-200[8]. On polyacrylamide gel electrophoresis in the presence of SDS[6] only a single protein band is obtained, corresponding to a mol. wt of approximately 50,000. Mol. wt determination by gel filtration in 6 M guanidine-hydrochloride yield 40,000 to 45,000. On the basis of these data, the toxin seems to be composed of 4 (or less probably 3) subunits with similar mol. wt.

On polyacrylamide gel electrophoresis, the purified native toxin gives 2 distinct bands with a smear in between. When this cylindrical gel is subjected to SDS electrophoresis on a slab gel in the 2nd dimension, the 2 bands and the smear of the protein give 1 single band, as expected from the proposed quarternary structure of the toxin. On omitting

Biological effects common to both the crude venom and the purified toxin

	Effects in vivo*	Effects in vitro
0–24 h	Convulsion of muscles Death by spasms of respiratory muscles	Hemolysis of human erythrocytes**
24–96 h	Loss of hair at the injection site	Cell wall damage of Bacillus megatherium***
	Destruction of: muscle, fat, connective } tissue-structure	

*20 g white mice, s.c. injection, dosage in the range of LD_{50}-values (see text). **Hemolysis was observed after application of 0.1 µg ml^{-1} of lyophilized toxin to blood containing agar gel media and in erythrocyte suspensions in 0.9% NaCl[3]. ***Spherical transformation of protoplasts was found in bacteria cultures in nutrient broth containing 5 mg ml^{-1} of crude venom. Growth times were 24–48 h at room temperature[7].

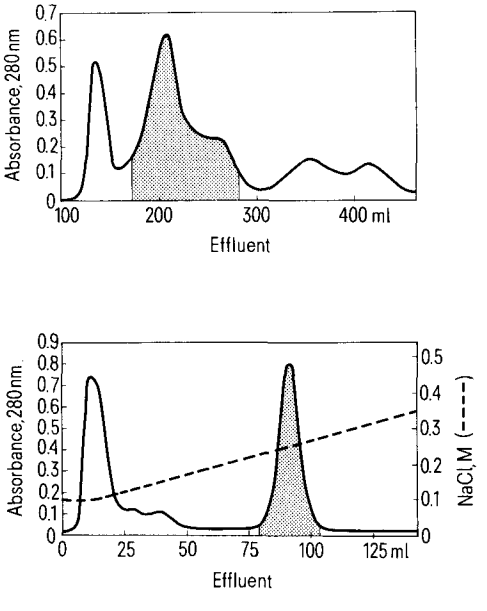

Fig. 1. Isolation of *Triturus cristatus* toxin. *A* Elution profile of crude venom chromatographed on Sephadex G-200. *B* Elution profile of Sephadex G-200 purified material after chromatography on DEAE Sephadex A-50. The shaded peaks contain the toxic protein.

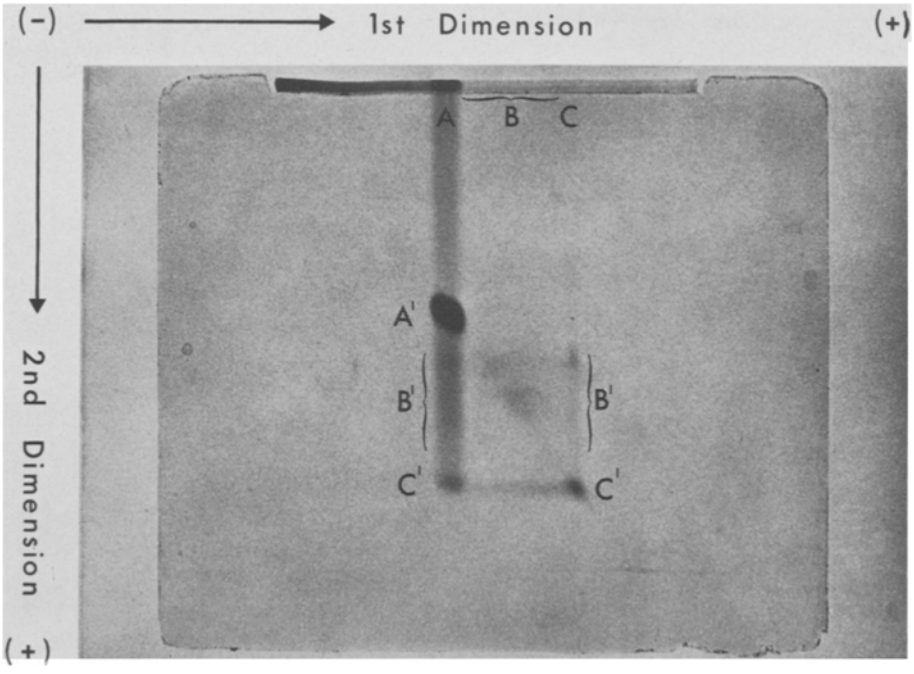

Fig. 2. 2 dimensional electrophoresis of purified *Triturus cristatus* toxin. Both dimensions: native toxin.

SDS in the 2nd dimension of polyacrylamide gel electrophoresis, the upper band A with the highest protein content splits up into the complete pattern of bands A′, B′, C′ obtained in the 1st dimension, band C forms B′ plus C′ and the smear B moves to 2 horizontal bands and a diagonal line, corresponding to the bands and the smear observed in the 1st dimension (figure 2). Apparently the components B and C are interconvertible and derive from the main component A, which contains the intact toxin. This is shown by incubation of the polyacrylamide slab on a blood agar slab. Hemolysis only occurs in an area corresponding to band A.

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Effect of theophylline on myocardial adenylate cyclase activity[1]

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Summary. Theophylline (0.01–10.0 mM) did not increase but rather decreased adenylate cyclase activity (AC) of guinea-pig auricles. Isoprenaline (1–100 μM) and sodium fluoride (0.3–10.0 mM) stimulated AC in a concentration-dependent manner.

We have recently shown[2] that the positive inotropic effect of theophylline in electrically driven left auricles from reserpine-pretreated guinea-pigs is consistently accompanied by an increase in cyclic AMP levels. The theophylline-induced increase in cyclic AMP, which was not impaired by propranolol, proceeded very rapidly: with 2 mM theophylline, cyclic AMP was elevated by about 25% within 5 sec. Under the same conditions, phosphodiesterase activity was inhibited by about 90%[3]. The conclusion from these data was that the theophylline-induced increase in cyclic AMP was due to the inhibition of the degradation of cyclic AMP and that the cyclic AMP turnover is a very fast process in the guinea-pig heart. However, the question arises whether the theophylline-induced increase in cyclic AMP proceeded so rapidly because theophylline also enhanced the formation of cyclic AMP via a stimulatory effect on myocardial adenylate cyclase. The present experiments were therefore designed to investigate whether theophylline increases adenylate cyclase activity of guinea-pig auricles. The effects of sodium fluoride and isoprenaline were studied for comparison.

Materials and methods. Guinea-pigs of either sex (220 to 300 g b.wt) were pretreated with reserpine (Serpasil® ampoules Ciba; 5 mg/kg i.p.; 18 h before sacrifice) to deplete cardiac catecholamine stores. Left auricles were isolated, suspended in aerated Tyrode solution (1.8 mM Ca^{++}; 5.4 mM K^{+}; 35 °C; pH 7.4) and stimulated electrically at 3 Hz as described previously[4]. After 30 min, the auricles were removed from the organ bath, blotted and weighed. Adenylate cyclase was prepared at 4 °C. 4 auricles were pooled, minced in a mortar for 2 min and suspended in 10 vol. (based on tissue weight) of a hypotonic medium consisting of 2.5 mM ATP, 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, and 2 mM Tris-HCl at a final pH of 7.4. The resulting