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31. ON THE TOXIN OF *TRITURUS MARMORATUS* LATR.

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Introduction

The range of the newt *Triturus marmoratus* includes Portugal, Spain and the southern parts of France. The amphibian is green with black speckles above and grey to brown with black or white dots beneath. It reaches a length of 16 cm and a weight of 10 g. Its toxin is supposed to be one of the most active newt toxins with a DLM of 1.8 mg/g white mouse (s.c.) (1). Since no chemical datas were available we studied some chemical and biochemical properties and compared them to the properties of other toxins.

Material and methods

Toxin: The newts were rinsed with destilled water, placed in a beaker and treated with 0.2 ml ether. The ether stimulated the excretion of the skin secretion. The product so obtained forms a characteristically smelling foam which was dissolved in dest. water and freeze-dried. A well fed mature newt yields 20-50 mg dry material. The first milking yielded entirely soluble products. After repeated milking the solubility of the toxin decreased.

For better comparison with other amphibian toxins this method was prefered to obtain the toxin by squeezing the paratoid glands.

Gel-chromatography: Sephadex G-200 was used in a column 80 × 2 cm. The void volume of 85 ml was determinated with blue Dextrane. 5 ml of a solution containing 1 per cent newt venom and 0.9 per cent sodium chloride was placed on the column. The elution was performed with saline. 2 ml fractions were taken.

Gel-electrophoresis: A disc electrophoresis system has been used (2). The small pore gel was prepared by dissolving 750 mg acrylamide (Fluka P 50135), 20 mg N,N'-methylene-bisacrylamide (Fluka P 55626), 0.06 ml 10 per cent solution of N,N,N',N'-tetramethylenethylenediamine (TEMED, Fluka 57580) in 10 ml TRIS-HCl buffer pH 8.9. After deaeration the polymerization was started with 7 mg ammonium persulfate. Glass tubes (10 × 0,5 cm) were filled with 1.2 ml solution to a level of 6 cm and allowed to polymerize under a layer of destilled water.

A large pore gel solution was prepared by dissolving 250 mg acrylamide, 6.2 mg N,N'-methylenebisacrylamide, 0.17 ml 10 per cent TEMED in 10 ml TRIS-HCl buffer pH 6.7, and starting with 3.5 mg ammonium persulfate. 0.2 ml of

this solution were placed on top of the small pore gel and polymerized again under a layer of dest. water. After the polymerization is complete the water is replaced by 20-50 μl of a 1 per cent solution of the toxin in TRIS-HCl buffer pH 6.7. After 90 min a sufficient amount of the sample was soaked into the large pore gel. The rest was removed.

Electrophoresis, staining and destaining has been done as described before (2, 3).

Hemolysis and enzyme assay: These determinations were done as described before (4, 5, 6, 7). Surface tension was measured stalagmometrically.

Results and discussion

The enzyme activities of the *Triturus marmoratus* toxin are similar to those of other amphibians (5, 6). E.g. the phosphatase (Fig. 1) is an unspecific phosphomonoesterase similar to acid prostatic phosphomonoesterase. The maximum of activity is at pH 5.0 (p-nitrophenylphosphate). An amylase similar to unk amylase could be detected (5). The arylamidase has an optimum of activity at pH 7.0-7.5 (L-leucine-β-naphthylamide). It hydrolyses (Table I) the β-naphthylamides of alanine, leucine, methionine and proline at a high rate. The hydrolysis rates of the β-naphthylamides of valine, glycine, lysine, arginine, isoleucine and cysteine decreases in the given sequence down to some per cents of

TABLE I — HYDROLYSIS OF L-AMINO ACID β-NAPHTHYLAMIDES (THE VALUE FOR L-LEUCINE β-NAPHTHYLAMIDE IS GIVEN AS 100)

Bombina species		*Triturus cristatus*		*Triturus marmoratus*	
leu	100	ala met pro	100	ala met pro	100
ala gly met pro	90	leu arg val hpro try	100 95 50 45 40	leu val hpro ser	100 80 55 40
lys arg his	45 40 20				
ileu	10	gly ser	30	gly his thr	15
glu	5	thr	25	lys	10
asp cys ser	2	glu his tyr	10	arg asp cys glu	10
		asp ileu cys	10	ileu try tyr	

the first group. The sequence of the hydrolysis rates of *Triturus cristatus* toxin differs from *T. marmoratus* toxin mostly in respect to the β-naphthylamides of lysine and arginine. The first mentioned toxin hydrolyzes these substrates much more readily than the latter. The natural substrates of this enzyme are of course not β-naphthylamides but peptides, it would be probably more correct to term this enzyme as peptidase.

Triturus marmoratus toxin hemolyses human erythrocytes down to dilutions of more than 1:10 millions. In this respect it is markedly more active than the toxins of *T. cristatus* or *Bombina variegata.* The hemolysing principle is considered to be a direct hemolysing protein by the same reasons (e.g. no phospholipase activity, inactivation by proteolytic enzymes) as discussed in a previous paper (6). Such proteins occur in snake venoms (8). In bee venom the direct hemolysing factor is a polypeptide with 26 amino acids (9). In both cases the factor was accompanied by phospholipase. The factors themselves were strongly basic.

In disc electrophoresis the hemolytic protein of *Triturus marmoratus* toxin migrates at pH 8.9 with appr. 80 per cent of the mobility of bromphenolblue (Fig. 2), it is therefore an acidic reacting substance. From Sephadex G-200 it is eluted by 1.9 times the void volume (Fig. 1). This corresponds an apparent molecular size of appr. 40.000. Similar data have been found for *Triturus cristatus* and *Bombina* toxins (6). *Triturus* toxins lower the surface tension markedly more than other proteins of similar mole size and electrophoretic behaviour (1). However, since the hemolytic activity of surface active substances does not always parallel the influence on the surface tension this is not necessary the only explanation for the strong hemolytic action of the *Triturus* toxin.

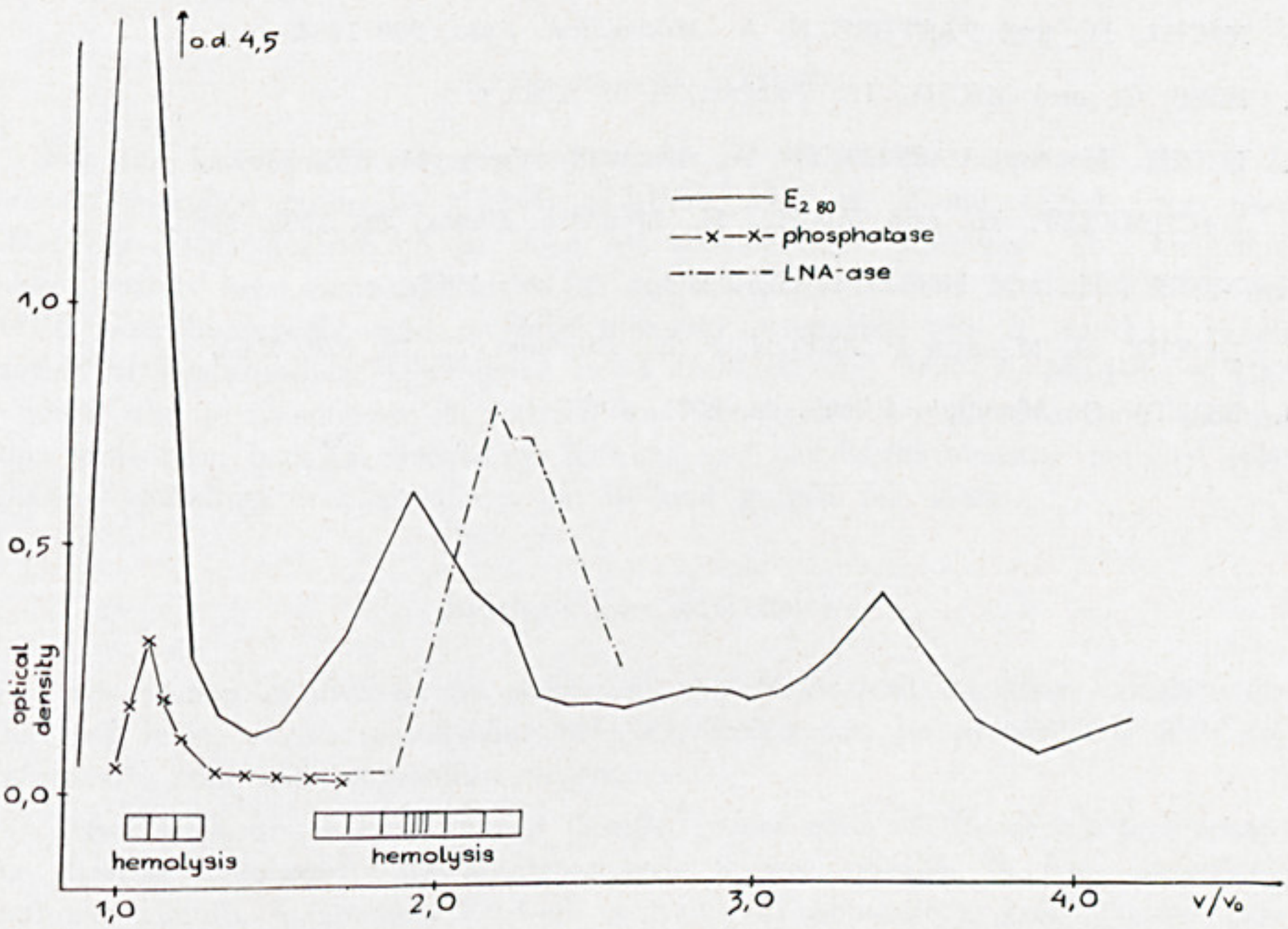

Fig. 1 — *Triturus marmoratus* — Chromatography on Sephadex G-200.

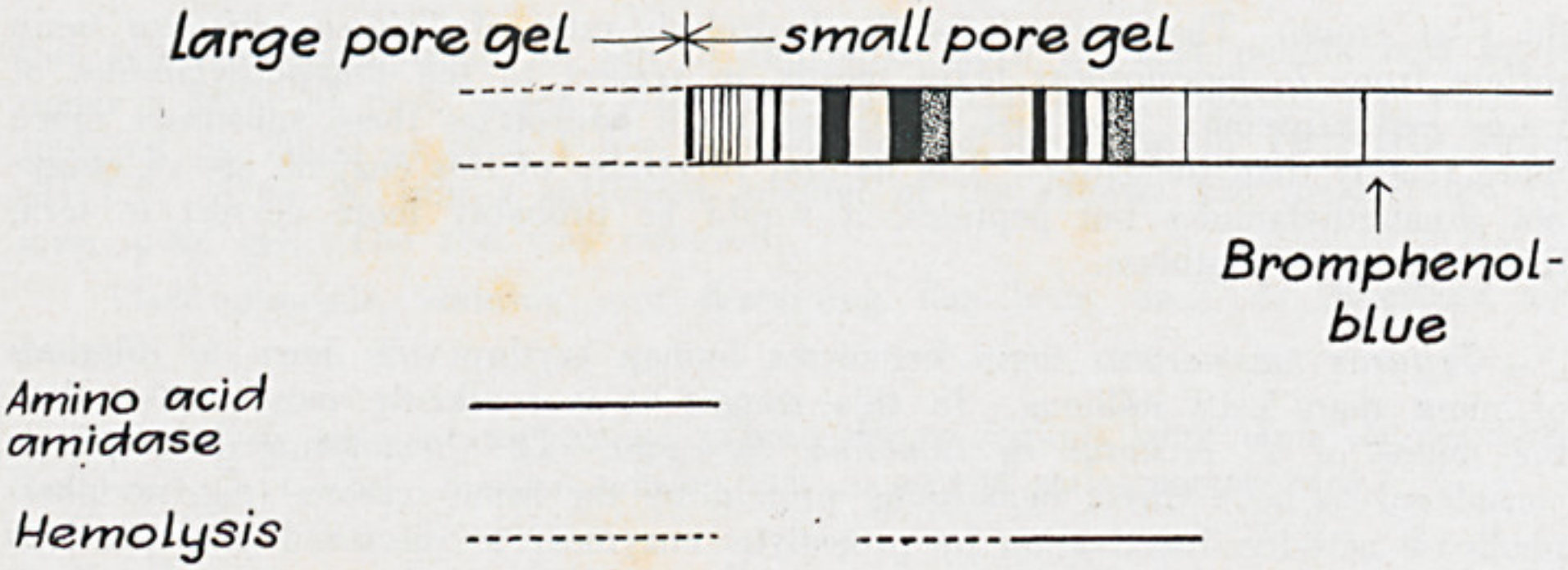

Fig. 2 — *Triturus marmoratus* — Electrophoresis in polyacrylamide.

Summary

Amylase, acidic phosphomonoesterase, arylamidase and a direct hemolysing protein were demonstrated in the toxin of *Triturus marmoratus* Latr. The enzymes and the hemolytic factor were characterized by chromatography on Sephadex, gel electrophoresis and substrate specificity.

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