# EFFECT OF TETANUS TOXIN ON PROTEIN COMPOSITION OF SYNAPTIC STRUCTURES OF THE RAT BRAIN AND SPINAL CORD

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It was shown by electrophoresis on polyacrylamide gel that the content of proteins with low electrophoretic mobility rises in a Triton extract of the fractions of synaptic structures from the spinal cord tissue of rats with local tetanus, whereas no change was found in the protein spectrum in the dodecyl sulfate extract. In experiments in vitro tetanus toxin stimulated the incorporation of lysine-$H^3$ into total proteins of cortical synaptosomes.

KEY WORDS: tetanus toxin; local tetanus; rat brain synaptosomes; electrophoresis of proteins in polyacrylamide gel; protein synthesis.

Tetanus toxin (TT) specifically damages synapses and thus blocks the secretion of mediators into the CNS [8, 10] and, in large doses, it also damages neuromuscular synapses [4, 15]. The primary link in the biochemical disturbances in the synapses in TT poisoning has not yet been established. TT has been shown to bind selectively with the fraction of brain synaptic membranes [1, 19], forming a complex with the gangliosides present in the membranes [1, 9, 21]. Whether TT (or its toxophore group) penetrates into the synaptoplasm or whether its harmful action is limited to disturbances in the presynaptic membranes is likewise unknown. The writers showed previously that in the initial stage of TT poisoning changes take place in the transport Na,K-ATPase activity of spinal cord membranes [5]. In experiments in vitro TT greatly inhibited contractions of actomyosin-like protein of synaptic origin [6]. Changes in protein metabolism in the synapses are an important indicator of the molecular pathology of synapses. No data on changes in the protein composition of the synapses in TT poisoning are to be found in the literature.

The object of this investigation was to study changes in the protein spectrum of synaptic structures of the brain and spinal cord tissues of rats poisoned with TT and during the action of TT in vitro.

## EXPERIMENTAL METHOD

Noninbred rats weighing 180-200 g were used Subcellular fractionation of the spinal cord and brain tissues of the rats was carried out by centrifugation in a sucrose density gradient as described earlier [12]. After centrifugation fractions rich in synaptic membranes, light and heavy synaptosomes, and "pure" mitochondria were collected, sedimented at 130,000 g (40 min), after which the residues were extracted in 0.1% Triton X-100 solution containing 0.01 M tris-HCl buffer, pH 8.4, for 16 h at 0-4°C. After centrifugation (105,000 g, 40 min) a Triton extract of the corresponding fractions was obtained, and the residues were solubilized in a 1% solution of Na dodecyl sulfate, containing 0.01 M tris-HCl buffer, pH 8.4, at 0-4°C (the DDS extract of structural proteins).

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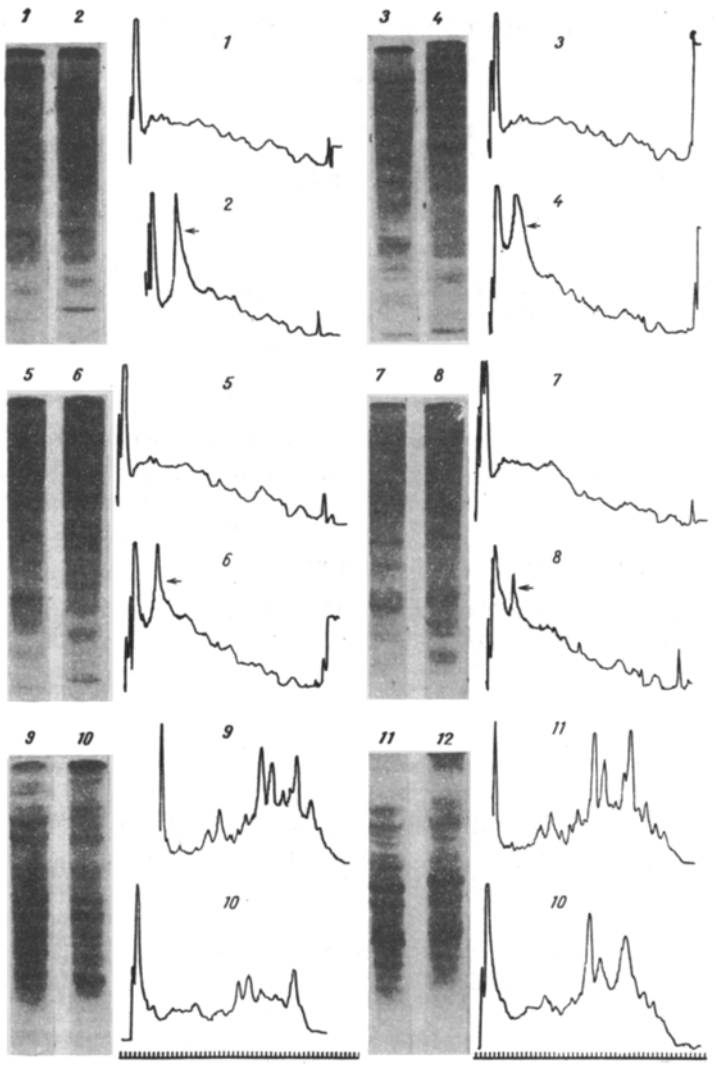

Fig. 1. Electrophoresis and densitometry of Triton and DDS extracts of synaptic structures from normal rats and rats poisoned with tetanus toxin. On chromatograms start at top, finish at bottom, direction of electrophoresis to anode; on densitograms start on left, finish on right. 1, 3, 5, and 7) Triton extracts of fractions of light synaptosomes (1), synaptic membranes (3), heavy synaptosomes (5), and mitochondria (7) from normal rats; 2, 4, 6, and 8) Triton extracts of corresponding fractions in TT poisoning; 9-12) DDS extracts of corresponding fractions from healthy rats.

Disk electrophoresis [16] in 7.5% polyacrylamide gel (PG) was carried out in 5 mM tris-glycine buffer, pH 8.3, at 0-4°C (Triton extract) or at 20°C (DDS extract) in the Reanal (Hungary) apparatus. The PG and working buffer contained detergent in the same concentration. Protein was applied in a dose of 100-150 $\mu$g per tube. The initial current was 1.5 mA/gel and the duration 20 min for the Triton extract, and 0.5 mA/gel and 40 min for the DDS extract, respectively. In the case of the DDS extract no concentrating gel was used. When the protein entered the gel the current was increased in both cases to 4 mA/gel, and electrophoresis continued for 1.5 h for the Triton extract and 3.5 h for the DDS extract. Bromphenol blue was used as the indicator dye. The PG was fixed with 7% TCA, washed with water, and stained with 0.2% Coomassie Brilliant Blue in a solution of $CH_3COOH-CH_3OH-H_2O$ (1:1:2), and then washed in the same solution (1:10:30). The fractions were estimated quantitatively by means of the Chromoscan (England) densitometer. The protein content was determined by Lowry's method. The residue of total rat cerebral cortical synaptosomes (mainly from the gray matter) was suspended in 0.8 ml Krebs–Ringer medium (NaCl

TABLE 1. Effect of Tetanus Toxin on Incorporation of Lysine-$H^3$ into Proteins of Cortical Synaptosomes of Rats in Vitro

| Statistical index | Specific radioactivity (in counts/min/mg protein) | | |
|---|---|---|---|
| | control | + TT (15 μg/ml) | + TT (inactivated, 15 μg/ml) |
| $M$ | 2754 | 4564 | 2341 |
| $\pm m$ | 168 | 241 | 250 |
| $n$ | 6 | 6 | 4 |
| % | 100 | 166* | 85 |

Legend: 1 Control – incorporation of lysine without addition of TT to samples. 2.* marks results of experiments for which $P < 0.05$ (compared with control); n – number of experiments.

124 mM, KCl 5mM, $MgSO_4$ 1.3 mM, $NaHCO_3$ 26 mM, $CaCl_2$ 0.75 mM, glucose 10 mM, tris-HCl, pH 7.4, 10 mM) and preincubated for 10 min at 37°C. Next 0.2 ml $H^3$-DL-lysine-HCL (specific radioactivity 0.5 mCi/mM), dissolved in the incubation medium, was added. The final concentration was 1 mg/ml protein and the radioactivity 4 μCi/ml. The samples were incubated for 30 min at 37°C. The reaction was stopped with 10% TCA (1 : 1) and a series of rinsings with 5% TCA (3 times) was carried out to remove unincorporated label. Nucleic acids were removed by heating to 80°C (20 min). Extraction was then carried out with 95% ethanol, saturated with sodium acetate, lipids were extracted with a mixture of ethanol and ether (2 : 1) on heating (6°C, 30 min), and the residue was dried with ether. The purified proteins were dissolved in 0.5 ml 0.5 M NaOH. Some of the solution was used to determine protein (up to 30% of protein was lost during the washing), the rest (0.2 ml) was transferred to the counting flask, to which 8 ml ethanol and 8 ml scintillation fluid – 0.1 g 2,5-diphenyloxazole (PPO) and 6.0 g 1,4-di[2-(5-phenyl)-oxazolyl] benzene (POPOP) in 1 liter toluene – were added. Radioactivity was counted in a Nuclear Chicago Mark II (USA) scintillation counter. The results were expressed as specific radioactivity after subtracting the background and the adsorption control (503 counts min/mg protein). The incubation conditions chosen were optimal for the study of local protein synthesis in synaptosomes [2]. Each experiment was carried out on synaptosomes isolated from six or seven rats.

TT was purified by gel-filtration on Sephadex G-200 [11] and inactivated at 120°C (20 min); the solution of the toxin thus obtained, in a concentration of 50-90 μg/ml, remained translucent. Local tetanus was produced by injecting 0.1 MLD at several points (0.1 ml per injection) into the muscles of the leg and thigh (so as to ensure the regular arrival of TT in the spinal cord along the regional neural pathway [4]). Tissues of the anterior horns of the lumbosacral enlargement [5] in TT poisoning were investigated after 72 h.

## EXPERIMENTAL RESULTS AND DISCUSSION

Triton extracts of fractions of the synaptic membranes, light and heavy synaptosomes, and mitochondria of the spinal cord contained 52, 64, 62, and 27% protein, respectively, whereas the DDS extracts of the same fractions contained 40, 35, 37, and 66% protein, respectively. The protein content in extracts of the fractions of synaptic structures from the spinal cord tissues of healthy and poisoned rats were virtually identical.

Anodic disk electrophoresis of proteins from synaptic structures of healthy rat spinal cord showed a high degree of heterogeneity for both Triton (up to 15 stained bands) and DDS extracts (up to 22-23 bands; Fig. 1). Heterogeneity of soluble, structural, and membrane-bound proteins of the synaptic structures of the cerebral cortex has been described previously [14, 18, 20, 22]. Specific proteins have also been shown to be present in the synaptosomes and synaptic membranes, not present in the phase spectrum of brain mitochondrial and microsomal proteins [3, 13, 17].

It follows from Fig. 1 that the greatest changes in protein composition in the Triton extracts (soluble and membrane-bound proteins) in TT poisoning were observed in the fractions of synaptic structures (especially the synaptic membranes fraction); in the mitochondrial fraction these changes were much less marked, evidently because of contamination with synaptosomes. These changes were expressed as an increase in the intensity of the stained proteins with an electrophoretic mobility of 0.1 in the synapses in tetanus poisoning. The experiments also showed that compared with normal, no significant changes were observed in the DDS extracts of the structural proteins from fractions of the synaptic structures in tetanus poisoning.

To continue the study of the changes in synaptic protein composition in tetanus poisoning, experiments were carried out to study the effect of TT on protein synthesis in the synaptosomes in vitro. As Table 1 shows, TT in a concentration of 15 μg/ml (1000 MLD for mice) considerably (by 66%) stimulated the incorporation of lysine-$H^3$ into total proteins of the synaptosomes of the rat cerebral cortex. Heat-inactivated

TT did not affect the protein-synthesizing system of the synaptosomes. The observed increase in the intensity of staining of some proteins after electrophoresis of the Triton extract of synaptosomes and synaptic membranes on PG found in the early stage of tetanus poisoning thus correlates with data showing an increase (evidently in the membrane component) of local protein synthesis in nerve endings as a result of the action of TT in vitro.

In the early stage of tetanus poisoning no significant morphological changes of synaptic structure took place and the most characteristic feature was the accumulation of synaptic vesicles close to the active zone of the presynaptic membrane [7, 8]. Changes in protein synthesis in the synapses therefore merit special attention for they could be an important link in the chain of biochemical disturbances in tetanus poisoning.

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