# IN VITRO RELEASE BY NEURAMINIDASE OF TETANOTOXIN FIXED ON BRAIN STRUCTURES

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After removal by neuraminidase of the n-acetylneuraminic acid from protagon isolated from bovine brain and from the "coarse mitochondrial fraction" isolated from guinea pig brain, the tetanotoxin previously fixed to them is released. Correlation is observed between the quantity of sialic acid and the quantity of tetanotoxin removed, and this effect is also dependent on the enzyme concentration. Preliminary treatment of protagon with neuraminidase reduces its ability to fix tetanotoxin.

A previous investigation [4] showed that treatment of a complex of protagon with tetanus toxin, isolated from brain tissue, with neuraminidase leads to the removal of n-acetylneuraminic acid (NANA) and release of the tetanus toxin.

In the investigation described below the removal of tetanus toxin by means of neuraminidase from the biologically active subcellular brain structures with which the toxin was first fixed in experiments in vitro was studied, and this effect was compared with that observed during the action of neuraminidase on a complex of protagon with tetanus toxin. The degree of fixation of tetanus toxin by protagon treated previously with neuraminidase was also studied in another series of experiments.

## EXPERIMENTAL METHOD

A lyophilized preparation of neuraminidase (a filtrate of Vibrio cholerae manufactured by N. V. Philips-Duphar, Amsterdam, The Netherlands) was used. Protagon was obtained by the method of Wilson and Cramer [14] in M. Sh. Promyslov's modification. The fraction of coarse mitochondria was isolated from guinea pig brain by the method of Schneider and Hogeboom [13]. Tetanus toxin was purified by Pillemer's method [12] and labeled with $I^{131}$ by the method developed previously [2, 3].

A suspension of protagon (25 mg), homogenized in 0.85% NaCl solution, or a suspension of the fraction of coarse mitochondria isolated from guinea pig brain (10-12 mg protein) in 0.32 M sucrose solution was treated with a solution of labeled tetanotoxin in the proportion of 1 μg to 1 mg protagon or mitochondrial protein. The toxin was incubated with the protagon in physiological saline (total volume 2 ml) for 45 min at 37°C. The toxin with the mitochondria was incubated in Gubler's medium [6] (total volume 3 ml) for 10 min at 37°C. After incubation the protagon was precipitated and washed three times by centrifugation for 15 min at 20,000 g and the mitochondria were centrifuged for 15 min at 13,000 g.

A solution of neuraminidase in 1 ml 0.1 M Na-phosphate buffer, pH 5.6, was added in doses of 5-25 mg per sample to the residues of protagon–tetanotoxin and mitochondria–tetanotoxin residues thus obtained. The samples were incubated for 1.5 h at 37°C and centrifuged for 1 h at 20,000 g. Eight samples were used in each test of the experimental and control series.

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TABLE 1. Action of Neuraminidase on Substrate–Tetanotoxin Complex

| Concentration of neuraminidase (mg/ml) | Fixed | Released by enzyme | Remained fixed | NANA liberated by enzyme (in mg/g dry substance) |
|---|---|---|---|---|
| | mg labeled toxin/g dry substrate | | | |
| | Protagon | | | |
| 0 | 0,4±0,014 | 0,04±0,006 | 0,34±0,01 | — |
| 5 | 0,4±0,014 | 0,09±0,003 $P<0,001$ | 0,27±0,01 $P<0,001$ | 1,3±0,1 |
| 10 | 0,4±0,014 | 0,15±0,017 $P<0,001$ | 0,2±0,02 $P<0,001$ | 2,6±0,21 |
| 25 | 0,4±0,014 | 0,24±0,014 $P<0,001$ | 0,12±0,006 $P<0,001$ | 7,0±0,46 |
| | Fraction of coarse mitochondria | | | |
| 0 | 0,51±0,016 | 0,08±0,0017 | 0,4±0,01 | — |
| 10 | 0,51±0,016 | 0,18±0,0042 $P<0,001$ | 0,23±0,021 $P<0,001$ | 3,06±0,23 |
| 20 | 0,51±0,016 | 0,42±0,03 $P<0,001$ | 0,1±0,019 $P<0,001$ | 9,2±0,51 |

Note: To calculate the dry weight of mitochondria their protein content was taken as 65% [5].

TABLE 2. Fixation of Tetanotoxin by Protagon Pretreated with Neuraminidase

| Concentration of neuraminidase (in mg/ml) | NANA removed by enzyme (in mg/g protagon) | Determination of tetanotoxin by radioindicator method | | Determination of tetanotoxin by a biological method | |
|---|---|---|---|---|---|
| | | amount fixed (in mg/g protagon) | % | amount fixed (in $LD_{50}$) | % |
| 0 | — | 0,25±0,01 | 100 | 5789 | 100 |
| 10 | 2,7±0,27 | 0,2±0,013 $P>0,01$ | 80 | 3948 | 71,3 |
| 25 | 7,3±0,21 | 0,14±0,015 $P<0,005$ | 56 | 2770 | 48 |

Note: The number of samples was eight.

The labeled toxin was determined as radioactive label with the UMF-1500 instrument, and the purified toxin was estimated by biological tests on mice in the usual way. The sialic acid content was determined by Gasuo's method [10] and protein by Lowry's method [11].

## EXPERIMENTAL RESULTS

Simultaneously with the removal of NANA, by the action of neuraminidase on protagon–tetanotoxin complex the previously fixed tetanotoxin is also released (Table 1). With an increase in the quantity of added neuraminidase the quantity of toxin released also increased. The largest dose of neuraminidase used removed 7 mg sialic acid (22,200 nmoles) from 1 g protagon, and in this case 0.24 mg (4.04 nmoles) of tetanotoxin, or about 70% of the toxin fixed on the protagon, was released. Only 0.12 mg (1.87 nmole) tetanotoxin, or 30% of the total toxin originally fixed, remained fixed with the residue of protagon.

By the action of neuraminidase on the complex of tetanotoxin with the freshly isolated fraction of coarse mitochondria, the toxin also was liberated from the complex and NANA removed; in this case also the effect depended on the amount of neuraminidase added to the complex and an increase in the quantity of tetanotoxin liberated was accompanied by an increase in the amount of NANA removed (Table 1). The removal of 9.2 mg (31,100 nmoles) NANA from 1 g (dry weight) of the coarse mitochondrial fraction was accompanied by the release of 0.42 mg (6.3 nmoles) tetanotoxin. The residue of toxin fixed on the mitochondria was about 20%. The fact will be noted that the ratio between the amounts of toxin and NANA (in nmoles) released from the protagon–toxin complexes and from the fraction of coarse mitochondria was of the order of about 1:5000.

In a separate series of experiments protagon was pretreated under the same conditions with similar doses of neuraminidase. The residue of protagon, washed to remove the liberated sialic acids, was incubated with labeled and purified tetanotoxin. After preliminary hydrolysis of the protagon with neuraminidase its ability to fix tetanus toxin was reduced (Table 2). For instance, 1 g protagon, from which 7.3 mg NANA had first been removed, fixed 45% less tetanus toxin than protagon incubated without neuraminidase and 70% less than the original protagon. Similar results were obtained in these experiments by the use of radioactive indicators and biological methods of determination of tetanotoxin. It is evident that under these conditions not all the NANA was removed. There is evidence to show [7] that removal of large quantities of NANA from biological substrates is observed after their incubation for several hours with neuraminidases.

The results of these experiments are in agreement with those obtained by Mellanby and van Heyningen [8], and they are further evidence that the bond linking tetanus toxin to gangliosides is effected through NANA not only in preparations of gangliosides but also in subcellular organelles isolated from the brain. The observed ability of neuraminidase to catalyze hydrolysis of gangliosides in actively functioning organelles from the brain, contained in the fraction of coarse mitochondria used in these experiments (synaptosomes, myelin fragments, and mitochondria), deserves attention. These organelles, especially synaptosomes, actively fix tetanospasmin [1, 9].

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