BBA 63336

## Cytotoxic, hemolytic and phospholipase contaminants of commercial neuraminidases

Neuraminidase (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) is frequently used in studies of the cell surface, since many of the electrophoretic, mitotic and receptor properties of cells are known to be related to surface sialic acid[1-4]. Commercially available neuraminidases include enzymes prepared from *Vibrio cholerae* culture filtrates, from influenzae virus and more recently from *Clostridium perfringens*. Preparations from the latter source have been of particular interest in studies[5,6] involving the relation of membrane-bound calcium to membrane sialoglycan structure, since the enzyme from *C. perfringens* is calcium indifferent and, in fact, is fully active in the presence of EDTA. This communication concerns the detection of contaminant activities in commercial preparations of *C. perfringens* neuraminidase capable of producing pronounced structural degradation of biological membranes. Most of the work was done with "NEUP" (lot 7LA, Worthington); in addition, 6 lot samples of "Type V" (Sigma) were examined. The material obtained from both companies was prepared from ATCC-10543 strain of *C. perfringens* by modifications of the method described by CASSIDY, JOURDIAN AND ROSEMAN[7]. No other lot samples were available from the NEUP neuraminidase supplier.

Previous studies[8,9] in this laboratory of surface sialic acid of suspension-cultured Chinese hamster cells (line CHO) utilized *V. cholerae* neuraminidase. Under the conditions employed, cells could be treated with neuraminidase in a balanced salt solution[10] without any detectable cell damage as judged by trypan blue impermeability and stable cell count during treatments and exponential growth without lag upon return to growth medium. In preliminary experiments concerning the relation of surface sialic acid and surface calcium of cultured cells and human erythrocytes, however, it was immediately apparent that the NEUP neuraminidase was both cytotoxic and hemolytic. These experiments were done with cells washed with calcium-free balanced salt solution and treated with NEUP neuraminidase in calcium-free balanced salt solution.

The cytotoxic and hemolytic effects were evident at NEUP concentrations of less than 1 $\mu$g/ml within the 30–45-min time periods (at 37°) that had been routinely used previously. The CHO cells became trypan blue permeable and swollen, and with further incubation shrank and gradually fragmented. The hemolytic effect could be demonstrated either by the release of hemoglobin from intact red blood cells or by observing with phase-contrast microscopy the "sphering", formation of small closed vesicles, and finally fragmentation of red blood cell ghost preparations. The effects on red blood cell ghosts were entirely similar to the usual effects that have been reported for many diverse hemolytic agents[11]. Both the hemolytic and cytotoxic properties of the NEUP neuraminidase preparation were reduced to less than 10% upon heating the preparation at 55° for 60 min.

Although the neuraminidase activity (neuraminidase specific activity was determined at room temperature as the initial velocity of release of sialic acid (using WARREN'S[12] TBA reaction) from excess neuramin-lactose) of "NEUP" was confirmed to be calcium indifferent, if calcium was present during treatment of either CHO cells

or red blood cells, the cytotoxic or hemolytic process was considerably inhibited. Calcium concentrations between 1 and 9 mM seemed to be equally effective in this regard. The quantitative aspects of these effects are illustrated in Fig. 1. Thus, the effects were the reverse of those expected if the contaminant were the major $\alpha$-toxin (a phospholipase) of *C. perfringens*, which requires calcium.

Since the final purification step described by CASSIDY, JOURDIAN AND ROSEMAN[7]

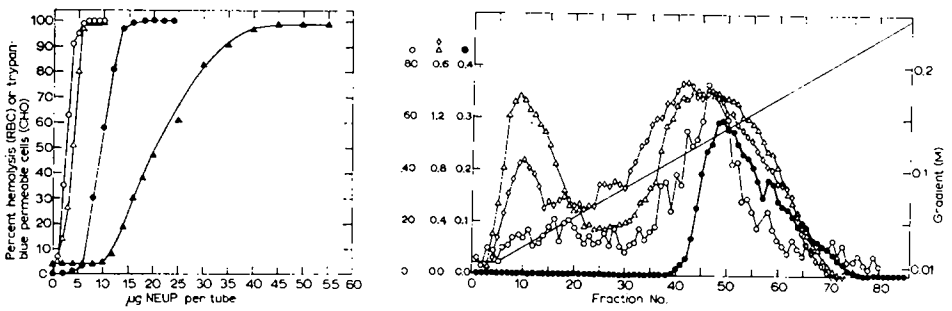


Fig. 1. Hemolytic and cytotoxic activities of NEUP neuraminidase. Hemolytic assays were done with $7 \cdot 10^7$ human red blood cells (washed 3 times with balanced salt solution lacking calcium) suspended in 10 ml balanced salt solution lacking calcium or containing 1 mM calcium and various amounts of neuraminidase. The suspension was incubated at 37° for 30 min, then centrifuged at 1000 × *g* for 5 min; the absorbance of the supernatant at 414 mμ was determined. Complete hemolysis was defined as the $A_{414\ m\mu}$ of the supernatant of $7 \cdot 10^7$ red blood cells lysed in 10 ml of water. Cytotoxicities for CHO cells (washed 4 times with balanced salt solution lacking calcium) were done with $4 \cdot 10^6$ cells per 5 ml, incubated for 60 min at 37°, after which the percentage of cells permeable to trypan blue was determined microscopically. ●—●, red blood cells, 1 mM calcium; ○—○, red blood cells, calcium absent; ▲—▲, CHO cells, 1 mM calcium; △--△, CHO cells, calcium absent. RBC = red blood cells.

Fig. 2. DEAE-cellulose gradient chromatography of NEUP neuraminidase. A 0.9 cm × 45 cm column of precycled and pre-equilibrated (with 0.01 M ammonium acetate) Whatman DE-52 microgranular, preswollen DEAE-cellulose was charged with 16 mg of NEUP dissolved in 0.01 M ammonium acetate. The material was then eluted with 400 ml of ammonium acetate, linear gradient, 0.01–0.3 M. Fractions (4 ml) were lyophilized and redissolved in 1 ml water containing 0.3% bovine serum albumin, and aliquots were assayed for hemolytic and cytotoxic activities (in a similar way to that used in Fig. 1) and for neuraminidase activity against neuramin-lactose. Aliquots for these assays were adjusted so that all fractions yielded values less than complete hemolysis and cytotoxicity and that released less than 10% of the sialic acid of the neuramin-lactose substrate within a 3-min incubation period. ○, cytotoxicity, trypan blue permeable CHO cells (%), calcium absent; □, hemolysis, $A_{414\ m\mu}$, 1 mM calcium; △, hemolysis, $A_{414\ m\mu}$, calcium absent; ●, neuraminidase activity, $A_{549\ m\mu}$.

consisted of DEAE-cellulose chromatography, a slight modification of their procedure was repeated. This was done (a) to ascertain whether the contaminants could be readily removed, and (b) to ascertain whether the cytotoxic and hemolytic properties behaved identically to the neuraminidase activity itself. The results of this experiment are illustrated in Fig. 2 and suggest that this method cannot readily separate all of the neuraminidase activity from all of the cytotoxic and hemolytic activities. The data indicate that the contaminant activities are, in fact, entities different from the neuraminidase and that more than one contaminant might be present.

Since the exotoxin spectrum of *C. perfringens* includes several phospholipases[13] potentially capable of effects on biological membranes, efforts were made to demonstrate phospholipase activity in the NEUP neuraminidase preparation. Attempts to

show breakdown of purified lecithins (synthetic $\beta,\gamma$-dipalmitoyl DL-$\alpha$-phosphatidyl choline, Sigma; plant lecithin and reduced egg lecithin, Applied Science Laboratory) were negative. However, if red blood cell ghosts were used as substrate and the red blood cell phospholipids were analyzed by thin-layer chromatography of a chloroform–methanol (2:1, v/v) extract of the reaction mixture (the methodology for preparing the ghosts, the reaction conditions and the chromatographic identification was identical to that used by KLIBANSKY *et al.*[14]), breakdown of phosphatidyl choline was readily demonstrable. Such an experiment is illustrated in Fig. 3. In similar experiments in which 1 mM calcium was included in the reaction mixture, only a slight inhibition was detected.

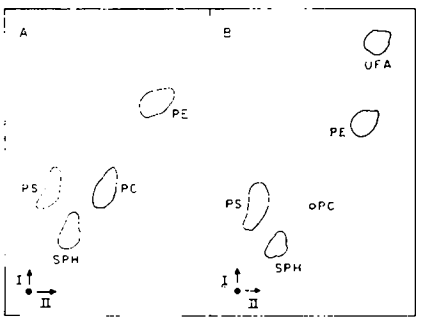


Fig. 3. Two-dimensional thin-layer chromatograms on silica gel G of chloroform–methanol extracts of $4 \cdot 10^9$ red blood cell ghosts. Solvent I: chloroform–methanol–water (65:25:4, v/v/v); and Solvent II: chloroform–methanol–30% $NH_4OH$ (14:16:1, v/v/v). A. Control extract. B. Extract from ghosts incubated with 0.5 mg NEUP neuraminidase, 37°, 2 h. Abbreviations: PC, phosphatidyl choline; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; SPH, sphingomyelin; UFA, unesterified fatty acids.

Comparison of a Type V preparation (lot 127B-8140) with NEUP neuraminidase indicated that, while the latter was about 10 times more active in neuraminidase activity, it was almost 100 times more potent as a hemolytic agent and over 100 times more potent as a cytotoxin. In addition, there appears to be at least a rough correlation between the cytotoxic, hemolytic and phospholipase activities. Three other Type V lots (127B-8130, 117B-8160 and 117B-8180) were very similar to lot 127B-8140 in terms of hemolytic potency. Two Type V lots (18B-8000 and 127B-8100) were less than one-half as hemolytic as the others. The 6 Type V lots varied in neuraminidase specific activity by a factor of three, but there was no correlation between this parameter and hemolytic potency.

The foregoing results suggest that commercially available neuraminidase from *C. perfringens* may not be satisfactory for investigations of membrane-bound sialic acid unless it can be further purified.

This work was performed under the auspices of the U.S. Atomic Energy Commission.

*Biomedical Research Group,*
*Los Alamos Scientific Laboratory,*
*University of California,*
*Los Alamos, N.M. (U.S.A.)*

PAUL M. KRAEMER

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Received April 5th, 1968