Purification of tetanus toxin by multi-membrane electrodecantation

It is desirable that a toxoid, to be used for prophylaxis against human and animal diseases, should be as pure as possible. Effective purification will reduce the incidence of reactions attributable to impurities in the toxoids and will minimize the number of undesirable antigens which may be present in relatively cruder preparations, and which may affect the antigenic response evoked in their recipients.

The toxin produced by *Cl. tetani* may be considerably purified by means of multi-membrane electrodecantation (M.M.E.D.)[1]. The method has a number of advantages such as the mild conditions to which the toxin is exposed (relatively low temperature; pH's near neutrality; absence of denaturing solvents), the high yield, the rapidity of the process and the relative simplicity of the apparatus.

In a typical experiment *Cl. tetani* (G.S. 761) was grown in a cellophane bag, such as was used by POLSON AND STERNE in the preparation of *Cl. botulinus* toxin[2], using a Hog's Stomach-Veal infusion medium[3]. There was no very marked improvement in the quantity of toxin produced as contrasted to that obtained with growth in the medium itself. However, the cellophane bag technique had the advantage that the only macro-molecular material present was produced by the *Cl. tetani* and none was derived from the medium. Filtrates containing 10^5 or more M.L.D. (for 20 g mice) per ml were obtained.

In the preliminary investigation, which is reported here, the toxin was purified by the removal of those constituents with electrophoretic mobility at the iso-electric point of the toxin (pH 5.1)[4]. The continuous flow, 3-cell model of M.M.E.D. apparatus was used for this purpose. It was operated in the horizontal position since paper electrophoresis of the crude toxin showed that there were both positively- and negatively-charged constituents at pH 5.1.

Three litres of the crude toxin-containing filtrate were dialysed against an acetate buffer (pH = 5.1; ionic strength = 0.05), containing Mg^{++}, for 48 hours, the buffer having been renewed at the end of 24 hours. A considerable portion of the pigmented material, present in the original filtrate, diffused into the dialysate. The toxin was passed through the apparatus at approximately 100 ml/h with a voltage gradient of approximately 4 volts/cm. The current was reversed twice during the experiment in order to minimize any accumulation of acid or alkali in either electrode compartment. The pH was shown to have remained constant throughout.

All the pigmented material migrated and collected at the bottom of the cells with the electrophoretically mobile contaminants. The extent of the purification was estimated both by paper electrophoresis and the measurement of toxicity, expressed as M.L.D./mg of protein nitrogen.

Paper electrophoresis was carried out in veronal buffer at pH 8.6 and ionic strength 0.05. The electrophoretogram showed a stationary band, 2 double bands moving towards the anode and a single band moving towards the cathode. By cutting up the paper strip, eluting and determining the toxicity, it was found that the activity was associated with the slower component of the slower double band. This is in accordance with the electrophoretic mobility, since it had the same speed as the β-globulin of a human serum under the same experimental conditions. The paper electrophoretogram of the purified toxin showed only the single toxin-containing band with a continuous, light, diffuse stain between it and the point of application on the paper. However, free electrophoresis indicated the presence of a non-charged component (the very low protein concentration prevented effective analysis by free electrophoresis, even using the Scale Method).

Based on the toxicity, expressed as the number of M.L.D. (for mice) per milligram of protein nitrogen (precipitated by trichloracetic acid), there was approximately an 8-fold purification by a single run through the M.M.E.D. apparatus (see Table I).

TABLE I

PURIFICATION OF TOXIN

Substance	*Toxicity M.L.E./ml*	*mgN/ml*	*Toxicity* M.L.D./mgN*
Original toxin	$8 \cdot 10^4$	0.0035	$2.3 \cdot 10^7$
Purified toxin	$7 \cdot 10^4$	0.00037	$1.9 \cdot 10^8$

* The high toxicity figures per milligram of protein nitrogen may be due to incomplete precipitation[5]. However, they serve as a comparison to demonstrate the extent of the purification.

The toxicity of the solution before and after M.M.E.D. was virtually the same, allowing for dilution by the buffer originally present in the apparatus. The proteins, which concentrated in the bottom of the three cells, contained progressively less toxin, varying from one-third to one-eighth of that present in the original solution.

It has also been possible to demonstrate that the crude toxin (even from dilute solutions) may be quantitatively recovered in a smaller volume in the M.M.E.D. apparatus, operated at a pH removed from the iso-electric point of the toxin (*e.g.* pH 7.0). Using a single separation cell (capacity500 ml) as a batch process, 95% or more of the toxin has been concentrated in 50 ml at the bottom of the cell within 4 hours with a potential gradient of approximately 4 volts/cm. The final concentration in the top of the cell was one-hundredth of that present originally (see Table II).

TABLE II

CONCENTRATION OF TOXIN

Substance	*Toxicity M.L.D./ml*
Original	$2 \cdot 10^4$
Bottom fraction	$2 \cdot 10^5$
Top fraction	$2 \cdot 10^2$

Further work using the M.M.E.D. to purify tetanus toxin from both the G.S. 761 and the "Harvard" strains of *Cl. tetani* (the latter producing significantly more toxin under the conditions of these experiments) is in progress. The effect of the purification on such properties as flocculation, antigenicity, etc. will also be investigated.

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Conversion of D-xylose to D-xylulose phosphate by extracts of *Pseudomonas hydrophila**

Phosphorylation studies, in which D-xylose was the pentose supplied, have been reported with cell-free extracts of *Pseudomonas hydrophila*[1], *Lactobacillus pentosus*[2] and *Aerobacter cloacae*[3]. The product of phosphorylation was first described only as an acid-stable pentose phosphate[1]. Later, using *L. pentosus*, LAMPEN[4] identified D-ribose-5-phosphate as the chief product of this system, stating, however, that D-xylose or D-xylulose phosphates could not be detected. Recent work has shown that the cell-free extracts used in phosphorylation studies of *P. hydrophila* and *L. pentosus* contained not only a kinase but also a specific xylose isomerase[5,6,7]. It, therefore, became uncertain whether D-xylose or D-xylulose was the actual substrate in the phosphorylation.

Attempts to identify the product of the phosphorylation have now revealed the presence of at least four acid-stable phosphate esters. This communication describes the identification of the first ester formed in the sequence of reactions now known to take place.

The enzyme preparation used was a fresh sonic extract of *P. hydrophila*[7]. Nucleoprotein was removed with $MnCl_2$[8] and nucleic acids and other extraneous protein with protamine sulphate[9,10]. This procedure was used to prevent the possible formation of pentose phosphate by enzymic hydrolysis of nucleic acids or of nucleoprotein. After centrifugation (14,000 r.p.m.), the supernatant solution was dialyzed against weak phosphate and then against weak bicarbonate buffers (pH 7.4–7.5) for a total of 48 h. The resulting product was used for all experimental work.

Phosphorylation experiments were carried out in an atmosphere of N_2–CO_2 in 15 Warburg vessels (27° C), each containing 0.02 *M* $NaHCO_3$, 0.01 *M* $MgCl_2$, 0.026 *M* ATP, 1.8 ml enzyme preparation and 0.018 *M* D-xylose. Conditions chosen were such that no free sugars remained at the termination of the experiments, as judged by paper chromatography of the products. Phosphorylation rates were measured by the method of COLOWICK AND KALCKAR[11]. After 90 minutes, the contents of all vessels were combined (48 ml), 0.6 ml glacial acetic acid was added and the solution was allowed to pass slowly (1–1.5 ml/min) through a large column of IR120 (H^+) ion exchange resin followed by several washings with deionized water. Protein flocculated out at the top of the column. The effluent (pH about 2.8) was collected at 0–4° C concentrated *in vacuo* at 30° C[12] to a volume of 50 ml, and treated with barium hydroxide. After removal of the adenosine phosphates at pH 8.2, the pH was quickly brought to 6.8. Four volumes of ethanol were then added to precipitate the barium salts of the sugar phosphates. The precipitate was thoroughly washed and dried.

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