Preparation of neuramin-lactose from bovine colostrum

Because of the interest in neuraminidase and its relation to infection by the influenza group of viruses, there is a need for a convenient, standard substrate for this enzyme. N-L, discovered by TRUCCO AND CAPUTTO[1,2], has been shown to be such a substrate[3–6]. Several methods for the preparation of this material have evolved which utilize ion-exchange resins, in some cases also making use of activated carbon[2,4,7]. However, these procedures all involve problems that make it difficult to produce pure N-L easily and quickly without hydrolyzing it. A need for a method that satisfied these conditions was recognized when one of us (L.W.M.) required substantial amounts of this compound for kinetic studies on the solubilized viral neuraminidase[8]. Such a process is hereby reported and involves only the following steps. The dialysate of fresh bovine colostrum was passed through a Dowex-1 column and the N-L was eluted with sodium chloride. This eluate was passed through an activated-carbon column and the desired material eluted with 60% ethanol. Lyophilization then followed after removal of the alcohol by vacuum evaporation.

500–1000 ml fresh or frozen bovine colostrum (obtained less than 1 day after parturition) was dialyzed against 5 volumes distilled water for 24 h at 4°, using a magnetic stirrer and Visking dialyzing tubing (23/32″). The outer water was changed and the dialysis allowed to proceed for another 24 h. The volume of the combined dialysates was reduced to 100–200 ml on a Rinco flash evaporator at a temperature less than 50°.

A 46.0 × 6.6 cm column of Dowex-1 resin, 200–400 mesh, was prepared by washing the resin successively with 2 column volumes each of 3 *N* HCl, 3 *N* NaOH and 3 *N* HCl, and then washed with water until the wash fluid was neutral and free of chloride ion as determined with $AgNO_3$. The colostrum dialysate was poured onto the column and washed through with 2 column volumes of distilled water. No neuraminic acid-containing compounds came through in the wash fluid, using this size column and up to 1 l colostrum, as determined by the method of WERNER AND ODIN[10] using Ehrlich's reagent, but much sugar was present in the eluate as measured by a modification* of the orcinol–H_2SO_4 procedure[11]. Then 3–4 column volumes of 0.1 *M* NaCl were passed through the column followed by the same amount of 1.0 *M* NaCl. All eluates were tested for sialic acid (the general name for neuraminic acid and derivatives) and for hexose (Fig. 1).

The fraction eluted with 0.1 *M* NaCl consistently contained a hexose:sialic acid molar ratio of 2:1 with little or no free sialic acid present as shown by the thiobarbituric acid procedure of WARREN[12]. This fraction was then passed through an activated-carbon column (Darco-60), consisting of an approx. 2-cm layer of filter-aid, over sintered glass, covered with 21.5 cm of a mixture of filter-aid and Darco-60 (2:3, wet volume) previously washed with HCl, NaOH and absolute ethanol. The layer of activated carbon was overlaid with another cm of filter-aid, and the column was also prewashed with 60% ethanol. (The 60% ethanol wash is absolutely necessary because at that concentration of alcohol a small amount of black soluble material is washed off the column. Once this material is removed, the column can be used over and over without any further appearance of this material and without any further

Abbreviation: N-L, neuramin-lactose.

* 0.5 ml of sample plus 0.5 ml 1.6% orcinol (recrystallized from benzene) plus 3.0 ml of 70% H_2SO_4; mix well and heat in an 80° water bath for 15 min; cool and read at 540 mμ.

washing except regeneration with water.) After the addition of sample, the column was washed with distilled water until the eluate was free of NaCl. This generally required 1–2 column volumes water. Then, 5 column volumes 60% ethanol were

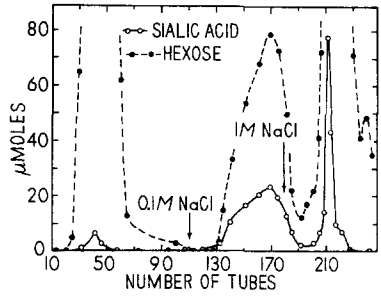

Fig. 1. Separation of neuraminic acid constituents of bovine colostrum dialysate on a Dowex-1-X4 column, Cl^- form, 200–400 mesh, using solutions of 0.1 *M* and 1.0 *M* NaCl as the eluting agents.

added to elute the adsorbed N-L. Usually, these eluate tubes were checked for their concentration of hexose and sialic acid (Fig. 2). All tubes containing N-L were combined and the alcohol removed by evaporation under reduced pressure (Rinco flash evaporator). The neutral, colorless water solution of N-L was lyophilized, yielding a white, hygroscopic solid, which was 95–100% pure according to the amount of hexose and sialic acid present. The yield could be as much as 0.56 g/l colostrum. Chromatography with the methyl ethyl ketone–acetone–water–formic acid (3:1:1:0.1) solvent system of HOGSTROM[9] revealed no additional spots when sprayed with aniline oxalate for reducing sugars, resorcinol reagent for sialic acid[13] or ninhydrin (0.4% in acetone). Treatment with viral neuraminidase followed by chromatography yielded two spots corresponding to lactose and sodium N-acetyl neuraminate.

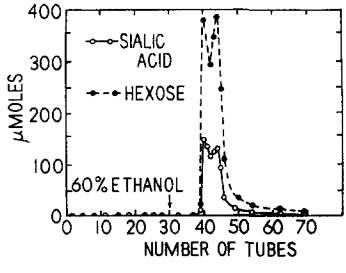

Fig. 2. Adsorption on Darco-60–filter-aid column and elution with 60% ethanol of the fraction eluted with 0.1 *M* NaCl off the Dowex-1 column.

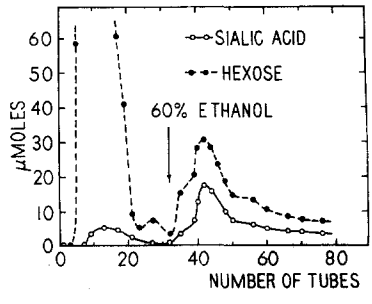

Fig. 3. Removal of salt and contaminating carbohydrate from the sialic acid peak eluted with 1.0 *M* NaCl by adsorption on a Darco-60–filter-aid column and elution with 60% ethanol.

The eluates which followed the addition of 1.0 *M* NaCl to the Dowex column proved to form several hexose-containing peaks but only one which contained neuraminic acid as well as much hexose. The fractions contained in this peak were combined and run through the Darco-60–filter-aid column under the same conditions as before.

After all the salt had been washed through the column, the 60 % ethanol was added to elute the desired material, temporarily termed neuramin-X (N-X).

Upon analysis it was found that much hexose-containing material had been washed away with the salt (Fig. 3). The eluates containing the sialic acid material were combined, evaporated under reduced pressure to remove the alcohol, and lyophilized, yielding a white hygroscopic solid. Analysis of this material showed it to contain sialic acid, glucose and galactose, but no hexosamine. It contains components which give a reaction with ninhydrin after acid hydrolysis and chromatography, and it does not appear to be susceptible to neuraminidase action. It absorbs strongly in the u.v., showing a peak at 262 mμ; chromatography with the above solvent system reveals three spots which absorb strongly in the u.v. One of these spots reacts to stains for reducing sugar, sialic acid, phosphate and amino acids (ninhydrin). Further studies on this material are now in progress.

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Concentration of unbound amino acids in human platelets

The presence of unbound amino acids in platelets has been noted by several workers[1–3]. To our knowledge amino acid concentrations in the platelets have not been determined quantitatively. The results of five analyses are reported herein.

The platelets were supplied by the Protein Foundation, Jamaica Plain, Massachusetts. They were separated from blood collected in acid citrate-dextrose solution by the ADL-Cohn Fractionator (A. D. Little and Company, Cambridge, Massachusetts) by the method of TULLIS *et al.*[4]. The platelets were washed with two 25-ml portions of a 1 % mildly acetylated albumin in 0.15 *M* NaCl solution, then eluted from the fractionator bowl with 5 ml of the same solution. (The albumin was acetylated to prevent its binding of tryptophan, Expt. 135 DI, Table I, ref. 5.) Aliquots of