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# Purification of Bovine Colostrum β-Galactoside α(2-6)Sialyltransferase to Near Homogeneity by Affinity Chromatography

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**Cytidine-5′-monophospho-*N*-acetylneuraminic acid:β-galactoside α2-6sialyltransferase was purified from bovine colostrum by two sequential affinity chromatography steps on CDP-ethanolamine-Sepharose and CDP-ethanolamine-(*N*-caproylamino-)-Sepharose, respectively. While the conditions for elution were those of Paulson *et al.* [J Biol Chem (1977) 252:3256-62], the ligand of the second affinity column was coupled to Sepharose by using 6-aminocaproic acid as linker. The ease of this procedure allows rapid synthesis of bulk quantities of ligand.**

**Highly purified preparations of sialyltransferase were obtained which moved on gradient gel electrophoresis as a single band of 76 kDa and on dodecylsulphate electrophoresis as a single band of 54 kDa. The product of the reaction between lactose and CMP-*N*-acetylneuraminic acid catalyzed by the purified sialyltransferase was identified by high-resolution 500 MHz $^1$H-NMR spectroscopy as Neu5Acα2-6Galβ1-4Glc.**

While several sialyltransferases have been purified and characterized (for review, see ref. 1), the biosynthesis, intracellular transport, subcellular localization and aspects related to gene expression of sialyltransferases are important topics for future research. The availability of a monospecific polyclonal antiserum against a sialyltransferase would greatly facilitate such studies; however, production of anti-sialyltransferase antisera and their specificity testing has been hampered by the small amounts of antigen present in tissues and body fluids and the low yields obtained by the affinity chromatography techniques so far described (for review, see ref. 2). Moreover, synthesis of the nucleotide-agarose affinity ligands as first described by Barker *et al.* [3] is a difficult and

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tedious procedure and has only been carried out in a few laboratories. Despite these drawbacks, affinity chromatography [2] must be considered the method of choice in purification of mammalian glycosyltransferases. Therefore, improvements of existing methodology should focus on simplifying the synthesis of affinity ligands. We have already developed a powerful and straightforward procedure for the purification of a galactosyltransferase without needing to use UDP-agarose [4,5]. In this work, we present a method which allows purification of a sialyltransferase based on the method worked out by Paulson *et al.* [6], but which circumvents the need to synthesise CDP-hexanolamine by introducing 6-aminocaproic acid as a spacer. Synthesis of this ligand is rapid and can be carried out using commercially available materials. An unexpected benefit of employing this ligand was a greater step purification factor, which eliminated the need for a final gel filtration stage, as used by Paulson *et al.* [6].

The linkage specificity of the purified sialyltransferase was confirmed by using lactose as acceptor substrate and identifying the product of sialylation by high-resolution 500 MHz $^1$H-NMR spectroscopy. Part of this work was presented at the 7th International Symposium on Glycoconjugates [7].

## Materials and Methods

### *Biological Material and Chemicals*

Bovine colostrum was obtained from farms near Berne, Switzerland, and was stored frozen at -20°C after the addition of sodium azide to 0.02% final concentration. Cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), cytidine 5′-diphosphoethanolamine hemipyridine salt (CDP-ethanolamine) and fetuin were purchased from Sigma (St. Louis, MO, USA). CMP-[$^3$H]Neu5Ac was obtained from New England Nuclear (Boston, MA, USA). Dr. K. Schmid (Boston, USA) generously supplied the $\alpha_1$-acid glycoprotein (AGP). Asialo-fetuin and asialo-AGP were prepared by mild acid hydrolysis according to the method of Schmid *et al.* [8]. *N-tert*-Butyloxycarbonyl-6-aminocaproic acid-*N*-hydroxysuccinimide ester was from Bachem, Switzerland. 2-(4-*tert*-Butylphenyl)-5-(4-biphenylyl)-1,3,4-oxodiazol (Butyl-PBD) was from Ciba-Geigy, Basel, Switzerland. All other chemicals were of reagent grade quality.

### *Sialyltransferase Assay*

The standard assay for sialyltransferase contained the following components in a final volume of 45 $\mu$l: 10 nmol CMP-[$^3$H]Neu5Ac (specific activity 10 Ci/mol); asialo-AGP, 4.4 mg; bovine serum albumin, 4.4 mg; sodium cacodylate buffer, pH 6.8, 20 $\mu$mol and 15 $\mu$l of enzyme sample. Assays were performed in plastic test tubes and initiated by the addition of the enzyme preparation. Incubation was at 37°C for 1-4 h during which time the reaction rate was linear. Incubation was stopped by addition of 0.5 ml of an ice-cold solution containing 5% (w/v) phosphotungstic acid and 15% (w/v) trichloroacetic acid. Precipitated protein was collected by suction filtration on Whatman GF/A glass fibre filters which were washed twice with the same solution to remove traces of CMP-[$^3$H]Neu5Ac. After

filtration, the filter loaded with the precipitated [$^3$H]Neu5Ac-glycoprotein was dried and counted in 4 ml 0.8% (w/v) butyl-PBD in toluene with a counting efficiency of 40%.

*Preparation of Affinity Ligand*

The affinity ligand was prepared in two steps by the reaction of CDP-ethanolamine with the *N*-hydroxysuccinimide ester of *N-tert*-butyl-oxycarbonyl-protected 6-aminocaproic acid. The protecting group was removed in the second step.

1) *Preparation of the active ester:* 0.1 mmol of CDP-ethanolamine was dissolved in 30 ml of dry dimethylsulphoxide (DMSO). The solution was brought to pH 8-9 by adding triethylamine. At this stage 0.11 mmol *N-tert*-butyl-oxycarbonyl-6-aminocaproic acid-*N*-hydroxysuccinimide ester was added, the flask tightly stoppered and the reaction allowed to proceed at room temperature for 20 h. The slight excess of active ester was then destroyed by adding 0.02 mmol ethanolamine and the reaction left to proceed for a further 4 h. DMSO was removed by lyophilization. No attempts were made at this stage to remove the unwanted by-products of the reaction (e.g. *N*-hydroxysuccinimide), as they do not interfere with the subsequent deprotection and purification steps.

2) *Removal of the tert*-butyl-oxycarbonyl protecting group: The residue from the above step was dissolved in 10 ml ice-cold trifluoroacetic acid (TFA) and left on ice for 30 min. TFA was removed on a rotary evaporator with a 20°C water bath, using a dry ice trap. Two 20 ml portions of ethyl acetate were added and removed by evaporation under the same conditions.

3) *Purification of affinity ligand:* The above residue was dissolved in water, the pH adjusted to 5.4 with concentrated ammonia solution and desalted in two 9 ml portions on a column (15 × 2 cm) of Bio-Gel P-2 (Bio-Rad, Richmond, CA, USA), previously equilibrated with distilled water. Fractions (4 ml) were collected and their absorbance at 271 nm determined after diluting 10 $\mu$l into 1 ml 30 mM NaOH. Fractions 8-10 from both runs were pooled. Fractions 12 and 13 also absorbed at 271 nm, but showed an absorption maximum at 260 nm and therefore corresponded to *N*-hydroxysuccinimide [9]. The cytidine-positive pool was then subjected to ion-exchange chromatography on a column of 8 ml AG 1-X2 (Bio-Rad), previously equilibrated with 10 mM pyridine/HCl, pH 5.4. After sample application, the column was washed with 50 ml of the equilibration buffer. A linear salt gradient, generated from the equilibration buffer and the same buffer containing 0.5 M LiCl, was applied and 1 ml fractions were collected at a flow rate of 20 ml/h. Absorbance at 271 nm was determined as above and the LiCl concentration was estimated from conductivity measurements. Fractions 20-27 were pooled. The overall yield, based on cytidine absorbance, was 77.8%. After lyophilization, the ligand was employed without further treatment for coupling to CNBr-activated Sepharose 4B.

*Coupling of Ligand to Sepharose 4B*

For the first affinity chromatography step, CDP-ethanolamine was employed, whereas the ligand synthesized as described above was used for the second affinity chromato-

graphy step. Both ligands were coupled to Sepharose 4B as described by Paulson *et al.* [6], at a concentration of 2 μmol ligand per ml gel. Coupling efficiency was over 96% in all cases.

*Purification of Sialyltransferase*

Purification of sialyltransferase from bovine colostrum was performed using the method of Paulson *et al.* [6], with the following modifications:
1) A 1 l column of CDP-ethanolamine-Sepharose 4B was employed for the first affinity chromatography step, providing a capacity for the chromatography of 10 l of colostrum.
2) CDP-ethanolamine-(*N*-caproylamino)-Sepharose 4B was used for the second affinity chromatography step. 35 ml of this adsorbent was sufficient for the chromatography of the ammonium sulphate-precipitated material from the first affinity column. Washing of the column was performed with 25 mM sodium cacodylate buffer containing 0.1 M NaCl and 25% (by vol) glycerol at pH 5.3 instead of pH 6.5, which proved to be essential.
3) Concentration on SP-Sephadex C-50 and gel filtration on Sephadex G-150 were omitted. If a more concentrated solution of sialyltransferase was required (e.g. for the preparation of sialyllactose), the sialyltransferase pool from the second affinity chromatography column was concentrated by placing it in a dialysis bag, which was then covered with Sephadex G-100 and kept at 4°C until a 5-10 fold concentration was reached. Glycerol was added dropwise with stirring to 50% by vol final concentration and the preparation stored at -20°C. For the preparation of pure enzyme to be used in the raising of antisera, the pooled sialyltransferase fractions eluted from the second affinity chromatography column were lyophilized and prepared for electrophoresis as described below. That part of the gel corresponding to the 54 kDa form of sialyltransferase (see Results) was excised and stored at -20°C, prior to use as antigen.

*Preparation of Sialyllactose*

The reaction mixture for preparation of sialyllactose contained the following: 1 ml 0.8 M lactose; 1 ml 0.5 M sodium cacodylate buffer, pH 6.8; 400 μmol CMP-[$^3$H]NeuAc (0.5 Ci/mol) and 200 μl (5.6 μg) purified sialyltransferase from the second affinity column.

The mixture was incubated at 37°C for 8 h and supplemented with an additional 5.6 μg sialyltransferase for a further 15 h. The incubation mixture was cooled to 4°C and applied to a column (100 × 2 cm) of Bio-Gel P-2 equilibrated with 50 mM ammonium acetate buffer, pH 5.4. Product and unused CMP-[$^3$H]NeuAc were not completely separated in this system, so the radioactive fractions were pooled and lyophilized, then applied to a 1 ml column of Bio-Rad AG 1-X8 (200-400 mesh, phosphate form) and washed with 4 ml distilled water. The column was eluted with 2 ml 5 mM potassium phosphate buffer, pH 6.8 and the eluate lyophilized. This material was dissolved in 50 mM ammonium acetate buffer, pH 5.4 and applied once more to the Bio-Gel P-2 column. Radioactivity eluted now as a single peak, at a position corresponding to a pentasaccharide. Absorbance of the pooled radioactive peak was found to be zero at 271 nm, indicating the absence of cytidine. The pool was lyophilized and transferred, via five separate washes of 0.5 ml double-distilled water and subsequent lyophilization, to a 1.5 ml Eppendorf microcentrifuge tube.

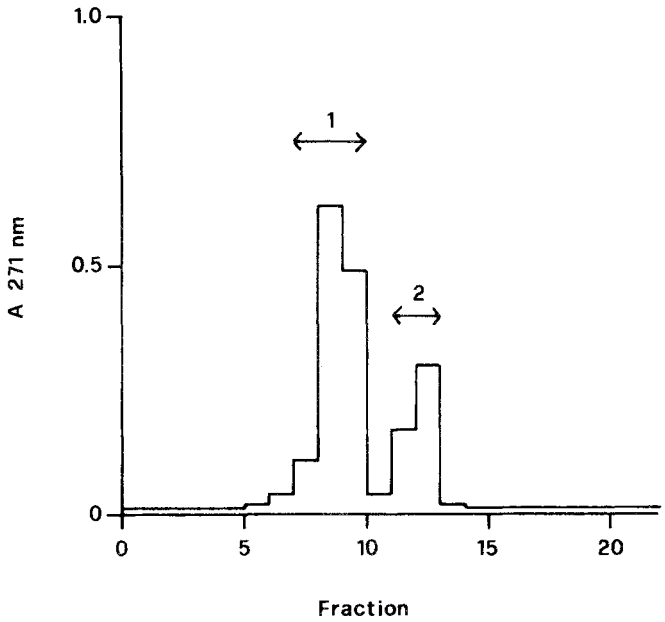


**Figure 1.** Bio-Gel P-2 chromatography of $N^2$-imino-(6-aminocaproyl)-CDP-ethanolamine. The reaction product was subjected to a Bio-Gel P-2 column (15 × 2 cm) after removal of the $N^6$-amino protecting group as indicated in the Methods section. Fraction size, 4 ml. Pool 1 corresponds to the product, pool 2 corresponds to *N*-hydroxysuccinimide.

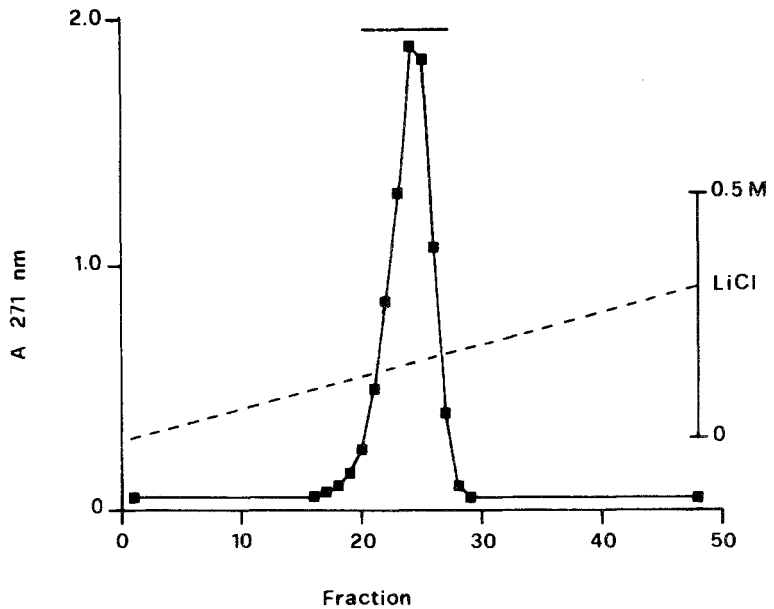


**Figure 2.** Ion-exchange chromatography of $N^2$-imino-(6-aminocaproyl)-CDP-ethanolamine. Pool 1 eluted from the Bio-Gel P-2 column (see Fig. 1) was passed over an 8 ml column of Bio-Rad AG 1-X2 and 1 ml fractions were collected. For further details, see Methods.

*High Resolution $^1$H-NMR Spectroscopy*

Prior to NMR spectroscopic analysis, the incubation product was repeatedly treated with $^2H_2O$ at p$^2$H 7 and room temperature, with intermediate lyophilization. Finally, the sample was redissolved in 400 $\mu$l $^2H_2O$ (99.996 atom % $^2$H, Aldrich, Milwaukee, WI, USA). A $^1$H-NMR spectrum of the product was recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysics, University of Nijmegen, The Netherlands), operating at 500 MHz at a probe temperature of 27°C. Further experimental details have been described previously [10,11]. Chemical shifts ($\delta$) are given in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) (indirectly to acetone in $^2H_2O$: $\delta$ = 2.225 ppm, at 27°C).

*Polyacrylamide Gel Electrophoresis*

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS/PAGE) was performed in 12.5% polyacrylamide gels, as described by Laemmli [12] and modified by Maniatis *et al.* [13]. Samples were prepared for electrophoresis by precipitation of protein with trichloroacetic acid in the presence of SDS as described [14], in an Eppendorf microcentrifuge tube, followed by centrifugation and washing in the same tube with 1 ml of ice-cold absolute ethanol. The pellet was dissolved in 62.5 mM Tris-HCl, pH 6.8 and the application buffer was added [12]. Samples were kept at 95°C for 5 min and allowed to cool before applying to the gel slot.

Pore gradient gel electrophoresis was performed on Pharmacia PAA 4/30 (4-30% acrylamide gradient) gels after dialyzing the samples for 2.5 h against 100 vol of buffer containing 0.18 M Tris, 0.16 M boric acid and 1.86 g/l disodium EDTA, pH 8.3. Gels were pre-run using the same buffer as for electrophoresis at 70 V for 1 h before applying the samples. Electrophoresis of the samples was then carried out at 300 V for 10 min followed by 150 V for 1 h.

## Results

*Synthesis of Ligand and Preparation of Affinity Chromatography Gel*

Extension of the CDP-ethanolamine molecule via an amide bond to the 6-aminocaproic acid moiety proceeded smoothly under the conditions described. Figs. 1 and 2 show the elution profiles of the ion-exchange and gel filtration steps used in purification of the ligand. The isolated ligand was successfully coupled via its terminal amino group to Sepharose 4B, as described by Paulson *et al.* [6], at a concentration of 2 $\mu$mol/ml of settled gel.

*Purification of Sialyltransferase*

Table 1 summarizes the preparation of sialyltransferase from bovine colostrum. Since the methods used were identical up to the second affinity chromatography step, our re-

**Table 1.** Purification of bovine colostrum β-galactoside: α 2-6 sialyltransferase.

| Step | Volume (ml) | Total Protein (mg) | Total Activity (U)[a] | Specific Activity (U/mg) | Yield (%) | Step Purification | Total Purification |
|---|---|---|---|---|---|---|---|
| Crude colostrum | 26300 | 216400 | 137 | 0.00006 | 100 | 1 | 1 |
| Dialyzed colostrum | 57000 | 165300 | 120 | 0.0007 | 88 | 1.15 | 1.15 |
| CDP-Sepharose I[b] | 4040 | 537.5 | 60 | 0.11 | 44 | 1538 | 1770 |
| Ammonium sulphate precipitation | 34 | 227 | 49 | 0.22 | 36 | 1.94 | 3429 |
| CDP-Sepharose II[c] | 35 | 0.228 | 22 | 98 | 16 | 453.8 | 1600000 |

[a] 1 U = 1 μmol of product formed/min, based upon assays using asialo-AGP.
[b] CDP-ethanolamine-Sepharose affinity chromatography; NaCl elution.
[c] CDP-ethanolamine-(aminocaproyl)-Sepharose affinity chromatography; specific elution with CDP.

sults correspond to those obtained by Paulson *et al.* [6]. However, the second affinity chromatography step, using CDP-ethanolamine-6-aminocaproyl-Sepharose in place of CDP-hexanolamine-Sepharose, resulted in a 16-fold higher purification factor and a total purification factor of 1.6 million-fold. An essential difference was the lower pH of the washing buffer for the second affinity chromatography column, since if a pH of 6.5 was used (as with CDP-hexanolamine-Sepharose), the enzyme detaches before its specific elution by CDP. In view of the high purification factor obtained, two further steps as indicated by Paulson *et al.* [6], namely concentration on SP-Sephadex and gel filtration on Separose G-150, were omitted. Affinity columns were regenerated before re-use, as described [2].

*Primary Structure of Sialyltransferase Product using Lactose as Acceptor*

To elucidate the primary structure of the incubation product, high-resolution $^1$H-NMR-spectroscopy was applied. The 500 MHz $^1$H-NMR spectrum of the sialylated lactose is depicted in Fig. 3. Relevant NMR parameters for this oligosaccharide (designated SL) are listed in Table 2. To facilitate comparison, the corresponding data for two well-characterized [10,15] reference substances, 3-sialyllactose (II$^3$Neu5Ac-Lac) and 6-sialyllactose (II$^6$Neu5Ac-Lac), have been included.

As usual for a reducing oligosaccharide, the $^1$H-NMR spectrum of SL is a superposition of the subspectra of the α- and β-anomers of the sialylated lactose. The reducing glucopyranose (Glc-I) is characterized by an H-1 signal at δ 5.223 ppm for its α-anomer ($J_{1,2}$ = 3.8 Hz), and an H-1 doublet at δ ~ 4.66 ppm, at the high-field side of the relatively intense HO$^2$H resonance (see Fig. 3), for its β-anomer. The H-2 signal of the β-form of Glc-I, at δ 3.309 ppm ($J_{1,2}$ = 8.0 Hz; $J_{2,3}$ = 8.4 Hz), is more clearly observable. The areas of the H-1(α) and H-2(β) signals point to an anomeric ratio α:β of about 7:10. The aforementioned values are in accord with those observed for the reference sialyllactoses (see Table 2). The Gal-II H-1 resonance is observed at δ 4.427 ppm. This chemical shift value, in conjunction with the coupling constant $J_{1,2}$ (8.0 Hz), is indicative of the β-type of linkage between galactose and glucose.

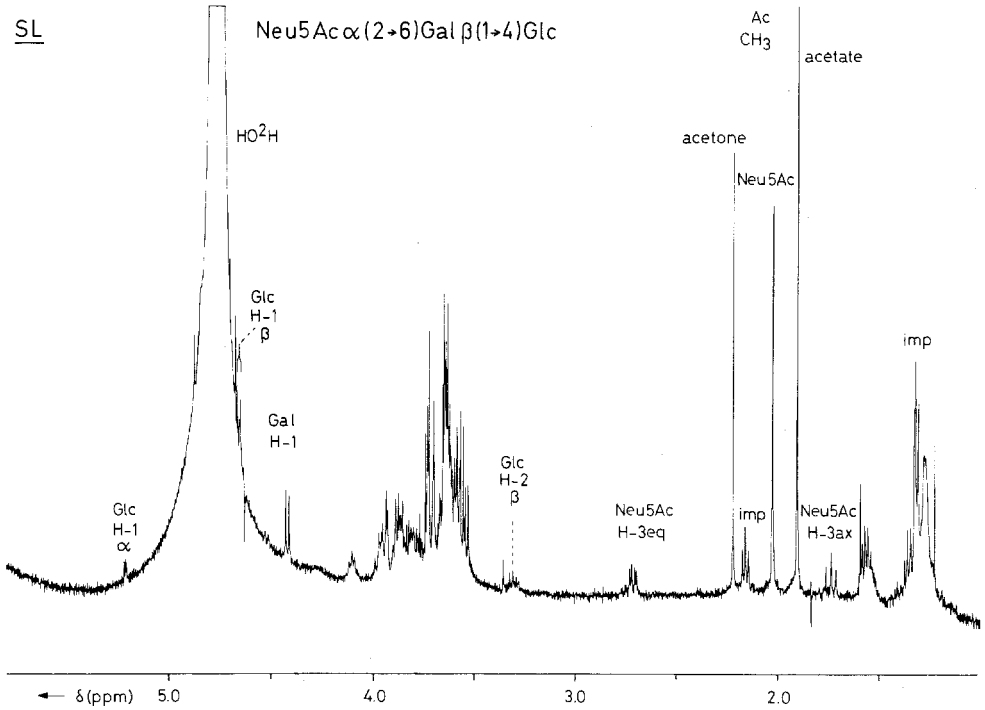


**Figure 3.** 500 MHz $^1$H-NMR spectrum of the *in vitro*-sialylated lactose. The spectrum was recorded in $^2H_2O$ solution, at p$^2$H 7 and 27°C. Sample concentration, 270 nmol/ml; spectral width, 2600 Hz; computer memory, 16 K data points; number of scans, 560.

The attached *N*-acetylneuraminic acid residue is characterized by its typically-shaped reporter-group signals, namely, the H-3ax triplet at $\delta$ 1.741 ppm, the H-3eq doublet of doublets at $\delta$ 2.714 ppm, and the *N*-acetyl methyl singlet at $\delta$ 2.029 ppm. The chemical shift values of H-3eq and Ac indicate that *N*-acetylneuraminic acid is $\alpha$-glycosidically-linked to an aglycon [10]. The set of chemical shifts of H-3ax and H-3eq points unambiguously to an $\alpha$(2-6)-linkage of *N*-acetylneuraminic acid to galactose that itself is $\beta$(1-4)-linked to glucose [10,15 and refs. cited therein] (compare Table 2). The $\alpha$(2-6)-linkage is corroborated by the rather upfield resonance position of Gal H-1.

In summary, a perfect agreement exists between the $^1$H-NMR spectral data of the *in vitro* sialylated lactose (SL), and those of II$^6$Neu5Ac-Lac (see Table 2). Therefore, on the basis of its 500 MHz $^1$H-NMR "identity card", the primary structure of SL could be established to be Neu5Ac$\alpha$2-6Gal$\beta$1-4Glc.

*SDS/PAGE of Purified Sialyltransferase*

Fig. 4 shows a silver-stained [16] SDS/polyacrylamide gel of the purified sialyltransferase. The gel has been purposely overloaded in order to show up any impurities. A

**Table 2.** $^1H$ Chemical shifts and coupling constants of structural-reporter groups of constituent monosaccharides for the *in vitro* sialylated lactose (SL), as compared to those for two reference sialyllactose isomers [10,15] Data were acquired at 500 MHz for neutral $^2H_2O$-solutions at 27°C.

| Residue | Reporter group | Coupling constant | Anomer | Chemical shift [ppm] and (coupling constant [Hz]) | | |
|---|---|---|---|---|---|---|
| | | | | II$^3$Neu5AcLac | II$^6$Neu5AcLac | SL |
| Glc-I | H-1 | $J_{1,2}$ | $\alpha$ | 5.220(3.8) | 5.225(3.8) | 5.223(3.8) |
| | | | $\beta$ | 4.661(7.9) | 4.667(8.1) | 4.662(8.0) |
| | H-2 | $J_{2,3}$ | $\beta$ | 3.281(8.5) | 3.308(8,4) | 3.309(8.4) |
| Gal-II | H-1 | $J_{1,2}$ | $\alpha$ | 4.530(7.8) | 4.429(7.8) | 4.427(8.0) |
| | | | $\beta$ | 4.528(7.8) | 4.429(7.8) | 4.427(8.0) |
| | H-3 | $J_{2,3}$ | $\alpha$ | 4.114(9.9) | {3.5-3.9[a] (n.d.)[b]} | {3.5—3.9[a] (n.d.)[b]} |
| | | | $\beta$ | 4.110(9.9) | | |
| | H-4 | $J_{3,4}$ | $\alpha,\beta$ | 3.959(3.3) | 3.936(3.2) | 3.934(3.2) |
| Neu5Ac | H-3ax | $J_{3ax,4}$ | $\alpha,\beta$ | 1.799(12.1) | 1.739(12.0) | 1.741(12.0) |
| | H-3eq | $J_{3eq,4}$ | $\alpha,\beta$ | 2.757(4.9) | 2.715(4.8) | 2.714(4.9) |
| | | $J_{3ax,3eq}$ | | (-12.4) | (-12.4) | (-12.4) |
| | Ac | — | $\alpha,\beta$ | 2.030 | 2.030 | 2.029 |

[a] Signal is hidden in the bulk of sugar-skeleton proton resonances.
[b] n.d. = not determined.

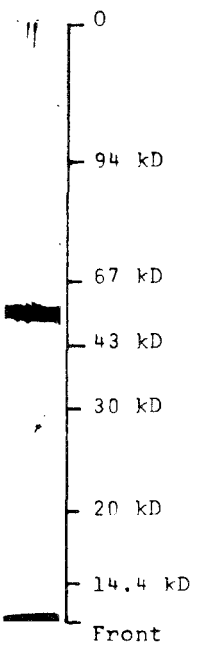


**Figure 4.** SDS-Polyacrylamide gel electrophoresis of purified sialyltransferase. 8 $\mu$g of highly purified sialyltransferase from the second affinity chromatography column were subjected to SDS/PAGE and silver-stained.

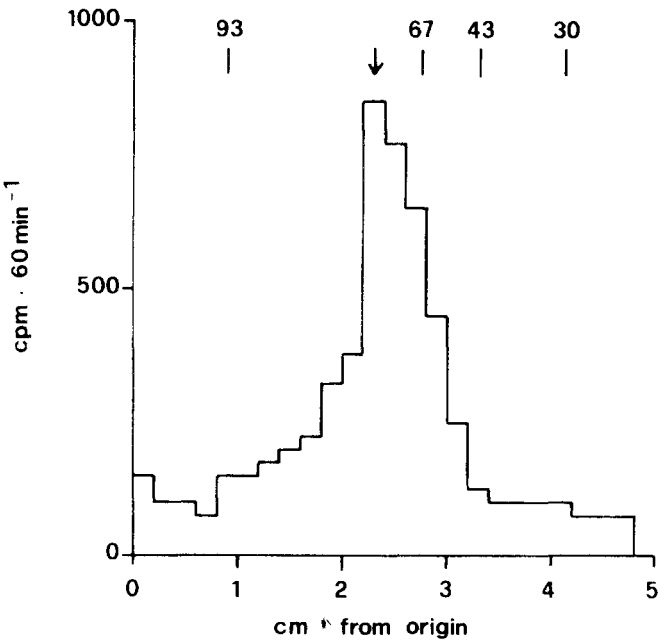


**Figure 5.** Polyacrylamide pore gradient gel electrophoresis of sialyltransferase. 0.56 μg sialyltransferase from the second affinity chromatography column in 80 μl electrophoresis buffer, containing 400 μg bovine serum albumin, 10% sucrose and a trace of bromophenol blue was applied to the central four wells of the gel. Protein standards were applied at the same time to a parallel gel in the double electrophoresis chamber. After electrophoresis the central 2.5 cm of the gel containing the enzyme was excised and cut into 2 mm strips from the origin, using a razor blade. Each strip was sliced into small cubes and placed together with 125 μl 1 M sodium cacodylate buffer, pH 6.8, containing 1 mg/ml bovine serum albumin into a 1.5 ml microcentrifuge tube. The strips were then left overnight at 4°C to elute sialyltransferase into the added buffer. 5 μl aliquots of the supernatant were then subjected to the standard assay. Arrows mark the positions of standard marker proteins on the parallel gel.

single band of mol wt 54 000 is seen, corresponding to the larger of the two forms previously reported [6]. Another form, of mol wt 43 000, previously described by Paulson *et al.* [6], is absent from the preparation. In addition, traces of impurities of mol wt 67 000 and 130 000 can be observed. Such highly-purified sialyltransferase preparations were extremely labile, even when stored under the conditions specified previously [6], and both the enzyme activity and the protein band at 54 kDa were lost within one to two weeks, suggesting the presence of a protease in the preparation. Attempts to stabilize it with a variety of protease inhibitors, including disodium-EDTA, p-hydroxymercuribenzoate, phenylmethane sulphonyl fluoride, leupeptin and pepstatin were unsuccessful.

### *Pore Gradient Gel Electrophoresis of the Purified Sialyltransferase*

Fig. 5 shows the migration of sialyltransferase under non-denaturing conditions on a pre-cast (Pharmacia) 4-30% polyacrylamide gradient gel. Details are given in the legend

to the Figure. Positions of marker proteins run on a parallel gel at the same time are indicated. The apparent molecular weight of the sialyltransferase under these conditions is 76 000, which is in reasonable accord with the value of 80 000 found using gel filtration [6].

## Discussion

Since the original report of the purification of bovine colostrum β-galactoside α2-6sialyltransferase by Paulson *et al.* [6], two additional reports on the purification of this enzyme have been published. The rat liver enzyme has been purified to near homogeneity using a variety of chromatographic steps [17] and to apparent homogeneity using CDP-hexanolamine-agarose [18]. In both cases, Triton X-100 extracts were used and the observed mol wt of the purified enzymes was approximately 40 000, suggesting limited proteolytic cleavage.

In order to meet our demands for a sufficient amount of the sialyltransferase, bovine colostrum was considered an appropriate enzyme source, as it is available in large quantities. However, efficient isolation of bulk quantities of this enzyme necessitated a simpler procedure for the synthesis of affinity ligands analogous to the ones described by Paulson *et al.* [6]. The preparation of a novel affinity ligand for sialyltransferase by the method described here has certain distinct advantages compared with the previous method for CDP-hexanolamine. The method only requires equipment and competence generally to be found in a biochemistry laboratory, the number of synthetic steps is reduced from 4 to 2, and the yield of product is relatively high, amounting to 78%. Coupling of the ligand to Sepharose 4B proceeds with high efficiency, providing the method described [6] is followed. Coupling of the amino group of the pyrimidine ring of cytidine to CNBr-activated Sepharose is unlikely [6], since no coupling occurs with cytidine, CMP or CDP, and coupling via the alkylamino group is quantitative.

Purification of sialyltransferase using the new ligand has both advantages and disadvantages. A purification factor of 1.6 million-fold is already achieved at the second affinity chromatography step, eliminating the need for two further steps of concentration and gel filtration (after which a purification of 440 000-fold was achieved [6] using CDP-hexanolamine). The reasons for such a high purification factor are uncertain, but could be due to a combination of: i) fewer unspecific interactions with the spacer arm, which contains an amido group (less contamination); ii) the longer spacer arm (increase in availability of ligand to the enzyme and consequently a more effective binding); iii) the different conditions necessary for elution (see Results).

A possible disadvantage of our method is that the sialyltransferase preparation is apparently more labile (J Paulson, personal communication) than that previously prepared [6]. Whether this is due to co-purification of a protease and/or increased lability as a result of higher purification, could not be ascertained. Stability can be improved by the addition of bovine serum albumin if high enzyme activity is required, as in the preparation of sialyllactose. When enzyme activity is less important, such as in the preparation of enough enzyme for use as antigen, stability of the protein is improved by immediate freezing, lyophilization, and storage at -20°C. In addition, it may (eventually) be possible to eliminate proteolytic degradation by using a suitable inhibitor or an additional chromatographic step, though this has not yet been accomplished.

High resolution $^1$H-NMR spectroscopy was successfully employed to characterize the primary structure of the sialylation product as II$^6$Neu5Ac-Lac, thus defining the newly-formed linkage as an $\alpha$(2-6)-bond between *N*-acetylneuraminic acid and galactose. Similarly, the product of the incubation of *N*-acetyllactosamine and UDP-galactose in the presence of a galactosyltransferase from calf thymus could be characterized [19] by 500 MHz $^1$H-NMR spectroscopy to be Gal$\alpha$1-3Gal$\beta$1-4GlcNAc. Previously, the application of 360 MHz $^1$H-NMR spectroscopy enabled establishment of the linkage and the branch specificity of an $\alpha$2-6sialyltransferase from colostrum with respect to asialo di- and triantennary glycopeptides from AGP [20], and also the $\alpha$(2-3)-linkage-specificity of a viral neuraminidase [21]. The present study illustrates once again the applicability of the NMR technique for the analysis of enzymic conversions of glycans, thanks to its non-destructiveness, its high resolving power and, particularly at 500 MHz, its fairly high sensitivity.

As shown in Fig. 4, sialyltransferase from the second affinity chromatography column is a highly purified preparation and contains only traces of contaminants of higher molecular weight. The observation of a single 54 kDa form of the sialyltransferase and the absence of a 43 kDa form [6] may be due either to different affinities of the two forms for our affinity chromatography column or to the fact that proteolytic degradation was purposely kept to a minimum by immediate freezing and lyophilization of sialyltransferase fractions from the second affinity chromatography column. The observation of an apparent molecular weight of 76 000 for sialyltransferase upon pore gradient gel electrophoresis (Fig. 5) is in keeping with the observation of an apparent molecular weight of 80 000 upon gel filtration of the SDS/PAGE 56 000 form [6] and supports the notion of an extended rather than globular structure for this form of sialyltransferase.

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