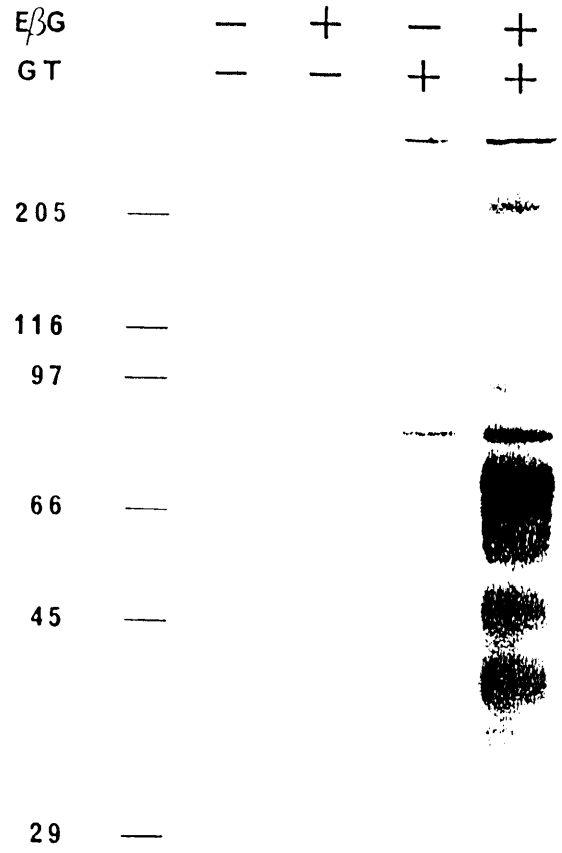


FIG. 2. Cell surface components of mouse N1E-115 neuroblastoma cells containing poly-*N*-acetyllactosamine glycans. The cells were treated with endo-β-galactosidase (EβG) and galactosyltransferase (GT) as indicated. After labeling the cells were extracted with 1% Triton X-100 in CMF-PBS for 5 min on ice, and the insoluble material was removed by centrifugation at 1000 *g* for 5 min at 4°.[14] The solubilized proteins (75 μg) were subjected to electrophoresis in an 8% polyacrylamide gel in the presence of SDS.[15] The gel was treated for fluorography[16] and then exposed to Kodak XAR film for 14 days at −70°. The positions and molecular weights (×10⁻³) of the reference proteins are indicated on the left-hand side of the gel.

partment of cells, where O-linked *N*-acetylglucosamine residues of glycoproteins are abundant and direct galactosyltransferase labeling has been successfully used for their identification.[17]

The sequential use of a glycosidase and a glycosyltransferase with defined specificities allows the labeling of a specific class of glycans, whereas

[17] G. D. Holt and G. W. Hart, *J. Biol. Chem.* **261**, 8049 (1986).

other methods such as galactose oxidase and mild periodate labeling[18] are less specific and therefore give a more general labeling of the surface components. The procedure described should be modifiable for the detection of other specific classes of glycans by replacing endo-β-galactosidase by other glycosidases such as different endo-β-*N*-acetylglucosaminidases, although the accessibility of the glycans to modification by the enzymes may be a restricting factor.

Acknowledgments

We thank Dr. J. Viitala for collaboration during the initial development of the method. This work was supported by the Swiss Nationalfonds (3.020-0.84), The Sigrid Jusélius Foundation, and the Academy of Finland.

[18] C. G. Gahmberg, this series, Vol. 50, p. 204.

[21] Preparation of Cytidine 5′-Monophospho-*N*-Acetylneuraminic Acid and Uridine 5′-Diphosphoglucuronic Acid; Syntheses of α-2, 6-Sialyllactosamine, α-2, 6-Sialyllactose, and Hyaluronic Acid

By ETHAN S. SIMON, ERIC J. TOONE, GARY OSTROFF, MARK D. BEDNARSKI, and GEORGE M. WHITESIDES

Introduction

Glycosyltransferases use nucleoside mono- and diphosphate sugars in the synthesis of glycoconjugates.[1-3] This chapter describes the preparation of two representative nucleoside phosphate sugars, cytidine 5′-monophospho-*N*-acetylneuraminic acid[4] (CMPNeuAc) and uridine 5′-diphosphoglucuronic acid[5] (UDPGlcUA). Both compounds are useful for the *in vitro* synthesis of complex oligosaccharides.

CMPNeuAc is one of the few important nucleoside phosphate sugars for which no chemical synthesis exists.[2] We present a multigram synthesis

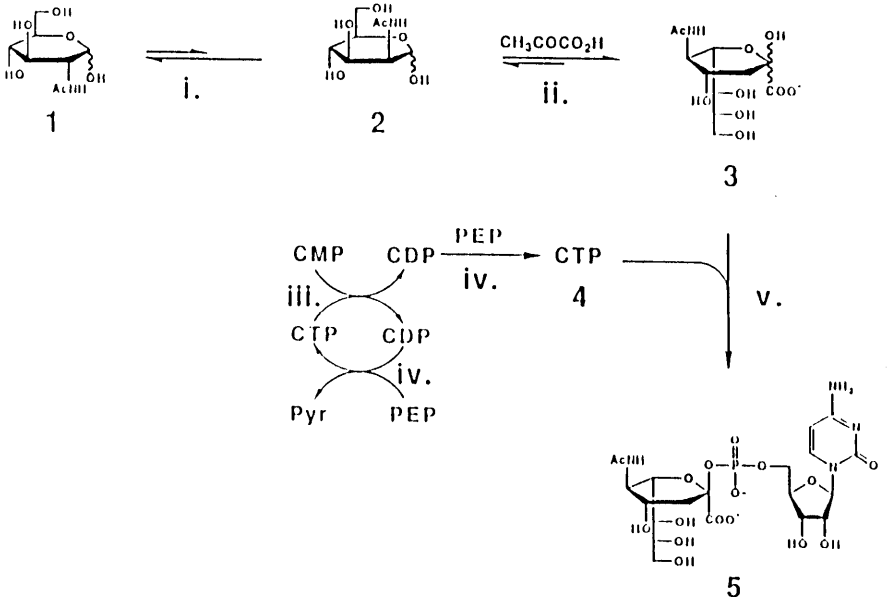
[1] T. A. Beyer, J. E. Sadler, J. I. Rearick, J. C. Paulson, and R. L. Hill, *Adv. Enzymol.* 52, 23 (1981).
[2] V. N. Shibaev, *in* "USSR Progress Khimiya Uglevodov." (I. V. Torgov, ed.), p. 149. Nauka, Moscow, 1985.
[3] O. Gabriel, this series, Vol. 83, p. 332.
[4] E. S. Simon, M. D. Bednarski, and G. M. Whitesides, *J. Am. Chem. Soc.* **110**, 7159. (1988). (Previous syntheses of CMPNeuAc are referenced in this paper.)
[5] For a discussion of UDPglucose dehydrogenase, see N. K. Kochetkov and V. N. Sibaev, *Adv. Carb. Chem. Biochem.* **28**, pp. 363–368 (1973).

for CMPNeuAc involving both chemical and enzymatic transformations, and we illustrate the use of the isolated material by the syntheses of α-2, 6-sialyllactosamine and α-2, 6-sialyllactose using glycosyltransferases. An enzymatic preparation of UDPGlcUA has been developed using UDPglucose dehydrogenase. Although product inhibition prevents the isolation of product, UDPGlcUA produced *in situ* by this method can be efficiently incorporated into the important biological polymer hyaluronic acid.

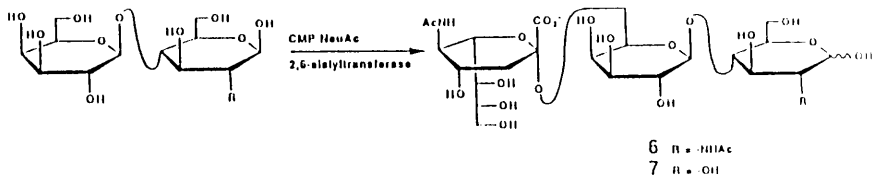
Preparation of CMPNeuAc

Discussion

This procedure describes a multigram-scale synthesis of CMPNeuAc from *N*-acetylmannosamine (ManNAc), pyruvate, cytidine 5′-monophosphate (CMP), and phosphoenolpyruvate (PEP) using four enzymes (Scheme I). ManNAc [produced in mole quantities by base-catalyzed epimerization of *N*-acetylglucosamine (GlcNAc)] and sodium pyuvate react to form *N*-acetylneuraminic acid (NeuAc) in a reaction catalyzed by NeuAc aldolase (*N*-acetylneuraminate lyase) enclosed in a dialysis membrane. CTP is generated *in situ* from CMP using adenylate kinase, pyruvate kinase, and PEP. The only purification step in the synthesis is the final separation of CMPNeuAc from the reaction mixture using ion-exchange chromatography. CMPNeuAc generated by this procedure is used to form sialyl conjugates, α-2, 6-sialyllactosamine and α-2, 6-sialyllactose, in reactions catalyzed by sialyltransferases (Scheme II).



SCHEME I. Generation of CMPNeuAc (**5**) from *N*-acetylglucosamine (**1**). i, $NaOH/H_2O$; ii, *N*-acetylneuraminate lyase; iii, adenylate kinase; iv, pyruvate kinase; v, CMP-*N*-acetylneuraminate synthase.



SCHEME II. CMPNeuAc generated by the procedure of Scheme I was used to form α-2, 6-sialyllactosamine (**6**) and α-2, 6-sialyllactose (**7**).

Procedure

Materials and Methods. NeuAc aldolase (*N*-acetylneuraminate lyase, EC 4.1.3.3, *Clostridium perfringens*, lyophilized powder) was purchased from Sigma Chemical Co. (St. Louis, MO).[6,7] CMPNeuAc synthase is isolated as described below according to the method of Higa and Paulson.[8] CMPNeuAc: Gal-β-(1 → 4)-GlcNAc (α2-6) sialyltransferase (EC 2.4.99.1) was from Genzyme Corporation (Boston, MA). All other enzymes were purchased from Sigma or Boehringer Mannheim (Indianapolis, IN) as suspensions in ammonium sulfate. GlcNAc and CMP were obtained from US Biochemical Corp. All other chemicals and biochemicals are reagent grade and are used without further purification.

In some reactions dialysis membranes contain the enzymes.[9] Dialysis membranes (Spectrapor, 12,000–14,000 MW cutoff, 16 mm, regenerated cellulose acetate) were obtained from Spectrum Medical Industries and are boiled in water for 15 min and then rinsed with water before use. To prepare an enzyme-containing bag for use in enzyme-catalyzed transformations, the tubing is tied shut at one end, a solution of enzymes in the

[6] The least expensive preparation of NeuAc aldolase ($100/500 U) is from Shinko American, Inc. (New York, NY). This enzyme preparation was not tried in this procedure.

[7] Study of NeuAc aldolase: Y. Uchida, Y. Tsudaka, and T. Sugimori, *J. Biochem (Tokyo)* **96**, 507 (1984). NeuAc aldolase has been cloned: Y. Ohta, M. Shimosaka, K. Murata, Y. Tsudaka, and A. Kimura, *Appl. Microbiol. Biotechnol.* **24**, 386 (1986).

[8] H. H. Higa and J. C. Paulson, *J. Biol. Chem.* **260**, 8838 (1985).

[9] M. D. Bednarski, H. K. Chenault, E. S. Simon, and G. M. Whitesides, *J. Am. Chem. Soc.* **109**, 1283 (1987).

reaction mixture is added, and the other end of the tubing is tied shut, taking care to exclude as much air as possible. Ion-exchange resin is washed with methanol or ethanol and water before use.

Water is distilled from glass in a Corning AG-1b still. Welding grade nitrogen is used without further purification. Evaporation of water is done at 0.1 Torr using a rotary evaporator equipped with a cold finger containing acetonitrile and dry ice, or by lyophilization. Centrifugation is carried out at 4° in a Sorvall RC-5B refrigerated centrifuge or, if noted, at room temperature in an IEC Model 428 desktop centrifuge. An LKB 2120 peristaltic pump connected to a Horizon pH controller affords pH control of certain reactions.

^1H (500 MHz) and ^{13}C (82 MHz) NMR spectra are referenced to 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) and dioxane, respectively. ^{31}P NMR spectra (121 MHz) are referenced internally to inorganic phosphate.

N-Acetylmannosamine (**2**).[10,11] A 6-liter Erlenmeyer flask equipped with a magnetic stirring bar is charged with 1 kg (4.5 mol) of GlcNAc (**1**) in 4.5 liters of water. The solution is adjusted to pH 11 with 10 *M* NaOH and stirred at room temperature for 3 days. The resulting pale yellow solution is neutralized to pH 7 with concentrated HCl, and water is removed by rotary evaporation to yield a solid. Heating the solid for 10 min on a steam bath (a hot plate should not be used) in 4 liters of boiling 95% ethanol dissolves most of **2**, whereas most of **1** does not dissolve. The solution is left at 4° for 3 hr, passed through a Büchner funnel, and rinsed with 250 ml of cold (4°) 95% ethanol. Removal of the ethanol in the filtrate by rotary evaporation at reduced pressure yields 143 g of a solid containing 88% of **2**, according to analysis by ^1H NMR spectroscopy[12]; **1** and ethanol are also present. The procedure is repeated twice using the recovered **1** to provide a total of 462 g of a crude solid containing approximately 88% of **2** (1.8 mol) according to analysis by ^1H NMR spectroscopy. Removal of all traces of ethanol prior to use of the material in enzymatic synthesis is accomplished by twice dissolving crude **2** in water (~1 g of **2** per 25 ml of water) followed by removal of liquids by rotary evaporation or by lyophilization.

N-Acetylneuraminic Acid (**3**). A solution containing 1.5 g (6 mmol) ManNAc (**2**), 6 g sodium pyruvate (96%, 52 mmol), and 25 mg sodium azide (to prevent bacterial growth in the solution) in 25 ml of distilled

[10] C. T. Spivak and S. J. Roseman, *J. Am. Chem. Soc.* **76**, 301 (1954).

[11] R. Kuhn and G. Baschang, *Justus Liebigs Chem. Ann.* **659**, 156 (1962).

[12] The ratio of ManNAc to GlcNAc was determined by comparing the area of the peaks corresponding to the anomeric proton of ManNAc (approximately δ 5.0, 4.9, 4.3, 4.2 ppm, α and β forms of pyranose and furanose rings) to the area of the peaks corresponding to the anomeric proton of GlcNAc (approximately δ 5.1, 4.6 ppm, α and β forms of pyranose ring).

water is placed in a 100-ml graduated cylinder containing a magnetic stirring bar. After the pH of the solution has been adjusted to pH 7.5 with 0.25 *M* NaOH, a dialysis bag (prepared as described above) containing NeuAc aldolase (5 U, 1 U converts 1 μmol of substrates to reactant per minute) and 10 mg bovine serum albumin dissolved in 4 ml of the reaction solution is added to the reaction vessel. The bag is allowed to stir freely in the solution at room temperature, and the solution is adjusted to pH 7.5 with 0.25 *N* NaOH approximately every 24 hr. After 5 days approximately 90% of **2** has been converted to **3**. The enzyme-containing bag is removed and stored at 4° (it was successfully reused in similar reactions during an interval of 2 months). The reaction solution is used without further purification in the synthesis of CMPNeuAc (see below). The solution may be stored at −10° for up to 2 months prior to its use without any decomposition. Excess pyruvate is used to drive the reaction because the equilibrium constant for ManNAc + pyruvate → NeuAc is approximately unity.[4]

Generation of CTP (**4**). The potassium salt of PEP[13] (4.75 g, 95% by enzymatic assay,[14] 22 mmol) is dissolved in 30 ml of water and placed in a 50-ml polypropylene centrifuge tube. The pH of the solution is adjusted to pH 8.5 with 5 *N* NaOH. CMP (4.65 g, 10 mmol) is added, followed by 1 ml of a basic solution (pH ~8) containing $MgCl_2 \cdot 6H_2O$ (1.3 *M*) and NH_4Cl (3.8 *M*) to precipitate any inorganic phosphate present.[15] (The inorganic phosphate generally arises from the preparation of PEP. If it is not removed, it precipitates during the reaction and removes from the solution the Mg^{2+} ions that are required for the activity of adenylate kinase.)

The reaction mixture is centrifuged (IEC centrifuge, 1,200 *g*, 5 min), and the supernatant is decanted into a clean tube containing a magnetic stirring bar. $MgCl_2 \cdot 6H_2O$ (3.0 g, 15 mmol) is added followed by a catalytic amount of CTP (100 mg, 0.2 mmol). The solution is degassed with nitrogen for 15 min, and 5 μl of 2-mercaptoethanol is added. A dialysis bag (prepared as described above) containing 1 ml of a suspension of adenylate kinase in ammonium sulfate (~700 U) and 0.5 ml of a suspension of pyruvate kinase (~1400 U) in ammonium sulfate dissolved in 2 ml of the reaction mixture is then added to the reactor. During the course of the reaction, performed at room temperature, nitrogen is slowly bubbled through the solution (~2 ml/min), and addition of 1 *N* HCl via a peristaltic pump maintains the pH of the solution near 8. A white precipitate, presumably the ammonium magnesium salt of inorganic phosphate, forms during the course of the reaction. After 9 days ^1H NMR and ^{31}P NMR

[13] B. L. Hirschbein, F. P. Mazenod, and G. M. Whitesides, *J. Org. Chem.* **47**, 3765 (1982).

[14] "Methoden der Enzymatischen Analyse," H. U. Bergmeyer, J. Bergmeyer, and M. Grassl, eds., 3rd Engl. ed., Vol. 6, p. 555. Verlag-Chemie, Weinheim, 1983.

[15] F. Baer, N. H. Grant, G. F. Lata, and H. E. Carter, *Biochem. Prep.* **2**, 25 (1952).

spectroscopy indicates that the ratio of CTP to CMP is 85:15; CDP is not detected. The pale yellow solution (pH 8.5) and the enzyme-containing bag are transferred to a 250-ml polypropylene bottle containing a magnetic stirring bar for use in the synthesis of CMPNeuAc. (The enzyme-containing bag is transferred so that hydrolyzed CTP would be recycled.)

Isolation of CMPNeuAc Synthase. The procedure of Higa and Paulson[8] was followed (with minor modifications) and is described below. All operations are conducted in a cold room (4°).

Ten fresh calves brains are obtained from a local slaughter house and transported on dry ice. The gray matter is roughly dissected (using forceps and a razor blade) and placed into approximately 1.5 liters of a 10 m*M* solution of Na_2HPO_4 (pH 10.5). The material is homogenized in a household blender, and the homogenate is centrifuged (12,000 *g*, 20 min). The supernatant is decanted and lyophilized. The powder (~30 g) is stored (for 2 days in this procedure) at −70°.

The following steps are carried out the day before the enzyme is to be used. The lyophilized powder is dissolved in 1.5 liters of 10 m*M* Na_2HPO_4 (pH 10.5) and homogenized (two 20-sec bursts with 30-sec intervals) using a Teflon pestle tissue grinder (55 ml glass chamber volume, Thomas Scientific) driven by a 5/8 inch electric drill mounted on a ring stand. The resulting mixture is centrifuged (12,000 *g*, 20 min), and the supernatant is carefully decanted. The enzyme-containing pellet is resuspended in 750 ml of water, and the solution is homogenized and centrifuged again. To extract the enzyme into solution, the pellet is resuspended in 500 ml of 0.4 *M* KCl, and the solution is homogenized and centrifuged. The supernatant is saved and stored on ice. The pellet is resuspended in 250 ml of 0.4 *M* KCl and the extraction procedure repeated. The supernatants from the two extractions are combined.

Most of the nucleoside phosphatases are precipitated with ammonium sulfate in the next step. Ammonium sulfate (147 g, 176 g/liter, 30% saturation) is added during a period of 30 min to the gently stirred supernatants from the previous step. The solution is stirred for an additional 30 min and then left on ice for approximately 3 hr. The solution is centrifuged (12,000 *g*, 20 min), and the supernatant is decanted and saved. The enzyme is precipitated by increasing the concentration of ammonium sulfate to 60% saturation by adding 175 g of $(NH_4)_2SO_4$ [198 g/liter of $(NH_4)_2SO_4$ was added] to the gently stirred supernatants from the previous step. The solution is stirred for an additional 30 min and then left on ice overnight. The solution is centrifuged (12,000 *g*, 20 min), and the enzyme-containing pellet is used in the next step.

Synthesis of CMPNeuAc. (5). The protein pellet containing cytidine 5′-monophosphoneuraminic acid synthase obtained after the second ammonium sulfate precipitation is dissolved in the solution of NeuAc and added to the solution of CTP. The mixture is adjusted to pH 8.5 with a solution of 1 *N* NaOH, and $MgCl_2 \cdot 6H_2O$ (1.42 g, 50 m*M*) is added. The reaction is stirred at room temperature, and addition of 1 *N* NaOH via a peristaltic pump maintains the pH of the reaction near pH 8.5. Copious amounts of a white precipitate form during the reaction, and after 2 days an additional 1.0 g of $MgCl_2 \cdot 6H_2O$ is added. After 3 days analysis by ^1H NMR spectroscopy indicates that 90–95% of **4** has been converted to **5**. The enzyme-containing bag is removed and dialyzed twice against 50 ml of H_2O for periods of 1 hr. The combined dialyzates are saved and used in the step below. After storing the bag in H_2O at 4° for 5 days, 70 U of adenylate kinase and 60 U of pyruvate kinase activity remain.[16] An excess of CTP is used in this step because phosphatases contaminate the preparation of the synthase.

Higa and Paulson report that CMPNeuAc synthase is most active near pH 9.5 in the presence of 30 m*M* $MgCl_2$ or near pH 7.5 in the presence of 5 m*M* $MgCl_2$.[8] We used different conditions (pH 8.5, 50 m*M* $MgCl_2$) that would also maintain the activity of adenylate kinase (which requires Mg^{2+} and is most active near pH 7.5). Other conditions may be satisfactory.

Purification of CMPNeuAc. The pH of the solution is kept alkaline (pH ~8–9) during the following steps to prevent hydrolysis of the acid-labile CMPNeuAc. The reaction mixture from the preceding step is centrifuged (25,000 *g*, 20 min) to remove precipitated salts and protein, and the supernatant is decanted and saved. The pellet is resuspended in 100 ml of 10 m*M* NH_4OH solution, and the mixture is centrifuged again; this procedure is repeated a third time.

Combination of the supernatants from the three centrifugation steps and dialyzates (see above) yields a solution which is adjusted to pH 9.5 with 1 *N* NaOH. Alkaline phosphatase (1 mg, ~1000 U) and $MgCl_2 \cdot 6H_2O$ (1.2 g) are added to the solution. A white precipitate forms during the reaction, and addition of 0.5 *N* NaOH maintains the solution near pH 9. After 30 min, analysis by ^1H NMR spectroscopy indicates that CTP, CMP, and PEP have been converted to cytidine, inorganic phosphate, and pyruvate. This cleavage step simplifies the subsequent ion-exchange chromatography. The solution is centrifuged (10,000 *g*, 10 min) to remove precipitated salts, and the pellet is resuspended in 25 ml of 10 m*M* NH_4OH and centrifuged again; this step is repeated a third time.

The combined supernatants are concentrated to 30 ml and purified by

[16] Assay procedures: "Methoden der Enzymatischen Analyse," H. U. Bergmeyer, J. Bergmeyer, and M. Grassl, eds., 3rd Engl. ed., Vol. 2, p. 248 (adenylate kinase), p. 303 (pyruvate kinase). Verlag-Chemie, Weinheim, 1983.

ion-exchange chromatography (Bio-Rad AG 1-X2, formate form, 5 × 20 cm; eluant: 50 m*M* ammonium bicarbonate followed by a gradient of ammonium bicarbonate[17]). The fractions containing CMPNeuAc, identified by thin-layer chromatography (0.25 mm layer of Merck silica gel 60 F254 on glass plates; eluant: *n*-butanol–acetic acid–water, 5:3:2), are pooled and concentrated to give a white powder. Excess ammonium bicarbonate is removed by dissolving the powder in 25 ml of H_2O and carefully adding cation-exchange resin (Dowex 50W-X8, H^+ form) to the stirred solution until the pH reaches 7; CO_2 is liberated in this step.

The solution is filtered, and the liquid is removed by lyophilization to yield the ammonium salt of CMPNeuAc (2.7 g, 3.7 mmol, 62% overall yield from **2**) in >95% purity; dipyruvate is also present. Spectral data agree with the literature[18] and with that of a sample from Sigma. ^{1}H NMR (500 MHz, D_1O): δ 1.55 (ddd, J = 13.2, 11.6, 5.7 Hz, 1*H*), 1.95 (s, 3*H*), 2.39 (dd, J = 13.2, 4.7 Hz, 1*H*), 3.35 (d, J = 9.7 Hz, 1*H*), 3.50 (dd, J = 11.8, 6.6, Hz, 1*H*), 3.79 (dd, J = 11.8, 2.4 Hz, 1*H*), 3.84–3.82 (m, 1*H*), 3.86 (d, J = 10.5 Hz, 1*H*), 4.18–4.11 (m, 3*H*), 4.20 (apparent t, J = 4.7–5.0 Hz, 1*H*), 4.25 (apparent t, J = 4.7–4.8 Hz, 1*H*), 5.88 (d, J = 4.7–4.8 Hz, 1*H*), 5.88 (d, J = 4.5 Hz, 1*H*), 6.02 (d, J = 7.6 Hz, 1*H*), 7.87 (d, J = 7.6 Hz, 1*H*). ^{13}C NMR (82 MHz, D_2O): δ 174.7, 174.2, 166.2, 157.7, 141.6, 100.1 (d, J = 7.8 Hz), 96.5, 89.1, 82.9 (d, J = 7.8 Hz), 74.2, 71.7, 69.6 (d, J = 9.9 Hz), 69.3, 68.8, 66.8, 64.8 (d, J = 5.3 Hz), 63.0, 51.8, 41.1 (d, J = 9.7 Hz), 22.1.

*(5-Acetamido-1-carboxy-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosyl)-(2,6)-β-*D*-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-α,β-*D*-glucopyranose (2,6-Sialyllactosamine,* **6***).* (In order to follow the progress of the reaction by ^{1}H NMR spectroscopy, the solvent for the reaction is D_2O.) A solution containing 43 mg of sodium cacodylate (200 μmol), 10 mg CMPNeuAc (14 μmol), and 17 mg *N*-acetyllactosamine (43 μmol; synthesized from glucose[19]) in 2 ml of D_2O is placed in a 15-ml polypropylene tube. The solution is adjusted to pH 6.8 with 0.4 *N* HCl in D_2O, and 0.1 ml (~17 mU) of a solution containing 2,6-sialyltransferase is added (the solution is used as supplied by Genzyme). The tube is capped and left at room temperature. ^{1}H NMR spectroscopy (500 MHz) indicates that the reaction is >90% complete after 2 days.

Purification by ion-exchange chromatography[20] yields approximately 3 mg (4 μmol) of **6**, identified by comparison of its ^{1}H NMR spectrum to that reported in the literature.[20] The ion-exchange resin (Dowex 1 × 2-400, 200–400 mesh, Cl^- form) is left in a solution of 0.1 *M* NaH_2PO_2 (pH 7) for 12 hr and then poured into a column (amount of resin: 1.5 × 8 cm). The column is rinsed with water. The reaction mixture is diluted to a volume of 10 ml with water and applied to the column. The column is eluted with 5 m*M* phosphate buffer (pH 6.8), and fractions containing sialyllactosamine (identified by thin-layer chromatography as in the synthesis of CMPNeuAc) are collected after approximately 50 ml of buffer has passed through the column.

*(5-Acetamido-1-carboxy-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosyl)-(2,6)-β-*D*-galactopyranosyl-(1,4)-α,β-*D*-glucopyranose (2,6-Sialyllactose,* **7***).* A solution containing 43 mg sodium cacodylate (200 μmol), 10 mg CMPNeuAc (14 μmol), and 800 mg lactose (155 μmol) in 2 ml of D_2O is placed in a plastic, 15-ml polypropylene tube. The solution is adjusted to pH 6.8 with 0.4 *N* HCl in D_2O, and 0.1 ml (~17 mU) of a solution containing 2,6-sialyltransferase is added. The tube is capped and left at room temperature. ^{1}H NMR spectroscopy (500 MHz) indicates that the reaction is 50% complete after 1 week. Purification by ion-exchange chromatography[20] as described for sialyllactosamine yields approximately 1 mg (2 μmol) of **7**, identified by comparison of its ^{1}H NMR spectrum to that reported in the literature.[20]

Preparation of Hyaluronic Acid

Discussion

This procedure describes the microgram-scale synthesis of hyaluronic acid from UDPGlcUA, prepared *in situ* from UDPGlc using NAD-dependent UDPglucose dehydrogenase, and UDPGlcNAc (Scheme III). A convenient multigram synthesis of UDPGlc from glucose, phosphoenolpyruvate, and UDP exists in the literature.[19] UDPGlcUA is prepared *in situ* to avoid problems of product inhibition.[21] The synthesis of hyaluronic acid is carried out using a group of membrane-bound enzymes purified from *Streptococcus zooepidemicus* using a modified procedure of Stoolmiller and Dorfman[22] (Scheme IV). Although the exact biosynthetic route to hyaluronic acid is unclear,[23,24] the nascent chains are attached to an obliga-

[17] A gradient mixer was used containing 1 liter of 50 m*M* ammonium bicarbonate in one chamber and 1 liter of 1 *M* ammonium bicarbonate in the other.

[18] J. Haverkamp, J.-M. Beau, and R. Schauer, *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 159 (1979).

[19] C.-H. Wong, S. L. Haynie, and G. M. Whitesides, *J. Org. Chem.* **47**, 5416 (1982).

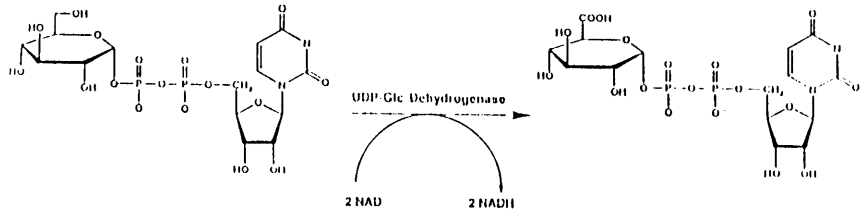
[20] S. Sabesan and J. C. Paulson, *J. Am. Chem. Soc.* **108**, 2068 (1986).

[21] H. Ankel, E. Ankel, and D. S. Feingold, *Biochemistry* **5**, 1864 (1966).

[22] A. C. Stoolmiller and A. J. Dorfman, *J. Biol. Chem.* **244**, 236 (1969).

[23] K. Sugahara, N. B. Schwartz, and A. J. Dorfman, *J. Biol. Chem.* **254**, 6252 (1979).

[24] P. Prehm, *Biochem. J.* **211**, 191 (1983).

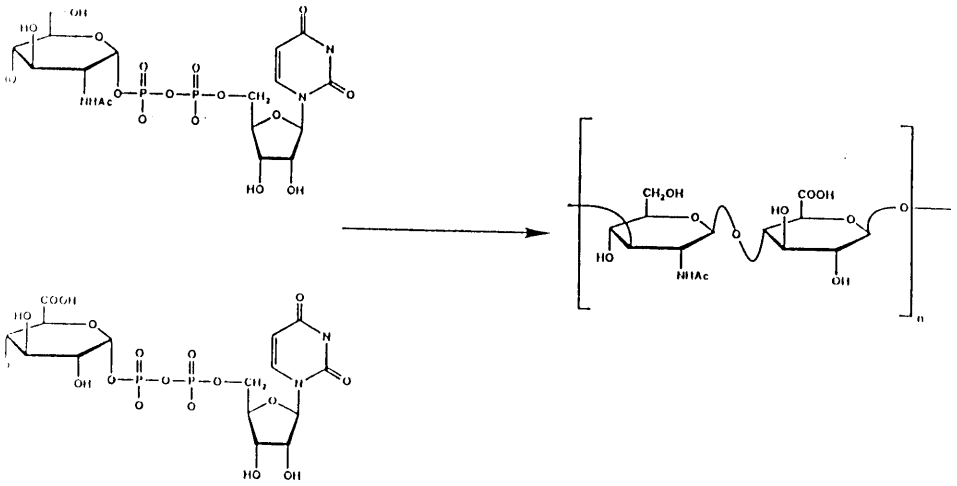


SCHEME III. Preparation of UDPglucuronic acid.

tory primer on the membranes.[23] The mechanism and signal for termination of polymerization and release of the polymer are not known.[24] Purification of the free product hyaluronic acid is carried out using precipitation and centrifugation, followed by dialysis.

Procedure

Materials and Methods. UDPGlc (sodium salt), UDPGlcNAc (sodium salt), UDPglucose dehydrogenase (Type VI from bovine liver), NAD, hyaluronidase (Type IV from bovine testes), trypsin (type III from bovine pancreas), and soybean trypsin inhibitor were purchased from Sigma. UDPGlc[^{14}C] (203.3 mCi/mmol, 20 μCi/ml) was purchased from New England Nuclear (Boston, MA). Tryptic soy broth was purchased from Difco (Detroit, MI). All other chemicals are reagent grade and are used without further purification. Water is distilled from glass in a Corning AG-1b still.



SCHEME IV. Preparation of hyaluronic acid.

Superspeed centrifugation during preparation of membranes is carried out at 20° in either a Sorvall RC-5B or a Sorvall RC-3B centrifuge. Ultracentrifugation is carried out at 0° in a Sorvall OTB 55 ultracentrifuge. Centrifugation during purification of hyaluronic acid is carried out at 10° in an Eppendorf 5415 benchtop centrifuge.

Dialysis tubing for removal of unincorporated monomer during hyaluronic acid purification is SpectraPor 2, 12,000–14,000 MW cutoff, and was purchased from VWR Scientific (Boston, MA). Dialysis tubing is soaked in distilled water for 30 min prior to use. ^{14}C detection is carried out in a Beckman LS 1801 scintillation detector, using Baker Aqualyte scintillant.

Preparation of Hyaluronic Acid-Synthesizing Membranes. Group C *Streptococcus zooepidemicus* cells (strain D1A1, Rockefeller University Culture Collection) are grown in a 20-liter Chemap fermenter in tryptic soy broth. The pH is maintained at 7.1 by addition of 5 *M* NaOH. The cells are harvested in mid log-phase growth, when the optical density at 530 nm reaches 2.0. The cellular hyaluronic acid capsule is removed by treatment with hyaluronidase (4 U/ml) at 37° for 20 min to facilitate centrifugation, then centrifuged at 10,000 rpm (11,950 *g*) for 15 min. The total cell pack is resuspended in 1 liter of 30 m*M* sodium/potassium phosphate buffer, pH 7.0, and treated with trypsin (1000 U/ml) at 37° for 20 min. Soybean trypsin inhibitor is added at a concentration of 120 μg/ml. The mixture is centrifuged at 10,000 rpm (10,240 *g*) for 10 min. The combined cell pack is washed by resuspension in 1 liter of 30 m*M* sodium/potassium phosphate buffer, pH 7.0, followed by repeated centrifugation at 10,000 rpm (10,240 *g*) for 10 min a total of 5 times.

The pellet is resuspended in 75 ml of 30 m*M* sodium/potassium phosphate buffer, pH 7.0, and dithiothreitol added to a final concentration of 5 m*M*. The total volume is split into two equivalent volumes, and each is sonicated at −5° (ice–salt slurry) with a 130 W sonicating probe.[25] Each fraction is sonicated through 12 1-min cycles, with 2 to 3-min intervals between each sonication to avoid overheating. The material is centrifuged at 10,000 rpm (10,240 *g*) for 10 min, and the pellet is resuspended in 75 ml of 30 m*M* sodium/potassium phosphate buffer, pH 7.0, with 5 m*M* dithiothreitol, split into two fractions, and sonicated through an additional 10 cycles. This is centrifuged at 10,000 rpm (10,240 *g*) for 10 min, and the pellet is resuspended in 75 ml of 30 m*M* sodium/potassium phosphate

[25] Heating and foaming are both substantial problems at this phase. Power levels are kept at the maximum permitted by foaming, usually 50–60% during the first 12 cycles, and 80–100% after the first centrifugation. Significant loss of activity will occur if the suspension temperature rises above 37°.

buffer, pH 7.0, with 5 m*M* dithiothreitol, split into two fractions, and sonicated through an additional 3 cycles.

After centrifugation at 10,000 rpm (10,240 *g*) for 10 min, the combined supernatants from the three sonication centrifugations are centrifuged at 42,000 rpm (78,000 *g*) for 1 hr. The pellet from this is resuspended in 15 ml of 30 m*M* sodium/potassium phosphate buffer, pH 7.0, with 5 m*M* dithiothreitol and centrifuged at 52,000 rpm (229,000 *g*) for 1 hr. The pellet is resuspended in 6 ml of 30 m*M* sodium/potassium phosphate buffer, pH 7.0, with 5 m*M* dithiothreitol and frozen in 1-ml aliquots. The suspension contains 8 mg/ml protein by the Lowry method. The suspension can be thawed and refrozen at least 3 times with no loss of activity

Preparation of Hyaluronic Acid. The following stock solutions are prepared:

Buffer: 0.5 *M* Na/K phosphate buffer, pH 7.0, 33 m*M* $MgCl_2$, 5 m*M* dithiothreitol
UDPGlcNAc: 5.8 m*M* (40 mg/ml)
UDPGlc: 6.2 m*M* (40 mg/ml)
β-NAD: 12 m*M* (80 mg/ml)
2,2,2-Trichloroacetic acid: 3.4 m*M* (5.6%, w/v)

A 1-ml reaction mixture contains the following: 100 *μ*l buffer; 100 *μ*l UDPGlcNAc; 100 *μ*l UDPGlc; 100 *μ*l *β*-NAD; 50 *μ*l UDP[^{14}C]Glc, and 500 *μ*l distilled water. UDPglucose dehydrogenase (2 mg, 0.4 U) is added, and the solution is mixed with a vortex mixer. The polymerization reaction is initiated by the addition of membrane suspension (50 *μ*l), and the system is incubated at 37°. The progress of the reaction is monitored by periodically removing aliquots and analyzing ^{14}C incorporation. A 100-*μ*l aliquot is removed and added to 1.0 ml of 5.6% trichloroacetic acid at 0° to precipitate the membranes. After standing for 30 min, the suspension is centrifuged at 15,000 rpm (14,700 *g*) for 15 min. The pellet is washed twice in 5.6% trichloroacetic acid by resuspension in 1 ml and centrifugation at 15,000 rpm (14,700 *g*). The washed pellet is digested in 150 *μ*l of 0.2 *M* NaOH for 24 hr at room temperature.

The combined supernatants are placed in dialysis tubing (10 mm flat width, ~8 inches in length) and dialyzed against tap water for 1,000 volumes, to remove unincorporated monomer. Free and bound hyaluronic acid is then determined as follows. One hundred microliters of the pellet–NaOH digest is added to 8 ml of scintillant and counted for 1 min. The result is multiplied by 1.5 to determine the activity of the total pellet. Three hundred microliters of dialyzed supernatant is added to 8 ml of scintillant and counted for 1 min, with the result multiplied by 10. Results of a sample reaction (Fig. 1) show that production of hyaluronic acid is linear

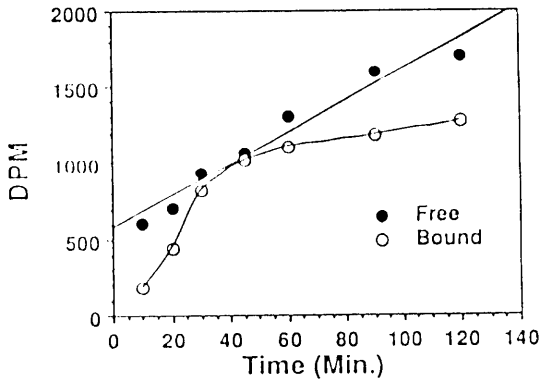


FIG. 1. Production of hyaluronic acid.

for at least 120 min. Although some UDPGlc is incorporated into the membrane system,[26] this incorporation levels off after 45 min.

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[26] A. C. Stoolmiller and A. Dorfman, *in* "Chemistry and Molecular Biology of the Intracellular Matrix" (E. A. Balazas, ed.), Vol. 2, p. 783. Academic Press, New York, 1970.