On the Biosynthesis of Alternating $\alpha$-2,9/$\alpha$-2,8 Heteropolymer of Sialic Acid Catalyzed by the Sialyltransferase of Escherichia coli Bos-12*

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Escherichia coli Bos-12 synthesizes a heteropolymer of sialic acids with alternating $\alpha$-2,9/$\alpha$-2,8 glycosidic linkages (1). In this study, we have shown that the polysialyltransferase of the E. coli Bos-12 recognizes an $\alpha$-2,8 glycosidic linkage of sialic acid at the nonreducing end of an exogenous acceptor of either the $\alpha$-2,8 homopolymer of sialic acid or the alternating $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid and catalyzes the transfer of Neu5Ac from CMP-Neu5Ac to this residue. When the exogenous acceptor is an $\alpha$-2,8-linked oligomer of sialic acid, the main product synthesized is derived from the addition of a single residue of $[^{14}\text{C}]$Neu5Ac to form either an $\alpha$-2,8 glycosidic linkage or an $\alpha$-2,9 glycosidic linkage at the nonreducing end, at an $\alpha$-2,8/$\alpha$-2,9 ratio of approximately 2:1. When the acceptor is the alternating $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid, chain elongation takes place four to five times more efficiently than the $\alpha$-2,8-linked homopolymer of sialic acid as an acceptor. It was found that the $\alpha$-2,9-linked homopolymer of sialic acid and the $\alpha$-2,8/$\alpha$-2,9-linked hetero-oligomer of sialic acid with $\alpha$-2,9 at the nonreducing end not only failed to serve as an acceptor for the E. coli Bos-12 polysialyltransferase for the transfer of $[^{14}\text{C}]$Neu5Ac, but they inhibited the de novo synthesis of polysialic acid catalyzed by this enzyme. The results obtained in this study favor the proposal that the biosynthesis of the alternating $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid catalyzed by the E. coli Bos-12 polysialyltransferase involves a successive transfer of a preformed $\alpha$-2,9-linked dimer of sialic acid at the nonreducing terminus of the acceptor to form an $\alpha$-2,9 glycosidic linkage between the incoming dimer and the acceptor. The glycosidic linkage at the nonreducing end of the alternating $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid produced by E. coli Bos-12 should be an $\alpha$-2,8 glycosidic bond and not an $\alpha$-2,9 glycosidic linkage.

Sialic acid generally occurs in vertebrate tissues as a constituent of oligosaccharide, glycoproteins, and glycolipid. In contrast to sialyl linkage in vertebrates which comprises a large panel of novel linkages and appears to have a regulatory role in broad spectra of biological process such as cell growth, differentiation, fertilization, and neuronal pathogenicity (2, 3), in bacteria, only three kinds of polysialic acid linkages, namely $\alpha$-2,8, $\alpha$-2,9, and alternating $\alpha$-2,9/$\alpha$-2,8 linkage have so far been identified as the major components of capsular polysaccharide (1, 4–6).

The biosynthesis of homopolymer of sialic acid with $\alpha$-2,8 glycosidic linkage has been studied extensively in Escherichia coli K1 and K235. These studies have shown that CMP-Neu5Ac is the immediate precursor, and the synthesis of the polymer takes place in two sequential steps: the transfer of a Neu5Ac residue from CMP-Neu5Ac to undecaprenyl phosphate to form undecaprenyl phosphate-neuraminyl derivative, which then serves as the acceptor for subsequent sequential transfer of a single unit of Neu5Ac from CMP-Neu5Ac to the C8 position of the acceptor to form the $\alpha$-2,8-linked polymer of sialic acid (7, 8). Ferrero et al. (9) and Reglero et al. (10) demonstrated that an $\alpha$-2,8-linked trimer or tetramer of sialic acids can serve as an exogenous acceptor for the transfer of Neu5Ac from CMP-Neu5Ac to increase its chain length, albeit less efficiently than the natural acceptor, the undecaprenyl phosphate derivative of sialic acid (11).

E. coli K1 synthesizes a homopolymer of sialic acids with $\alpha$-2,8 glycosidic linkage, whereas E. coli Bos-12 synthesizes a heteropolymer of sialic acids with alternating $\alpha$-2,9/$\alpha$-2,8 glycosidic linkages (1). Alignment of the primary structures of polysialyltransferase from both sources reveals a 92% sequence homology (12, 13). It has been postulated that a single polysialyltransferase from E. coli Bos-12 catalyzes the formation of both $\alpha$-2,9 and $\alpha$-2,8 glycosidic bonds to form the alternating $\alpha$-2,9/$\alpha$-2,8 glycosidic bond, to form the unique E. coli Bos-12 sialic acid polymer (13). The mechanism of this enzymatic reaction remains obscure. In this report, we provide evidence in support of the proposal that the biosynthesis of the alternating $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid catalyzed by the polysialyltransferase of E. coli Bos-12 involves a successive transfer of a preformed $\alpha$-2,8-linked dimer of sialic acid at the nonreducing terminus of the acceptor to form an $\alpha$-2,9 glycosidic linkage between the incoming dimer and the acceptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—E. coli Bos-12 (016:K92:NM) was provided by Dr. John B. Robbins of the National Institutes of Health, Bethesda, MD, and was maintained in trypticase soy broth as described (1). E. coli bacteriophage 492 was purchased from the American Type Culture Collection and propagated on E. coli Bos-12 in LB medium as described (14). Dithiothreitol, colominic acid, and CMP-Neu5Ac were purchased from Sigma Chemical Co. Uniformly labeled CMP-$[^{14}\text{C}]$Neu5Ac was purchased from NEN Life Science Products. Oligomeric polysialic acid with $\alpha$-2,8 ketosidic linkages were purchased from Calbiochem. Silica Gel G TLC plates were purchased from Whatman. Capsular polysaccharide from E. coli Bos-12 was purified as described (1). Meningococcal C polysaccharide was a gift from Dr. E. C. Gotschlich of the Rockefeller University, New York. O-Acetyl groups of the meningococcal C polysaccharide were removed by mixing the capsular polysaccharide with 10 mg/ml with an equal volume of 0.1 M NaOH and were kept for 30 min at 4 °C followed by the addition of an equal volume of 0.1 M HCl (15).

**Isolation of Polysialyltransferase Complex and Polysialyltransferase Assay**—A heavily encapsulated strain of E. coli Bos-12 (016:K92:NM) was grown in trypticase soy broth to a stationary phase culture as

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Biosynthesis of Sialic Acid α-2,9/α-2,8 Heteropolymer

FIG. 1. Chemical structure of the alternating α-2,8/α-2,8-linked heteropolymer of the E. coli Bos-12 polysialic acid and its periodate degradation products. Reproduced from Ref. 1 with permission from Biochemistry.

Results from the incorporation of radiolabeled monosialic acid into polymers. The reaction mixtures for enzymatic activity assay contained 50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 1 mM dithiothreitol, 1 mM CMP-Neu5Ac, 0.9 μM CMP-[14C]Neu5Ac (27 mCi/mmol), membrane protein (12 mg/ml), and exogenous acceptor (7.5 mg/ml). At the end of the reaction, supernatants that contained polysialic acids from both endogenous and exogenous acceptors were spotted onto 20 × 20-cm, 250-μm-thick Silica Gel G thin layer plates and developed by a mobile phase made up of n-propyl alcohol, 25% aqueous ammonia, and water at a ratio of 6:1:2.5. The radiolabeled polysialic acids were visualized and quantified by autoradiography using a Bio-Imaging Analyzer BAS 1500 (Fuji Photo Film Co., Ltd., Japan). The pellets that contained the enzyme complex and residual endogenous polysialic acids were also analyzed for incorporation of radiolabeled Neu5Ac.

Isolation of Oligosialic Acid with Alternating α-2,9/α-2,8 Glycosidic Linkage—Approximately 4.5 mg of capsular polysaccharide isolated from E. coli Bos-12 was partially hydrolyzed with 5 × 10⁷ plaque-forming units of bacteriophage φ92 lysate, which contains endo-N-acetyleneuraminidase (Endo-N)¹ activity, at 37 °C for 1 h as described (14). At the end of the reaction, the reaction mixture was passed through a Dowex 50-X8 column in H⁺ form and filtered through a 0.22-μm filter prior to fractionation by the Mono Q HR5/5 anion exchanger. Oligosaccharides were collected according to their degree of polymerization (DP), desalted, and lyophilized.

High Performance Liquid Chromatography (HPLC) of Polysialic Acids—The DP of oligomeric sialic acids was analyzed by either ion exchange or gel permeation HPLC using either a Mono Q HR 5/5 anion exchanger column (5 × 50 mm, Amersham Pharmacia Biotech) or a Superdex peptide PE 7.5/300 column (7.5 × 300 mm, Amersham Pharmacia Biotech), respectively. Sialic acid oligomers separated by TLC and recovered were redissolved in the initial buffer, 5 mM Tris-HCl, pH 8.0, and subjected to ion exchange chromatography. The column was eluted with the initial buffer for 10 min at a flow rate of 500 μl/min followed by a linear gradient from 0 to 500 mM NaCl within 200 min with the same flow rate (17). In gel-permeable chromatography, samples were dissolved in 20 mM ammonium bicarbonate and loaded onto the column. The column was eluted with the same buffer used to dissolve the sample at a flow rate of 200 μl/min for a period of 70 min. The effluent was monitored either by the absorbance at 214 nm or by the radioactivity and plotted against the elapsed time.

Linkage Analysis—Periodate oxidation specifically cleaves C-C

¹ The abbreviations used are: Endo-N, endo-N-acetyleneuraminidase; DP, degree of polymerization; HPLC, high performance liquid chromatography.
Polysialyltransferase Requires Either a Homopolymer with α-2,8 Bonds or a Heteropolymer with Alternating α-2,9/α-2,8 Glycosidic Linkages as Exogenous Acceptor—The addition of an alternating α-2,9/α-2,8 heteropolymer of sialic acid to the polysialyltransferase complex enhanced the transfer of [14C]Neu5Ac to the endogenous and the exogenous acceptors (Fig. 2, △ compared with ◊). The stimulatory effect of colominic acid, which is an α-2,9-linked homopolymer of sialic acid, and the alternating α-2,9/α-2,8 heteropolymer (Table II) as acceptors. The extent of [14C]Neu5Ac transferred to the exogenous acceptor was quantitated from the chromatogram of either TLC or anion exchange HPLC chromatography. Linkage analysis of the sialyl residue newly incorporated into the acceptor was examined by periodate oxidation, which cleaves C-C bonds having vicinal hydroxyl groups, e.g., the α-2,9-linked sialic acids (18).

TABLE II

| Inhibitor:acceptor ratio | Neu5Ac incorporated/exogenous acceptor% | Inhibition |
|--------------------------|----------------------------------------|------------|
| -2,9 homopolymer/α-2,8 homopolymer| 0.1:1 | 0.56 | 0 |
|                          | 1:1 | 0.22 | 100 |
|                          | 10:1 | 0 | 100 |
| -2,9 homopolymer/α-2,9/α-2,8 heteropolymer| 0.1:1 | 6.33 | 0 |
|                          | 1:1 | 5.57 | 15 |
|                          | 10:1 | 1.56 | 75 |

See Footnote a of Table I.

b See Footnote b of Table I.

RESULTS AND DISCUSSION

The biosynthesis of the heteropolymer of sialic acid (Fig. 1) catalyzed by E. coli Bos-12 polysialyltransferase was investigated using CMP-[14C]Neu5Ac as the donor of sialic acid and exogenous polymers of sialic acids with different chain length and different glycosidic linkages (the α-2,8 homopolymer, the α-2,9 homopolymer, and the alternating α-2,9/α-2,8 heteropolymer) as acceptors. The extent of [14C]Neu5Ac transferred to the exogenous acceptor was quantitated from the chromatogram of either TLC or anion exchange HPLC chromatography. Linkage analysis of the sialyl residue newly incorporated into the acceptor was examined by periodate oxidation, which cleaves C-C bonds having vicinal hydroxyl groups, e.g., the α-2,9-linked sialic acids (18).

Polysialyltransferase Requires Either a Homopolymer with α-2,8 Bonds or Heteropolymer with Alternating α-2,9/α-2,8 Glycosidic Linkages as Exogenous Acceptor—The addition of an alternating α-2,9/α-2,8 heteropolymer of sialic acid to the polysialyltransferase complex enhanced the transfer of [14C]Neu5Ac to the endogenous and the exogenous acceptors (Fig. 2, △ compared with ◊). The stimulatory effect of colominic acid, which is an α-2,9-linked homopolymer of sialic acid (Fig. 2, ◊), was four to five times less than that found for the alternating α-2,9/α-2,8 heteropolymer of sialic acid, whereas the α-2,9-linked homopolymer of sialic acid (Fig. 2, △) not only failed to serve as an acceptor but had an inhibitory effect on the de novo endogenous synthesis of polysialic acid (Fig. 2, ◊).
and 3 of the transfer of $[^{14}\text{C}]\text{Neu5Ac}$ into the endogenous and exogenous acceptors. The results showed that the amount of $[^{14}\text{C}]\text{Neu5Ac}$ transferred to the $\alpha$-2,8 homopolymer (Fig. 2, E) or oligomer (Fig. 3, lanes 3–6) was less than 1 nmol/nmol of exogenous acceptors. However, unlike the $\alpha$-2,9-linked homopolymer or oligomer of sialic acid (Table II), the $\alpha$-2,8-linked oligomer or the polymer did not inhibit the transfer of $[^{14}\text{C}]\text{Neu5Ac}$ to the endogenous acceptor, at the concentration used.

The radiolabeled $\alpha$-2,8-linked oligomeric acceptors were analyzed further by anion exchange HPLC to determine the extent of chain elongation catalyzed by the polysialyltransferase. The chromatogram in Fig. 4, A–D, shows that with a trimer, tetramer, or pentamer as the acceptor, the main product resulted from the addition of a sialyl residue to form the tetramer (83%), pentamer (78%), and hexamer (75%), respectively. Minor products resulting from the addition of two or three sialyl residues to the acceptor were also noted. When the acceptor was a hexamer, in addition to the heptamer (51%), products resulting from the addition of two or three sialyl residues to form an octamer (38%) and a nonamer (15%) became more significant.

On the other hand, when the $\alpha$-2,9/$\alpha$-2,8-heteromer was the acceptor (Fig. 2, A) the transfer of $[^{14}\text{C}]\text{Neu5Ac}$ to the acceptor was much more efficient. Of the total of 8.37 nmol transferred to the endogenous plus the exogenous acceptors (Table I), 7.21 nmol or 86% ended up with the external acceptor. Assuming that the alternating $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid has an average chain length (DP) of 150 (1), it corresponds to 4.51 nmol of $[^{14}\text{C}]\text{Neu5Ac}$ incorporated/nmol of acceptor. It should be remembered that the polysialic acids isolated from bacteria are not expected to have a uniform chain length and that the DP used in the calculation is an average chain length. Thus, the molar ratio of $[^{14}\text{C}]\text{Neu5Ac}$ incorporated/acceptor is not expected to be an integral number.

The amounts of radioactivity found to be associated with the pellets that contain the enzyme complexes were found to be less than 10–20% of that found in the soluble supernatant (see “Experimental Procedures”) in all cases.

The Polysialyltransferase Activity Is Inhibited by the Polymer and Oligomer of Sialic Acid with an $\alpha$-2,9 Glycosidic Linkage at the Nonreducing End—The homopolymer of $\alpha$-2,9-linked sialic acid not only failed to serve as an acceptor, but inhibited the transfer of $[^{14}\text{C}]\text{Neu5Ac}$ to the endogenous acceptor by about 60% (Fig. 2, M, and Table I). At an inhibitor:acceptor molar ratio of 10:1 the polysialyltransferase-catalyzed transfer of $[^{14}\text{C}]\text{Neu5Ac}$ to the $\alpha$-2,8-linked homopolymer and to the $\alpha$-2,9/$\alpha$-2,8-linked heteropolymer of sialic acids was inhibited by 100% and 75%, respectfully, by the $\alpha$-2,9-linked homopolymer of sialic acid (Table II).

The effect of an oligomer of sialic acid with an $\alpha$-2,9 linkage at the nonreducing end on the polysialyltransferase was investigated. For this purpose, the $\alpha$-2,9/$\alpha$-2,8 heteropolymer of sialic acid was digested with Endo-N, which specifically cleaves the $\alpha$-2,8 glycosidic linkage within the polysialyl chain, resulting in the formation of oligosaccharides that end up with an $\alpha$-2,9 glycosidic linkage at the nonreducing end but with internal $\alpha$-2,8 glycosidic linkages (14). Fig. 5A shows an anion exchange HPLC chromatogram of oligosaccharides obtained from the Endo-N digestion of a 2,9/$\alpha$-2,8 heteropolymer of sialic acid. The chain length of oligosaccharides increases successively from left to right of the chromatogram with an interval DP value of 2. Oligosaccharides with lactone were observed as the peaks with a retention time corresponding to oligosaccharides with an odd DP value number caused by a reduction in a
negative charge of a carboxyl group after lactonization reaction (19).

Fig. 5B shows that the oligosaccharides with a DP value ranging from 2 to 8 with an α-2,9 glycosidic linkage at their nonreducing end were not only unable to serve as an exogenous acceptor for the chain elongation mediated by the polysialyl-transferase, but they inhibited the de novo endogenous synthesis of polysialic acid catalyzed by the enzyme. The extent of inhibition by the α-2,9-terminated oligomer of sialic acid, calculated from the amount of [14C]Neu5Ac incorporated into the endogenous polymeric form of sialic acid, was 70–80% (Fig. 5B, lanes 2–5 compared with lane 1). In the absence of inhibitors, the alternating α-2,9/α-2,8-linked heteropolymer of sialic acid with an α-2,8 linkage at the nonreducing end enhanced the transfer of [14C]Neu5Ac to the polymeric acceptor by about 10-fold (Fig. 5B, lane 1 compared with lane 6). The polysialyl-transferase of E. coli Bos-12 appears only to recognize an α-2,8 linkage at the nonreducing end in the acceptor to transfer Neu5Ac.

To eliminate the possibility that the inhibition could be caused by attachment of the O-acetyl group or by formation of lactone, all polysaccharides used were treated with 0.1N NaOH as described under “Experimental Procedures.”

Linkage Analysis of the Sialic Acid Added to the Acceptor—Periodate, which cleaves C-C bonds having vicinal hydroxyl groups (e.g. the C7-C8 bond of the α-2,9-linked sialic acid), was employed to determine the newly formed linkage between [14C]Neu5Ac and the acceptor, the α-2,9/α-2,8 heteropolymer of sialic acid (Fig. 1 and refs. therein). This radioactive disaccharide derivative was examined further by thin layer chromatography and was shown to possess the requisite mobility on the chromatogram (Fig. 6, inset). Periodate oxidation did not release a trisaccha-
Periodate Oxidation

FIG. 6. Glycosidic linkage analysis of newly added sialyl residues on the capsular polysaccharide of E. coli Bos-12. 14C-Labeled capsular polysaccharide from E. coli Bos-12 was oxidized with 2-fold molar excess of sodium metaperiodate for 18 h at 25 °C and quenched by ethylene glycol. The reaction mixture was applied to a Superdex peptide gel filtration column to separate radiolabeled material from ethylene glycol and salt. Samples recovered from the gel filtration column prior to analyses by Mono Q anion exchange chromatography were reduced by sodium borohydride and desalted by the Superdex peptide gel filtration column. Samples recovered from the gel filtration column were analyzed for radioactivity incorporated into the acceptor, which resulted in a monomeric product with 24–29% periodate oxidation.

The amount of Neu5Ac incorporated into the acceptor, which resulted in a monomeric product with 24–29% periodate oxidation.

The amount of Neu5Ac incorporated was obtained by liquid scintillation counting of effluent from ion exchange chromatography and recalculated from the specific activity of CMP-Neu5Ac. The amount of CMP-Neu5Ac present in the reaction mixture was 74.2 nmol. The stock solution of CMP-[14C]Neu5Ac with specific activity 0.256 μCi/nmol was diluted with nonradioactive CMP-Neu5Ac 12-fold to a final specific activity 0.0215 μCi/nmol.

The percentage of newly formed glycosidic linkage was obtained by dividing the amount of Neu5Ac released after periodate oxidation by the amount of Neu5Ac incorporated. This value was used to assign α-2,9 linkage.

| Chain length | Product | Amount of Neu5Ac incorporated | Amount of Neu5Ac released after periodate oxidation | Newly formed glycosidic linkage |
|--------------|---------|-------------------------------|----------------------------------------------------|--------------------------------|
| 4            | 5       | 0.17                          | 0.05                                               | 29.4                           |
| 5            | 6       | 0.39                          | 0.10                                               | 25.6                           |
| 6            | 7       | 0.33                          | 0.08                                               | 24.2                           |
| 6            | 8       | 0.18                          | 0.11                                               | 61.1                           |

* Chain length was defined as described in Fig. 4 and Footnote a of Table I.

* The amount of Neu5Ac incorporated was obtained by liquid scintillation counting of effluent from ion exchange chromatography and recalculated from the specific activity of CMP-Neu5Ac. The amount of CMP-Neu5Ac present in the reaction mixture was 74.2 nmol. The stock solution of CMP-[14C]Neu5Ac with specific activity 0.256 μCi/nmol was diluted with nonradioactive CMP-Neu5Ac 12-fold to a final specific activity 0.0215 μCi/nmol.

* The percentage of newly formed glycosidic linkage was obtained by dividing the amount of Neu5Ac released after periodate oxidation by the amount of Neu5Ac incorporated. This value was used to assign α-2,9 linkage.

linker dimer nor a monosaccharide; NeuAc7, which would have resulted, had the last residue added to the acceptor and formed either an α-2,8-linkage or an α-2,9-linked sialic acid at the nonreducing end. In the former case the last two residues would have been α-2,8/α-2,8-linked sialic acids, whereas in the latter case it would have been α-2,8/α-2,9-linked sialic acids. These results favor the proposal that the polysialyltransferase from E. coli Bos-12 catalyzed the transfer of a preformed α-2,8-linked dimer of sialyl residue to the nonreducing end of the acceptor to form an α-2,9 linkage between the acceptor and the α-2,8-linked dimer.

When the acceptor molecule was the α-2,8-linked homo-oligomer of sialic acid, the results obtained were much more complicated (Fig. 5, A–D). Upon periodate oxidation, the tetramer, the pentamer, the hexamer, and the heptamer resulting from the addition of one residue of Neu5Ac to the respective acceptor released a monomeric product with 24–29% of the radioactivity incorporated into the acceptor, which moved in the position of NeuAc7 on the chromatogram. This portion of the product was assigned to have an α-2,9-linkage at the nonreducing end (Table III).

When the octamer resulting from the addition of two residues of Neu5Ac to the hexamer was subjected to periodate oxidation, the tetramer, the pentamer, and the hexamer released a monomeric product with 24–29% periodate oxidation.

The amount of Neu5Ac incorporated was obtained by liquid scintillation counting of effluent from ion exchange chromatography and recalculated from the specific activity of CMP-Neu5Ac. The amount of CMP-Neu5Ac present in the reaction mixture was 74.2 nmol. The stock solution of CMP-[14C]Neu5Ac with specific activity 0.256 μCi/nmol was diluted with nonradioactive CMP-Neu5Ac 12-fold to a final specific activity 0.0215 μCi/nmol.

The percentage of newly formed glycosidic linkage was obtained by dividing the amount of Neu5Ac released after periodate oxidation by the amount of Neu5Ac incorporated. This value was used to assign α-2,9 linkage.

| Chain length | Product | Amount of Neu5Ac incorporated | Amount of Neu5Ac released after periodate oxidation | Newly formed glycosidic linkage |
|--------------|---------|-------------------------------|----------------------------------------------------|--------------------------------|
| 4            | 5       | 0.17                          | 0.05                                               | 29.4                           |
| 5            | 6       | 0.39                          | 0.10                                               | 25.6                           |
| 6            | 7       | 0.33                          | 0.08                                               | 24.2                           |
| 6            | 8       | 0.18                          | 0.11                                               | 61.1                           |

* Chain length was defined as described in Fig. 4 and Footnote a of Table I.

* The amount of Neu5Ac incorporated was obtained by liquid scintillation counting of effluent from ion exchange chromatography and recalculated from the specific activity of CMP-Neu5Ac. The amount of CMP-Neu5Ac present in the reaction mixture was 74.2 nmol. The stock solution of CMP-[14C]Neu5Ac with specific activity 0.256 μCi/nmol was diluted with nonradioactive CMP-Neu5Ac 12-fold to a final specific activity 0.0215 μCi/nmol.

* The percentage of newly formed glycosidic linkage was obtained by dividing the amount of Neu5Ac released after periodate oxidation by the amount of Neu5Ac incorporated. This value was used to assign α-2,9 linkage.

| Chain length | Product | Amount of Neu5Ac incorporated | Amount of Neu5Ac released after periodate oxidation | Newly formed glycosidic linkage |
|--------------|---------|-------------------------------|----------------------------------------------------|--------------------------------|
| 4            | 5       | 0.17                          | 0.05                                               | 29.4                           |
| 5            | 6       | 0.39                          | 0.10                                               | 25.6                           |
| 6            | 7       | 0.33                          | 0.08                                               | 24.2                           |
| 6            | 8       | 0.18                          | 0.11                                               | 61.1                           |

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* The percentage of newly formed glycosidic linkage was obtained by dividing the amount of Neu5Ac released after periodate oxidation by the amount of Neu5Ac incorporated. This value was used to assign α-2,9 linkage.

linker dimer (see above). The remainder of the octamer formed by the addition of two residues of NeuAc to the hexamer which are resistant to periodate oxidation would have to consist of an α-2,8/α-2,8-linked sialic acids at the terminus.

The E. coli Bos-12 polysialyltransferase appears to be able to catalyze the synthesis of both an α-2,9-sialyl linkage and/or an α-2,8 glycosidic linkage at the nonreducing end when the acceptor is an oligomer or a polymer of α-2,8-linked sialic acid.

Proposed Mechanism of Biosynthesis—The genomic structure of the E. coli Bos-12 polysialyltransferase has been elucidated (12, 13). The deduced amino acid sequence of this enzyme shares an extensive homology with that of the E. coli K1 polysialyltransferase, which catalyzes the biosynthesis of an α-2,8 homopolymer of sialic acid. Whereas the K1 polysialyltransferase catalyzes the synthesis of an α-2,8 homopolymer of sialic acid by transferring a sialyl residue to the nonreducing end of the polymer, the Bos-12 polysialyltransferase synthesizes the alternating α-2,9/α-2,8 heteropolymer of sialic acid by either transferring a preformed α-2,8-linked dimer of the sialyl residue to the nonreducing end of the acceptor to form an α-2,9 linkage or by sequentially transferring a monomer of sialic acid with the alternating formation of α-2,9 and α-2,8 linkages. The former mechanism is favored because the resulting polymer will end with an α-2,8-glycosidic linkage that is amenable to further elongation catalyzed by the Bos-12 polysialyltransferase. If, on the other hand, an α-2,9 and an α-2,8 linkage are alternatively formed, the synthesis will terminate upon formation of the α-2,9 linkage at the nonreducing end. The findings that both the α-2,9-linked homopolymer of sialic acid and the α-2,8/α-2,9-linked oligomer of sialic acid with the α-2,9 linkage at the nonreducing end not only failed to serve as exogenous acceptors for the transfer of [14C]Neu5Ac from CMP-[14C]Neu5Ac catalyzed by the Bos-12 polysialyltransferase, but acted as inhibitors of the enzyme, support this proposal.

Finally, it remains to be established whether the two steps proposed for the biosynthesis of the alternating α-2,9/α-2,8 heteropolymer of sialic acid involves one enzyme or two enzymes. We are currently working on this problem.

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