The Synthesis of Neuramin Lactose by Preparations of Rat Mammary Gland and Its Relation to the Synthesis of Lactose*

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SUMMARY

Lactose and neuramin lactose were produced by incubating subcellular particles from rat mammary gland in a system containing glucose, uridine diphosphate galactose, and cytidine monophosphate N-acetylenuraminic acid. The $K_m$ values for glucose in the synthesis of neuramin lactose were fairly constant for three similarly prepared enzyme preparations (0.25, 0.17, and 0.22 mM), but were widely dispersed in the synthesis of lactose (0.93, 1.8, and 18 mM). For UDP-galactose the $K_m$ value in the synthesis of neuramin lactose was 0.01 mM; in the synthesis of lactose it was 0.15 mM. The above mentioned system for the synthesis of neuramin lactose was compared with another in which lactose substituted for UDP-galactose plus glucose. The $K_m$ value for lactose was 5.6 mM. The $K_m$ value for CMP-N-acetylenuraminic acid in the system containing UDP-galactose plus glucose was 0.066 mM. The rate of production of neuramin lactose in the system containing lactose was lower than in the system containing UDP-galactose plus glucose. In the latter system an intermediate particle-lactose complex was isolated by centrifugation; on incubation of this complex with CMP-N-acetylenuraminic acid, neuramin lactose was produced. The formation of this intermediate complex is probably common for the synthesis of lactose and neuramin lactose; on addition of CMP-N-acetylenuraminic acid to a system synthesizing lactose, neuramin lactose was produced while corresponding amounts of lactose disappeared. No indication was obtained that the enzyme system which produces neuramin lactose from UDP-galactose plus glucose could be solubilized. No success was achieved in attempts to separate the particles which synthesize lactose from those which synthesize neuramin lactose from UDP-galactose plus glucose. The neuramin lactose produced from lactose and that produced from UDP-galactose plus glucose were chromatographically identical.

Neuramin lactose is a component of the milk from most species in which it has been investigated; it is a quantitatively important component in human and rat milks at the beginning of the lactation period. Mechanisms for the biosynthesis of lactose and NL, are known. Watkins and Hassid (1) showed that preparations from mammary gland catalyze a reaction between uridine diphosphate galactose and glucose to produce lactose (Reaction 1), whereas Jourdain, Carlson, and Roseman (2) showed that particulate preparations from rat mammary gland catalyze the synthesis of NL according to Reaction 2.

Glucose + UDP-galactose $\rightarrow$ lactose + UDP (1)

Lactose + CMP-N-AN $\rightarrow$ NL + CMP (2)

In 1964 Carubelli et al. (3) observed that slices of rat mammary gland incubated in a medium with $^{14}$C-glucose produced NL whose lactose moiety was of similar specific activity as that of lactose itself; this was so at periods of incubation of 15 sec and 1 min. In our laboratories (see below) it was found that slices of rat mammary gland also produced lactose sulfate and neuramin lactose sulfate with their lactose component of similar specific activity as that of lactose. Comparable observations were made with the products isolated from homogenized mammary glands obtained from lactating rats that were given injections of $^{14}$C-glycerol, were also of similar specific activities. These findings are not incompatible with Reactions 1 and 2 as an adequate representation of the mechanism of production of NL, but to apply this sequence requires the assumption of some conditions on the size of the intracellular pools of lactose or NL to explain the similarity of specific activities of both compounds. The study of these conditions should prove of interest since it has been reported that nothing is known about the pool of lactose inside the cell (5), and probably the same is correct with respect to the intracellular pool of NL.

The present work shows that, for the formation of neuramin lactose, Reactions 3a and 3b should be considered besides Reactions 1 and 2.

$\text{Particle} + \text{glucose} + \text{UDP-galactose}$ $\rightarrow$ $\text{particle-lactose} + \text{UDP}$ (3a)

$\text{Particle} + \text{glucose} + \text{UDP-galactose}$ $\rightarrow$ $\text{particle-lactose} + \text{UDP}$ (3b)

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1 The abbreviations used are: NL, neuramin lactose; CMP-N-AN, cytidine 5'-monophospho-N-acetylenuraminic acid; N-AN, N-acetylenuraminic acid.
Particle-lactose + CMP-N-AN → NL + CMP + particle

Particle + glucose + UDP-galactose + CMP-N-AN →

\[
\text{particle} + \text{NL} + \text{CMP} + \text{UDP}
\]

The overall reaction 3 is proposed because the conditions have been found in which NL was produced at a faster rate when glucose and UDP-galactose were used as precursors of NL than when lactose was the precursor. The separation in Reactions 3a and 3b accounts for the observation that after incubation of glucose and UDP-galactose with mammary gland particles, some of the lactose remained attached to the particles, from which it could not be separated by washing; NL was produced by incubating this particle-lactose complex with CMP-N-AN.

**EXPERIMENTAL PROCEDURE**

**Materials**

UDP-galactose, N-AN, CTP, Sephadex G-50, UTP, and DEAE-cellulose were purchased from Sigma; Bio-Gel P-30 was obtained from Bio-Rad Laboratories (Richmond, California); \(^{14}\text{C}\)-glucose and UDP-\(^{14}\text{C}\)-galactose were purchased from New England Nuclear; lactose and glucose were purchased from E. Merck (Darmstadt). The components of lactose synthetase (A and B proteins) were obtained from bovine skin milk according to the method of Brodbeck and Ebner (6).

**Analytical Methods**

Protein was assayed with the Folin-Ciocalteu reagent (7) with crystalline bovine serum albumin as the standard. Hexoses were determined by the anthrone method (8). N-AN was determined by the method of Aminoff (9). Radioactive determinations were carried out in a gas flow counter with a background of less than 1 cpm.

**Preparation of Labeled \(^{14}\text{C}\)-Lactose**

For this purpose lactose synthetase was prepared according to the method of Brodbeck et al. (10) up to the step called “Fraction 3”; to eliminate rests of lactose a modification was introduced in that the enzyme preparation was washed twice with 75% ammonium sulfate prior to dissolving it in 0.02 M tris-chloride buffer (pH 7.4). The incubation mixture for the synthesis of lactose contained 5 \(\mu\)moles of uniformly labeled \(^{14}\text{C}\)-glucose (3.5 \(\mu\)Ci per \(\mu\)mole), 10 \(\mu\)moles of UDP-galactose, 100 \(\mu\)moles of tris-chloride buffer (pH 7.0), 100 \(\mu\)moles of MnCl\(_2\), and 1.0 ml of enzyme preparation. The total volume was 1.6 ml. The incubation period was 4 hours and the temperature was 37°C. The incubation system, inactivated by immersion in a boiling water bath for 90 sec, was centrifuged and the supernatant fraction was centrifuged at 55,000 \(x\) g for 10 min; the precipitate was discarded and the supernatant fraction was centrifuged at 55,000 \(x\) g during 30 min; the precipitate was washed twice with 0.25 M sucrose and finally suspended in this solution so that particles from 1 g of mammary gland were suspended in 0.2 ml. Despite several attempts at standardization, the stability of the neuramin lactose synthetase activity of this preparation was erratic. After 1 week at \(-15^\circ\) the remaining activity varied from 90 to 20% of that determined at the day when the enzyme was prepared.

**Determination of Enzyme Activities**

With UDP-\(^{14}\text{C}\)-galactose—This procedure was used for the determination of lactose synthetase and neuramin lactose synthetase (UDP-galactose plus glucose co-substrates). The incubation mixture contained, in a total volume of 0.17 ml, 5 \(\mu\)moles of Tris-chloride buffer (pH 7.4), 0.25 \(\mu\)mole of UDP-\(^{14}\text{C}\)-galactose (40,000 cpm), 0.65 \(\mu\)mole of CMP-N-AN, 0.25 \(\mu\)mole of MnCl\(_2\), 2.5 \(\mu\)moles of MgCl\(_2\), 0.25 \(\mu\)mole of UTP, 5 \(\mu\)moles of glucose, and 0.025 ml of the suspension of mammary gland particles described above. After 6 min at 37°C the reaction was stopped by adding 0.5 ml of ethanol and immersing the tubes in a boiling water bath for 15 sec. Then 0.5 ml of a mixture of ethanol-water, 3:2 (v/v), containing 5 \(\mu\)moles each of lactose and galactose was added; the total suspension was centrifuged and the clear supernatant fraction was passed through a column (1 \(\times\) 10 cm) of Dowex 1-formate. Prior to use, the resin was washed with 2 volumes of a solution 0.01 M Tris-chloride buffer (pH 7.4) and 1 ml of distilled water. In control runs \(^{14}\text{C}\)-lactose passed through columns so prepared was quantitatively recovered. The other reaction product, NL, was eluted with 1 M formic acid. Fractions of 3 ml were collected, and aliquots of each of them were dried and counted. NL can be removed from the column with 0.1 M formic acid, but, since no interference was observed when it was eluted with 1 M formic acid, this last concentration was preferred to keep NL as concentrated as possible. Blanks containing no glucose were run to correct for small quantities of \(^{14}\text{C}\)-galactose liberated during the incubation. The production of NL was linear for 6 min when the incubation mixture described above was used.

Preparation of CMP-N-AN

The enzymatic synthesis of CMP N-AN was carried out with a crude extract of swine submaxillary gland prepared according to the method of Roseman (12); the incubation system was that of Aroé, Maccioni, and Caputto (13); the CMP-N-AN obtained was deproteinized by passing it through a column of Sephadex G-50 and then was purified in a column of DEAE-cellulose according to the procedure of Warren and Blacklow (14); LiCl was eliminated by passage through a column of cellulose.\(^3\)

**Enzyme Preparation**

Inguinal mammary glands from rats in the 3rd to 5th day after parturition were cut with scissors and then ground at 4°C for 5 min in a mortar containing glass powder and 1 ml of 0.25 M sucrose per g of tissue. The ground tissue was suspended in a total of 4 ml of cold 0.25 M sucrose per g of mammary gland and centrifuged at 4,500 \(x\) g for 10 min; the precipitate was discarded and the supernatant fraction was centrifuged at 55,000 \(x\) g during 30 min; the precipitate was washed twice with 0.25 M sucrose and finally suspended in this solution so that particles from 1 g of mammary gland were suspended in 0.2 ml. Despite several attempts at standardization, the stability of the neuramin lactose synthetase activity of this preparation was erratic. After 1 week at \(-15^\circ\) the remaining activity varied from 90 to 20% of that determined at the day when the enzyme was prepared.

\(^3\) We are indebted to Dr. H. J. Maccioni for giving us information on this column before publication.
With 14C-Lactose—For the determination of neuramin lactose synthetase (lactose cosubstrate), the reaction mixture contained, in a volume of 0.17 ml, 2 pmoles of 14C-lactose (150,000 cpm), 5 μmoles of Tris-chloride buffer (pH 7.4), 2.5 μmoles of MgCl₂, 0.26 μmole of MnCl₂, 0.06 μmole of CMP-N-AN, and 0.05 ml of the suspension of mammary gland particles described above. After incubation at 37° for 5 hours, it was initially processed in the same way as in the assay with UDP-14C-galactose for the determination of the synthesized NL (see above), but the NL obtained from the resin column was further purified by paper electrophoresis in 0.05 M sodium tetraborate, pH 9.2 (15). This last procedure eliminated a contaminant which accounted for 10 to 20% of the radioactivity recovered from the column. The method with 14C-lactose was tested between 24 and 5 hours of incubation, being the production of NL approximately proportional to the incubation time.

Isolation of 14C-Lactose and Its Derivatives after Incubation of Rat Mammary Gland Slices with 14C-Glucose

The incubation and isolation procedures to obtain lactose and NL were essentially those described by Carubelli et al. (3) in their Experiment C. From the same incubation system was also obtained lactose sulfate and neuramin lactose sulfate which were eluted (from the same column in which the previous compounds were isolated) with 2.0 M formic acid and 4.0 M formic acid-0.2 M ammonium formate, respectively. The products were purified until the specific activities after two following steps were equal, as follows: 14C-lactose and 14C-glucose, by successive paper chromatography using 1-butanol-pyridine-water as solvent (11) and electrophoresis in 0.05 M sodium tetraborate at pH 9.2 (15); 14C-neuramin lactose, by paper chromatography in ethyl acetate-pyridine-water, 10:5:6 (v/v) (16) as solvent and by electrophoresis in 0.05 M ammonium acetate-acetic acid, pH 4.65; 14C-lactose sulfate, by the method of Barra and Capputto (17), but a final step was added in which 14C-lactose sulfate was purified by paper electrophoresis in 0.05 M ammonium acetate-acetic acid, pH 4.65; 14C-neuramin lactose sulfate (18), by removing the formic acid with ether and eliminating the ammonium formate by passing the solution through a column of Dowex 50-H⁺ and drying. The residue was run successively by paper chromatography and electrophoresis as with 14C-lactose sulfate.

RESULTS

Synthesis in Vitro of Labeled Lactose and Its Derivatives

After incubation of mammary gland slices with the medium containing 14C-glucose for 20 min, the products were isolated and purified as described under “Experimental Procedure.” The similarity of the specific activities of lactose and the lactose moiety of NL was confirmed (Table I); furthermore, the specific activity of the lactose component of neuramin lactose sulfate was not different from that of free lactose, although that of lactose sulfate was somewhat higher. The specific activity of glucose remained very high relative to that of lactose and its derivatives at the end of the experiment; this discarded the possibility that an equilibrium had been reached at 20 min. Confirming a previous report by Bartley, Abraham, and Chaikoff (19), we found that the specific activity of the glucose recovered at the end of the incubation period was very similar to that added originally.

### Table I

Radioactivity of lactose and lactose derivatives isolated after incubation of mammary gland slices with 14C-glucose

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incorporation into Total compound</th>
<th>Lactose component</th>
<th>N-AN component</th>
</tr>
</thead>
<tbody>
<tr>
<td>14C-Lactose</td>
<td>20,240</td>
<td>19,100</td>
<td>0,650</td>
</tr>
<tr>
<td>Neuramin Lactose</td>
<td>20,238</td>
<td>19,100</td>
<td>0,650</td>
</tr>
<tr>
<td>Neuramin lactose sulfate</td>
<td>31,314</td>
<td>20,200</td>
<td>11,109</td>
</tr>
<tr>
<td>Lactose sulfate</td>
<td>28,330</td>
<td>28,330</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>948,740</td>
<td>0</td>
<td>948,740</td>
</tr>
</tbody>
</table>

* N-AN was hydrolyzed from the rest of the molecule in 0.05 N sulfuric acid at 80° for 1 hour. Sulfuric acid was removed with barium hydroxide. In the case of NL, N-AN was separated from lactose by electrophoresis in 0.05 M ammonium acetate-acetic acid, pH 4.65; in the case of neuramin lactose sulfate, N-AN was separated from lactose sulfate in a Dowex 1-formate column.

### Table II

Synthesis of neuramin lactose with particulate preparation when either 14C-lactose or UDP-14C-galactose plus glucose were used as sialyl acceptor

The incubation system was 2.5 μmoles of MgCl₂, 0.25 μmole of MnCl₂, 5 μmoles of Tris-chloride (pH 7.4), 0.05 μmole of CMP-N-AN, 0.25 μmole of UTP, and 0.025 ml of enzyme preparation (containing 0.25 mg of protein). The final volume was 0.15 ml; incubation time, 6 min; temperature, 37°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Incorporation into</th>
<th>Lactose</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>UDP-14C-galactose + glucose</td>
<td>2678</td>
<td>223</td>
</tr>
<tr>
<td>14C-Lactose</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-14C-galactose + glucose + lactose</td>
<td>2901</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>14C-Lactose + UDP-galactose + glucose</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>UDP-14C-galactose + glucose</td>
<td>3562</td>
<td>297</td>
</tr>
<tr>
<td>UDP-14C-galactose + 14C-lactose + glucose</td>
<td>300</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* The amounts added were 0.1 μmole of UDP-14C-galactose (30,000 cpm), 0.1 μmole of 14C-lactose (30,000 cpm), 5 μmoles of glucose, 5.5 μmoles of lactose, and 0.1 μmole of UDP-galactose.

Synthesis of Neuramin Lactose with Particulate Preparations

Velocity of Formation When Either 14C-Lactose or UDP-14C-galactose Plus Glucose Were Used as Sialyl Acceptor—Table II shows the results of experiments in which particulate preparations were incubated with CMP-N-AN and, respectively, 14C-lactose or UDP-14C-galactose plus glucose. The concentrations and specific activities of 14C-lactose and UDP-14C-galactose were equal. CMP-N-AN and glucose concentrations were, respec-
tively, 0.33 mM and 33 mM. In the conditions used, the production of NL from UDP-14C-galactose plus glucose was around 30 times faster than from 14C-lactose. Furthermore, the presence of unlabeled lactose at concentrations 55-fold higher than that of UDP-14C-galactose produced only a 36% decrease in the labeling of NL. Added lactose did not inhibit the production of 14C-lactose, which is in agreement with the findings of Palmiter (5) with particles from mouse mammary gland; with soluble bovine milk enzyme Babad and Hassid (20) had previously found a substantial inhibition in the synthesis of lactose by adding lactose at the same concentration used in Experiment 1 of Table II.

The faster production of NL from the mixture UDP-14C-galactose plus glucose than from 14C-lactose could not be attributed to an activating effect of the mixture since the presence of glucose plus UDP-galactose had no effect in the labeling of NL from 14C-lactose. Also it could not be attributed to an inhibiting effect of the 14C-lactose preparation since the synthesis in the presence of the three precursors did not show significant difference from that with just UDP-14C-galactose plus glucose (Table II, Experiment 2).

The Michaelis constant for glucose (K_{glucose}) in the synthesis of NL obtained with several enzyme preparations, similarly prepared, were satisfactorily close, but this did not occur in the synthesis of lactose; for three different preparations the K_m values were, for the synthesis of lactose and NL, respectively, 0.93 and 0.23 mM; 1.80 and 0.17 mM; 18.0 and 0.22 mM. Andrews (21) also found a wide variation of the K_m for glucose of purified lactose synthetase from human milk depending on the concentration of added α-lactalbumin; the values reported changed from 80 to 3 mM when, respectively, none or 400 μg of α-lactalbumin were added. Since, on the other hand, it has been shown that α-lactalbumin can be separated from the particles under a great variety of conditions (22, 23), we attribute the dispersion of K_m, which synthesizes the lactose moiety of NL. K_m values for glucose and UDP-galactose in both syntheses were determined. The incubation mixtures at different concentrations of each substrate were the same for both syntheses.

The enzymatic assays were as described under "Experimental Procedure" except for A and B where the specific activity of UDP-14C-galactose was 2.1 × 10^6 cpm per μmole and for D where the concentration of CMP-N-AN was 1 mM.

### Table III

- **Two-step synthesis of neuramin lactose**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Incorporation into</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First step</strong></td>
<td><strong>Second step</strong></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>UDP-galactose (0.5 μmole) + 14C-glucose (0.11 μmole, 450,000 cpm)</td>
<td>None</td>
</tr>
<tr>
<td>UDP-galactose (0.5 μmole) + 14C-glucose (0.11 μmole, 450,000 cpm)</td>
<td>CMP-N-AN</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>UDP-14C-galactose (0.16 μmole, 80,000 cpm) + CMP-N-AN (0.25 μmole)</td>
<td>Glucose</td>
</tr>
<tr>
<td>UDP-14C-galactose (0.16 μmole, 80,000 cpm) + glucose (5 μmole)</td>
<td>None</td>
</tr>
<tr>
<td>UDP-14C-galactose (0.16 μmole, 80,000 cpm) + glucose (5 μmole)</td>
<td>CMP-N-AN</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
</tr>
<tr>
<td>UDP-14C-galactose (0.16 μmole, 80,000 cpm) + glucose (5 μmole)</td>
<td>None</td>
</tr>
<tr>
<td>UDP-galactose (0.16 μmole) + glucose (5 μmole)</td>
<td></td>
</tr>
<tr>
<td>UDP-galactose (0.16 μmole) + 14C-lactose (0.008 μmole, 4,000 cpm)</td>
<td></td>
</tr>
</tbody>
</table>

a The purification procedures for lactose and NL were similar to the method described under "Experimental Procedure" for determination of enzyme activity with UDP-14C-galactose. Final separation of 14C-lactose from other radioactive material was achieved by electrophoresis in 0.05 M sodium tetraborate, pH 9.2 (15).

b After separation of particles in the first step, 14C-lactose found in the supernatant fraction had 5985 cpm.
for glucose in our preparations to possible variations in their content of α-lactalbumin. When the $K_m$ values obtained with the same enzyme preparation as neuramin lactose synthetase and lactose synthetase were compared, the $K_{glucose}$ for neuramin lactose synthetase was lower in every case. The $K_{UDP-galactose}$ was also lower for the synthesis of NL than for the synthesis of lactose (Fig. 1). The ratio $K_{UDP-galactose}$ (lactose synthetase) to $K_{UDP-galactose}$ (NL synthetase) was close to 15. The value of 0.15 mM for $K_{UDP-galactose}$ in the synthesis of lactose found in the present work was in the range of the values reported by other workers, which were 0.5 mM for purified milk enzyme (20) and 0.06 mM (24) for a rat mammary gland homogenate.

$K_m$ Values for Substrates of Synthesis of Neuramin Lactose—Fig. 1 shows the Lineweaver-Burk plots for the different substrates from which NL can be synthesized. $K_{UDP-galactose}$ and $K_{glucose}$ were lower than $K_{lactose}$. The ratio $K_{UDP-galactose}$ to $K_{lactose}$ was 0.0018 and the ratio of $K_{glucose}$ to $K_{lactose}$ was close to 0.037. The $K_{CMP-N-AN}$ (lactose cosubstrate) was reported by Carlson and Jourdieh (25) as 0.5 mM. The $K_{CMP-N-AN}$ (UDP-galactose plus glucose cosubstrates) found in the present work was close to 10-fold lower (Fig. 1).

Two-step Synthesis of Neuramin Lactose—Since apparently the lactose moiety of NL synthesized from UDP-galactose plus glucose did not enter the pool of free lactose, the possibility was investigated that lactose remained attached to the particles until NL was formed. For this purpose a synthesis of NL carried out in two steps was attempted (Table III). In the first step, particles from mammary gland were incubated with UDP-galactose plus 14C-glucose or with UDP-14C-galactose plus either CMP-N-AN or glucose (see Experiments 1 and 2 in Table III). After 5 min of incubation, the particles were diluted to 10 ml with cold 0.14 M KCl, centrifuged, washed, and resuspended in a salt solution of identical composition with that used in the first step. This suspension was then incubated by itself or after addition of the substrate omitted in the first step. The three experiments summarized in Table III showed that a certain amount of lactose remained attached to the particles after incubation with the precursors. In Experiment 3, after the first incubation and separation of the particles, the 14C-lactose obtained from UDP-14C-galactose plus glucose was measured in the supernatant fraction and in the washed particles. Approximately 15% of the total lactose synthesized remained attached to the particles (see Footnote b in Table III). Incubation of the particle-lactose complex with CMP-N-AN (in the second step) transformed approximately one-third of the lactose into NL. The radioactivity attached to the particles was quantitatively released by adding ethanol at 75% final concentration and immersing for 15 sec in a boiling water bath. Experiment 2 also showed that incubation of UDP-14C-galactose plus CMP-N-AN in the first step did not result in the production of NL after addition of glucose in the second step; consequently, it was concluded that a galactose-N-AN-containing intermediate either was not formed or was not attached to the particles. The latter possibility was not investigated further. Radioactive lactose added to the incubation system in similar amounts to the total lactose synthesized from UDP-14C-galactose plus glucose was not substantially attached to the particles (Experiment 3, Table III).

Fractionation of Particles—In attempts to elucidate whether or not the particles carrying lactose synthetase activity can be separated from those carrying NL synthetase (UDP-galactose plus glucose cosubstrates), they were centrifuged in sucrose solution in a linear gradient of densities between 1.04 and 1.26. The procedure was that described by Coffey and Reithel (26) but with 30-ml centrifuge tubes in a Spinco model L centrifuge, rotor SE-25-1; the amount of homogenate layered per tube was correspondingly diminished to 1.5 ml. After centrifugation, fractions of 2 ml were obtained from the bottom of the centrifuge tube by syphoning, diluted with 4 ml of water, and centrifuged at 100,000 $\times$ g for 60 min. Each pellet was suspended in 0.2 ml

![Fig. 2. Particle centrifugation in a linear sucrose gradient. The density varied from 1.04 to 1.26. $\bullet$ -- $\circ$, lactose synthetase; $\bigcirc$ -- $\bigcirc$, NL synthetase; and $\bullet$ -- $\bigcirc$, protein concentration. Enzymatic assays were carried out with labeled UDP-14C-galactose as described under "Experimental Procedure."](http://www.jbc.org/)

![Fig. 3. The effect of adding CMP-N-AN on lactose (○--○) and NL (●--●) synthesis. Each point is the average of three determinations. The incubation system was similar to that described under "Experimental Procedure," but 0.05 μmole of glucose, 0.002 μmole of UDP-14C-galactose (15,000 cpm), and 0.005 ml of enzyme preparation were used. CMP-NANA: cytidine 5'-monophospho-N-acetylneuraminic acid.](http://www.jbc.org/)
of 0.25 M sucrose, and protein concentration, lactose synthetase, and NL synthetase were determined. In Fig. 2 results are plotted as the percentage of the total against the fraction number. It appeared that particles which in total accounted for more than 50% of the proteins were devoid of synthetase activities and that separation of lactose synthetase from NL synthetase was not achieved.

Effect of Addition of CMP-N-AN on Synthesis of Lactose and Neuramin Lactose—CMP-N-AN was added in increasing amounts to a system that produced lactose. Fig. 3 shows that in such an experiment increasing amounts of NL were produced when corresponding amounts of lactose disappeared. At low concentrations of CMP-N-AN the results on the actual decreases of lactose were erratic, and it was difficult to ascertain if there was equivalence between the lactose and NL that, respectively, disappeared or was synthesized. This was probably due to the inherent errors in the determination of small differences between large quantities of lactose. In those assays in which CMP-N-AN was above 0.1 mM the equivalence appeared well established.

Attempts to Solubilize Neuramin Lactose Synthetase—Several attempts were made to solubilize NL synthetase following some of the procedures which were successful in solubilizing lactose synthetase (23, 26). Table IV shows an instance in which, by using Triton X-100, the NL synthetase (lactose cosubstrate) was recovered in disrupted particles or in the pellet obtained from disrupted particles; the NL synthetase was recovered in higher proportion than lactose synthetase itself. The NL synthetase (UDP-galactose plus glucose cosubstrates) disappeared completely in preparations in which 30% of the NL synthetase (lactose cosubstrate) remained attached to the disrupted particles. Addition of B protein or A and B proteins to intact particles in quantities which enhanced the production of lactose 50 to 100% had little or no effect on the production of NL from UDP-14C-galactose and glucose.

When B protein or the mixture of A and B proteins was added to disrupted particles or to the pellet or supernatant fractions from disrupted particles the lactose synthesis increased in every case, whereas NL synthetase (UDP-galactose plus glucose cosubstrates) in no case was reactivated.

Identity of Reaction Products—"Fingerprint" experiments were carried out. The NL produced by reaction of UDP-galactose, glucose, and CMP-N-AN and that produced from lactose and CMP-N-AN were chromatographically identical with each other and with the major NL isomer present in rat mammary gland when run on paper in the ethyl acetate-pyridine-water system (16). The identity of both NL with the major NL isomer of the rat mammary gland was corroborated by paper chromatography in 1-butanol-pyridine-water (11) and by electrophoresis in 0.05 M sodium tetraborate, pH 9.2, and 0.1 M acetic acid-0.1 M ammonium acetate, pH 4.65. Jourdian et al. (2) had previously identified as 3'-NL the product formed from lactose and CMP-N-AN by the rat mammary gland particulate preparation.

**DISCUSSION**

The similarity of the specific activities of lactose and the lactose moiety of NL obtained after incubation of mammary gland slices with 14C-glucose can be readily explained by applying the sequence of Reactions 3a and 3b (see above). According to this sequence, 14C-glucose does not enter the pool of free lactose before entering that of NL; assuming that lactose and NL are end products in the metabolism of the mammary gland and, consequently, produced at rates commensurate with their concentrations in milk, they necessarily must be secreted with the same specific activity. The similarity of the specific activity can also be explained if the synthesis occurs according to Reactions 1 and 2; in this case it has to be assumed that the measurements had all been carried out when the intracellular pools of either lactose or NL, or both, became negligible compared with the amounts synthesized from the labeled precursor, since at this point the dilution undergone by the precursor at any one of those pools becomes of no consequence. At present there is

**TABLE IV**

Attempts of solubilization of neuramin lactose synthetases

The particulate fraction was obtained as described under "Experimental Procedure" but resuspended in 0.125 M sucrose. Disrupted particles were obtained by adding 2.0% (w/v) of Triton X-100 to the particulate fraction. Disrupted particles which had been standing 1 hour at 4° were centrifuged at 55,060
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no information on the sizes of those pools and so no conclusion can be offered on the possibility that Sequence 1 and 2 may explain the data on specific activities.

Reaction 2 derives from the work of Jourdian et al. (2) on the synthesis of NL; the need to postulate the over-all Reaction 3 with Steps 3a and 3b arise from (a) the observation that the synthesis of NL proceeds at higher speed when UDP-galactose plus glucose were used as precursors instead of lactose at the same concentration; the affinity constants for UDP-galactose and for glucose were lower than the constant for lactose in the synthesis of NL. Free, unlabeled lactose at a concentration 55-fold higher than added UDP-\(^{14}\)C-galactose (600-fold higher than the lactose formed) (Table I) had little diluting effect over the labeled NL formed. All these observations are incompatible with the hypothesis that free lactose is a necessary intermediate in the synthesis of NL from UDP-galactose plus glucose. (b) It was shown (Table III) that the synthesis of NL proceeds in two steps; in the first, a particle-lactose complex was formed, and, in the second, in the presence of CMP-N-AN, NL was produced. These findings relate the synthesis of NL to other syntheses carried out by mulitienzyme complexes, in which the substrates are subjected to successive transformations while they are kept bound to the enzyme complex (25).

Assuming that Reactions 3a and 3b correctly represent the fast synthesis of NL in the presence of UDP-galactose plus glucose, the possibility should be considered that each of the corresponding transferases is the same as those for Reactions 1 and 2. In the following discussion on this question the kinetic constants of Reactions 1 and 3a are compared in the same incubation system; free lactose is considered the product of Reaction 1, and the lactose moiety of NL, the product of Reaction 3a. It has not been possible to compare Reactions 2 and 3b under identical conditions because addition of UDP-galactose plus glucose to the incubation mixture of Reaction 2 dilutes substantially the initial \(^{14}\)C-lactose. The kinetic constants of the \(\beta\)-galactosyltransferases of Reactions 1 and 3a are significantly different, but this does not discard the possibility that one and the same enzyme intervenes in both reactions. Attempts to separate the transferases by isopycnic centrifugation did not succeed. Determination of the lactose missing after addition of CMP-N-AN to a lactose-synthesizing system was accounted for by the NL synthesized (Fig. 3). These observations indicate that the galactosyltransferases for both syntheses are identical, but the lower values of \(K_{\text{UDP-galactose}}\) for Reaction 3a appear at first as conclusive evidence against the identity. However, it is known that addition of \(\alpha\)-lactalbumin to the galactosyltransferase of Reaction 1 increases its activity and lowers its \(K_m\) for glucose (21). In our experiments, erratic values for \(K_{\text{glucose}}\) of Reaction 1 were obtained when similarly prepared particle preparations were used; it is conceivable that this variation was due to the different amounts of \(\alpha\)-lactalbumin in each preparation. The lower \(K_{\text{glucose}}\) and \(K_{\text{UDP-galactose}}\) values for Reaction 3a could be explained assuming that the galactosyltransferase was maximally activated where the synthesis of NL occurred. This may be so because at those positions the transferase is saturated with \(\alpha\)-lactalbumin or because some other activating factor (sialyltransferase?) is present there. Palmiter (29) has postulated the existence of a third factor in the lactose synthetase system to explain the higher stability of the particles relative to complexes of soluble A and B proteins.

Reaction 3b is probably the same described by Carlson and Jourdian (25) as occurring with an endogenous acceptor which "appears to be lactose but it cannot be removed from the particulate preparation by repeated washing and does not exchange with \(^{14}\)C-lactose." At present it cannot be decided whether or not the sialyltransferase of Reaction 2 is the same as the one in Reaction 3b. The major product in both reactions was the same. Addition of lactose to a system that synthesized NL from UDP-\(^{14}\)C-galactose plus glucose inhibited the synthesis of labeled NL approximately 25%; since the synthesis of lactose was not inhibited, it appears that the effect was due to inhibition of the sialyltransferase. However, it was not ascertained that the inhibition was competitive to decide on the identity of the sialyltransferases. The possibility of obtaining the enzyme for Reaction 2 but not that for Reaction 3b after treatment with Triton X-100 and other detergents does not show that they are different enzymes because of the complex nature of the reaction in which 3b was tested. It is possible that separation of the enzymes of Reaction 3a created conditions under which 3b cannot proceed. The \(K_{\text{CMP-N-AN}}\) (UDP-galactose plus glucose cosubstrates) found in the present work was approximately 10-fold lower than the \(K_{\text{CMP-N-AN}}\) (lactose cosubstrate) reported by Carlson and Jourdian (25). Since there is no known explanation for changes in \(K_m\) values for a substrate of a single sialytransferase, this difference is at present the strongest evidence to suggest that Reactions 2 and 3b are catalyzed by different enzymes.

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