Lactose Synthesis

VI. LABELING OF LACTOSE PRECURSORS BY GLYCEROL-1,3-C\textsuperscript{14} AND GLUCOSE-2-C\textsuperscript{14}\textsuperscript{*}

R. G. Hansen, Harland G. Wood, Georges J. Peeters, Birgit Jacobson, and JoAnne Wilken

From the Department of Biochemistry, Michigan State University, East Lansing, Michigan, the Department of Biochemistry, Western Reserve University, Cleveland, Ohio, and the Veterinary School of the University of Ghent, Belgium

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It is generally agreed that blood glucose is the major source of carbon for the synthesis of both hexose portions of lactose by the mammary gland (1, 2), but the exact mechanism of lactose synthesis remains in doubt. An abridged outline of some possible pathways of labeled carbon in the biosynthesis of lactose is presented in Fig. 1. In Reaction A of Fig. 1, uridine diposphogalactose is the galactosyl donor and glucose 1-phosphate the galactosyl acceptor. The enzyme catalyzing this reaction has been described (3), but so far a confirmation of this work has not been reported. Hexokinase (4, 5), phosphoglucomutase (6), uridine triphosphate glucose 1-phosphate pyrophosphorylase (7), and uridine diposphogalactose 4-epimerase have all been found in mammary gland (8, 9).

A second postulated pathway involves the condensation of free glucose and UDP-galactose (Fig. 1, Reaction B). This mechanism was proposed to account for the results obtained in tracer experiments with the perfused isolated cow udder (10) and in vivo when the labeled substrate was injected into the pudic artery of cows (11, 12). In both types of experiments, it was found that the glucose moiety of the lactose differed from the galactose. With glycerol-1,3-C\textsuperscript{14} (12), the galactose contained much more C\textsuperscript{14} than the glucose and the carbon atoms in positions 4 and 6 of the galactose were much more heavily labeled than the carbon atoms in positions 1 and 3. The glucose was approximately equally labeled in carbon atoms 1, 3, 4, and 6 and had a C\textsuperscript{14} pattern similar to the blood glucose. The excess labeling in carbon atoms 4 and 6 of galactose has been tentatively explained by proposing (12) that a transaldolase type of exchange of triose phosphate-C\textsuperscript{14} occurs with fructose-6-P, which introduces a high degree of labeling into carbon atoms 4 and 6 (Fig. 1, Reaction C). Ljungdahl et al. (13) have shown such an exchange using purified transaldolase from yeast. The difference in labeling of the glucose and galactose is readily explained by Mechanism B because the free glucose would not be subject to the exchange reaction and thus would not acquire heavy labeling in carbon 4 and carbon 6.

An alternative proposal was also made (12), namely that there may be a particular architecture within the cells that permits compartmentalization of certain enzymes. Thus, substrate pools may arise which are not in equilibrium. Therefore, glucose and galactose with different labeling could occur even though both arise from hexose phosphate precursors. In Fig. 1, Reactions D and E are presented, each giving rise to glucose-6-P to indicate such compartmentalization. If the hexose phosphates of Pathway E were not subject to the transaldolase type of exchange, then lactose could be synthesized via Reaction A (Fig. 1) in which the glucose and galactose moieties would have different C\textsuperscript{14} patterns.

Another mechanism is indicated by Reaction F of Fig. 1, in which there is direct synthesis of UDP-hexoses from 3-carbon precursors or, alternatively, a transaldolase-like exchange with UDP-galactose. Such a mechanism could account for the preferential labeling of the galactose moiety of lactose by glycerol-1,3-C\textsuperscript{14} and it would not require compartmentalization of pools of hexose phosphates to account for the observed C\textsuperscript{14} patterns. This possibility is of particular interest in view of the recent report of Smith, Galloway, and Mills (14) that UDP-dihydroxyacetone is a product of the metabolism of Diplococcus pneumoniae. The existence of such a compound definitely presents the possibility that exchange may occur at the uridine hexose level.

The present investigation represents an attempt to study these different mechanisms and to determine whether there are separate nonequilibrated pools of hexose phosphate that are involved in lactose synthesis. The UDP-hexoses and hexosephosphates have been isolated from the tissue of the mammary gland after injection of glycerol-1,3-C\textsuperscript{14} into the pudic artery of a cow or perfusion of glycerol-1,3-C\textsuperscript{14} and glycerol-1,3-C\textsuperscript{14} and glucose-2-C\textsuperscript{14} through a lactating mammary gland. The total radioactivity and distribution of C\textsuperscript{14} in the 6-carbons of these lactose precursors has been determined. It has been found that the hexoses of the UDP-glucose and UDP-galactose contain a much higher C\textsuperscript{14} activity than the glucose 6- or glucose 1-phosphates. It is concluded that there may be a new biosynthesis of UDP-hexose and compartmentalization and nonequilibration of hexose phosphates in the mammary gland, but that some of the compartments are in nonsecretory tissues of the gland and not involved in lactose synthesis. The results are in accord with the proposal that free glucose is the source of the glucosyl moiety of lactose.

EXPERIMENTAL PROCEDURE

In Experiments 1 and 2 (in vivo), cows were treated surgically to permit injection of labeled glycerol directly into the arterial...
supply of the lactating mammary gland. In Experiments 3 and 4 (in vitro), an isolated cow’s udder was perfused with blood containing labeled glycerol in one experiment and a mixture of labeled glycerol and labeled glucose in the other. In Experiments 1 and 2, Holstein cows were used that weighed approximately 500 kg and were producing approximately 20 liters of milk per day. The cows were given oxytocin and milked; then 0.5 mmoles of glycerol-1,3-C\textsuperscript{14} (0.5 mc) in 25 ml of 0.9% NaCl solution was injected at constant rate during a period of 8 minutes into the left pudic artery. A description of the procedure has been given previously (12). The cows were killed, and the left half of the udder was removed, dissected into small pieces, and dropped into liquid air for freezing. After the termination of the injection, 6 minutes elapsed before all the tissue was in the liquid nitrogen and an additional 6 minutes elapsed before the freezing was complete as judged by cessation of boiling of the nitrogen. The frozen tissue was wrapped in cheesecloth and powdered with a hammer before extraction with 2 volumes of 0.6 M perchloric acid.

In Experiment 2 (the second experiment in vivo), part of the powdered tissue was treated by a procedure based on that used by Dounce (15) to obtain a "nuclei fraction." Portions of tissue, 200 g each, were placed in 500 ml of ice-cold H\textsubscript{2}O and brought to pH 5.8 with 1 M citric acid and then homogenized in a large Waring Blender at high speed for 10 minutes in the cold room. The temperature was maintained at less than 8°C and the pH between 5.8 and 6.2. The homogenate was centrifuged in 500-ml tubes at high speed for 10 minutes to separate fractions. The sedimented fraction has been designated "nuclei fraction," but it contained sedimented material in addition to the nuclei. It represents a fraction in which nuclei were more concentrated than in the supernatant fraction.

In the third experiment, 2.5 g of sodium acetate were added to 9 liters of heparinized and oxygenated blood, and the blood was perfused at 37°C through the left half of a lactating cow's udder (16) that normally yielded 12 liters daily. Peeters and Massart (16) have found that the oxygen consumption of the perfused gland is maintained constant over a longer period of time if acetate is added to the blood as well as glucose. After 30 minutes, when the output of blood reached 1000 ml per minute, 0.2 mmoles of glycerol-1,3-C\textsuperscript{14} (0.5 mc) was added to the blood, and the perfusion was continued for 15 minutes. The perfused half of the udder was disconnected and rapidly dissected into small pieces and held in ice. To remove as much of the milk as possible, 3.3 kg of the tissue were ground in a meat grinder previously cooled to 0°C and collected in 6 liters of ice-cold 0.9% NaCl solution. The suspension in the NaCl solution was stirred for 1 minute. It was then filtered through gauze over a Buchner funnel. The tissue was resuspended in 3 liters of ice-cold 0.9% NaCl solution, stirred a few seconds, and again filtered. The filter cake was added to 6 liters of 0.6 M ice-cold perchloric acid. Sixteen minutes were required for these manipulations after cessation of the perfusion. The tissue was homogenized with a Waring Blender and centrifuged. The perchlorate was removed as the potassium salt from the supernatant solution, the pH was adjusted to 6.6, and the solution was lyophilized.

In Experiment 4, 8.75 liters of blood containing 3.5 g of sodium acetate were perfused through the right half of an udder, and after 10 minutes when the output of blood reached a relatively high value, glycerol-1,3-C\textsuperscript{14} and glucose-2-C\textsuperscript{14} were added to the circulating blood. The glycerol-1,3-C\textsuperscript{14} (0.5 mc) and glucose-2-C\textsuperscript{14} (0.25 mc) were added in 50 ml of 0.9% NaCl solution at the rate of 5 ml per 70 seconds. When the addition of the isotope was completed, the perfusion was stopped and the tissue was cut into thin slices. The slices were slowly washed in cold 0.0% NaCl solution to remove excess blood and milk; then to obtain immediate freezing (17) after blotting with a dry towel, we placed them in isopentane that was cooled in liquid nitrogen. The frozen tissue was wrapped in several layers of cheese cloth and powdered with a hammer. It was then extracted with 2 volumes of 0.6 M cold perchloric acid, and the extracts were treated as before.

**Isolation of Nucleotide Hexoses**—The lyophilized powder was dissolved in water, and the nucleotides were adsorbed on charcoal, leaving the bulk of the sugar phosphates in solution. The nucleotides were eluted from the charcoal with 15% aqueous pyridine, and the pyridine was removed by extraction with chloroform. The nucleotides were then adsorbed from the aqueous phase on a Dowex 1-formate column and eluted by the gradient system described by Hurlbert et al. (18). Appropriate fractions containing the UDP-glucose and UDP-galactose were combined and again adsorbed on charcoal to remove the formate. After elution with pyridine and removal of the pyridine, the solution was lyophilized. The residue was dissolved in water, and an aliquot was assayed enzymically as described by Brett-hauer et al. (19). This procedure employs UDP-glucose dehydrogenase and permits estimation of the UDP-glucose and UDP-galactose after the addition of UDP-galactose-4-epimerase. Carrier glucose and galactose were added to the nucleotide mixture which was then hydrolyzed in 1 N sulfuric acid at 100°C for 15 minutes, neutralized with barium hydroxide, filtered, and lyophilized. Glucose and galactose were separated on a cellulose column, crystallized to constant specific activity, and then degraded according to procedures described previously (12, 20).

**Isolation of Sugar Phosphates**—The supernatant solution from the Norit treatment was concentrated by lyophilization. The hexose phosphates were precipitated from an aqueous solution at pH 8 by addition of barium acetate and 4 volumes of ethanol.

![Fig. 1. Suggested pathways of carbon in the biosynthesis of lactose from glycerol-1,3-C\textsuperscript{14} and glucose-2-C\textsuperscript{14}](http://www.jbc.org/). Abbreviations used are: DIOH acetone-P, dihydroxyacetone-P; G-6-P, glucose-6-P; G-1-P, glucose-1-P; TA, transaldolase.)
The precipitate was only partially soluble in water. The barium ions were removed by adsorption on Dowex 50-H+, which lowered the pH to approximately 2 and also accomplished complete solution of the esters. After centrifugation, the solution was made basic (pH 8.5) before being placed on a Dowex 1-C+ column for separation of the esters. Elution was accomplished with ammonium hydroxide, ammonium chloride, potassium chloride, and sodium borate in the sequence and proportions described by Khym and Cohn (21). The separate compounds were located by the anthrone reaction and appropriate fractions were concentrated. Cations were removed by Dowex 50-H+ before removal of the boric acid by vacuum distillation with methanol (22). After three such methanol treatments, the residues containing the phosphate esters were dissolved in a small quantity of water.

Glucose 6-phosphate was determined spectrophotometrically with glucose 6-phosphate dehydrogenase from yeast (20). Carrier crystalline barium glucose 6-phosphate was added, and the resulting mixture was recrystallized twice. After hydrolysis with phosphatase and deionization, the glucose was further purified on a cellulose column, crystallized to constant activity, and then degraded as previously described (12).

Glucose 1-phosphate was converted to glucose 6-phosphate with crystalline phosphoglucomutase and then estimated with glucose 6-phosphate dehydrogenase (23). Dipotassium glucose 1-phosphate was added as a carrier, and the product was recrystallized twice. After hydrolysis with phosphatase, the glucose was further purified on a cellulose column.

Fructose diphosphate present in the diphosphate fraction from the Dowex column was determined by enzymic assay with aldolase and triosephosphate dehydrogenase (24). After hydrolysis and dilution with carrier fructose, the fructose was chromatographed on a cellulose column. The phenylalanine was prepared and crystallized to constant specific activity. The osazone was degraded by periodate oxidation (25), yielding carbon atoms 1, 2, and 3 as the osazone of mesoxaldehyde, carbon atoms 4 and 5 as formate, and carbon 6 as formaldehyde.

Isolation of Lactose—The aqueous ethanol supernatant from which the barium hexose phosphates had been removed was held in the deep freeze. Upon standing, a precipitate formed that contained most of the lactose. This precipitate was dissolved in H2O, barium was removed by filtration after the addition of sulfuric acid, and then the acetic acid was removed by vacuum distillation. Residual ions were removed by the addition of a mixed anion-cation resin (Amberlite MB-3). The lactose was then separated from other contaminants by preparative paper chromatography. After aqueous elution and concentration, the lactose was recrystallized twice without dilution by dissolving the precipitate in a minimal volume of hot water and adding ethanol to approximately 90% concentration.

A critical part of the experimental procedure is the accuracy of the dilution with unlabeled carrier compounds to permit crystallization of the metabolites. In all cases, the enzymic assays employed to estimate the amount of each compound present were checked thoroughly with known compounds. This included quantitative recovery of standards added to the mammary gland fractions. The purity of the crystalline carrier phosphate esters was checked by enzymic assay and served as the basis for the dilution. In each case except for fructose 1,6-diphosphate, the purification was accomplished by recrystallization of the phosphate esters as well as recrystallization of the free sugar or a derivative. This procedure was necessary to remove the small quantities of radioactive contaminants that were present in most fractions as obtained from the various columns.

### RESULTS AND DISCUSSION

**Amount of Phosphate Esters**—The amount of UDP-glucose isolated from mammary gland varied from 12 to 50 μmoles per kg fresh weight of tissue, the UDP-galactose from 2.4 to 12.1, and the glucose 6-phosphate from 5.8 to 30.6 (Table I). However, there were losses during the isolation. The specific activity of the metabolites was of primary concern and quantitative recovery was not attempted. It is noted that the percentage of galactose in the uridine hexoses varied from 16 to 20%. The equilibrium value for UDP-galactose 4-epimerase is 25% UDP-galactose and 75% UDP-glucose (26, 27). However, there is no reason to expect the UDP-glucose and UDP-galactose to be at the equilibrium values in a dynamic system.

**Specific Activity of Hexoses of Nucleotides and Hexose Phosphates**—Table II shows that the specific activities of the nucleotide hexoses were in all cases at least 10 times that of the glucose 6-phosphate. This was true in the experiments in vivo (Experiments 1 and 2) and also with the isolated and perfused mammary gland (Experiments 3 and 4). In Experiment 1, glucose 1-phosphate and fructose-1, 6-diphosphate were also isolated and they contained 955 and 1070 c.p.m. per μmole, respectively. These values are similar to that of the glucose 6-phosphate from the same experiment.

The surprisingly low activity of the hexose phosphates as compared to the hexoses of the uridine nucleotides indicated that there might be compartments in which the nucleotides were synthesized. The possibility of nonequilibrated pools in the nuclei was of particular interest since UTP-glucose 1-phosphate pyrophosphorylase has been reported to be associated with the cell nucleus (28). It seemed possible that UDP-glucose and UDP-galactose were formed in the nucleus and then through a transaldolase-like reaction (Fig. 1, Reaction F) acquired C14 from a 3-carbon compound formed from the glycerol-1,3-C14. Therefore, in Experiment 2, a fraction in which nuclei were concentrated was compared with the “supernatant fraction.” If the UDP hexoses were formed in the nucleus, it was expected that hexose phosphates with high C14 activity would occur in the nuclei. Table II, Experiment 2, shows that the nucleotide hexoses were labeled similarly in all fractions and varied from

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Tissue fraction of</th>
<th>UDP-G</th>
<th>UDP-Gal</th>
<th>Glucose-6-P</th>
<th>Glucose-1-P</th>
<th>Fructose-1,6-diphosphate</th>
<th>Proportion of uridine hexoses as</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total</td>
<td>19.2</td>
<td>5.9</td>
<td>10.3</td>
<td>0.9</td>
<td>1.3</td>
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<td>22</td>
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<td>2</td>
<td>Total</td>
<td>49.6</td>
<td>12.1</td>
<td>15.2</td>
<td></td>
<td></td>
<td>N/A</td>
<td>20</td>
</tr>
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<td>“Nuclei fraction”</td>
<td>1.2</td>
<td>0.3</td>
<td>30.6</td>
<td></td>
<td></td>
<td>N/A</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>“Supernatant fraction”</td>
<td>8.9</td>
<td>3.1</td>
<td>30.2</td>
<td></td>
<td></td>
<td>N/A</td>
<td>26</td>
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<tr>
<td>3</td>
<td>Total</td>
<td>12.3</td>
<td>2.4</td>
<td>6.7</td>
<td></td>
<td></td>
<td>N/A</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Total</td>
<td>32.8</td>
<td>6.0</td>
<td>5.6</td>
<td></td>
<td></td>
<td>N/A</td>
<td>16</td>
</tr>
</tbody>
</table>

### Table I

Amount of phosphate esters isolated from cow's udder

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Tissue fraction of</th>
<th>UDP-G</th>
<th>UDP-Gal</th>
<th>Glucose-6-P</th>
<th>Glucose-1-P</th>
<th>Fructose-1,6-diphosphate</th>
<th>Proportion of uridine hexoses as</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total</td>
<td>19.2</td>
<td>5.9</td>
<td>10.3</td>
<td>0.9</td>
<td>1.3</td>
<td>N/A</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Total</td>
<td>49.6</td>
<td>12.1</td>
<td>15.2</td>
<td></td>
<td></td>
<td>N/A</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>“Nuclei fraction”</td>
<td>1.2</td>
<td>0.3</td>
<td>30.6</td>
<td></td>
<td></td>
<td>N/A</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>“Supernatant fraction”</td>
<td>8.9</td>
<td>3.1</td>
<td>30.2</td>
<td></td>
<td></td>
<td>N/A</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Total</td>
<td>12.3</td>
<td>2.4</td>
<td>6.7</td>
<td></td>
<td></td>
<td>N/A</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Total</td>
<td>32.8</td>
<td>6.0</td>
<td>5.6</td>
<td></td>
<td></td>
<td>N/A</td>
<td>16</td>
</tr>
</tbody>
</table>
The glucose 6-phosphate had the same specific activity in the “nuclei fraction” and “supernatant fraction,” that is 1,238 versus 1,243. However, the specific activity of glucose 6-phosphate from the total tissue was unaccountably lower. A duplicate isolation was made with essentially the same result. However, the results of Experiment 2 provided no evidence that there are high activity pools of hexose derivatives in the nuclei.

Since milk contains hexose phosphates (29), it was considered that the C14 of the tissue hexose phosphates might be diluted by unlabeled hexose phosphates present in the milk not removed from the tissue. It also was considered that dilution of C14 might occur by metabolic changes that occurred after the termination of the injection. In the experiments in vivo, the heart probably continued to beat for some time after the cows were killed and, therefore, unlabeled blood was pumped into the udder and the glycerol-1,3-C14 was washed out. It thus was possible that low activity hexose phosphates were synthesized subsequent to the termination of the injection. For these reasons, a perfusion experiment was performed in which the tissue was cooled quickly and, in addition, ground and washed to remove the milk. It is seen in Experiment 3, Table II, that the nucleotide hexoses again were labeled much more highly than the glucose 6-phosphate, thus resembling the experiments in vitro.

It remained possible that there was dilution of the C14 of the glucose-6-P through metabolism of endogenous C12 compounds during the grinding and washing of the tissue. In Experiment 4, (a) to maintain intact cells before freezing, (b) to retain the labeled substrate in the tissue and (c) to reduce the freezing time, the tissue was sliced and then frozen in isopentane. This procedure provides very rapid freezing (17). Again, the glucose-6-P had a much lower specific activity than the hexoses of the uridine nucleotides. It therefore seems quite certain that the values observed for the glucose-6-P of the udder are true values.

Experiment 4 was of additional interest because in this case the C14 was entering at both the triose-P level from glycerol-1,3-C14 and also at the glucose-6-P level from glucose-2-C14. Thus, even though the C14 was entering the uridine hexoses from glycerol via Pathway F of Fig. 1, the hexose-P should also acquire C14 from glucose via the hexokinase reaction. However, the glucose-6-P had a low activity even when glucose-C14 was present. These results indicate that there is present in the tissue of the mammary gland a pool of glucose-6-P that turns over slowly and thus does not acquire C14 rapidly from either glucose or glycerol. This pool with a low turnover probably is not a precursor of lactose. It may be associated with nonsecretory cells such as in connective tissue which may have a lower metabolic rate than the secreting cells.

It is of interest that the UDP-galactose consistently had a somewhat greater specific activity than the UDP-glucose (see Table II). Although these differences were not great, they were consistent throughout this work. In this connection, Kosterlitz (30) has reported that galactose-1-P is more acid-labile than glucose-1-P and, therefore, UDP-galactose is presumably more labile than UDP glucose. Although there was no indication of hydrolysis during our isolation of UDP-galactose, the amount of galactose present would have been underestimated if such hydrolysis of UDP-galactose had occurred. In this case, the dilution of galactose by the added carrier would have been less than calculated and, therefore, the estimated specific activity of the UDP-galactose would have been too high. Although the hydrolysis would cause an error in the estimation of total activity, it would not alter the C14 distribution patterns. It will be seen (Table IV) that there is some indication that the UDP-glucose and UDP-galactose do have different C14 distribution patterns.

### Table II

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Procedure</th>
<th>Tissue fraction of udder</th>
<th>Compounds isolated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>UDP-G, UDP-Gal, Glucose-6-P</td>
</tr>
<tr>
<td>1</td>
<td>Injection in vivo</td>
<td>Total</td>
<td>11,950 12,220 1,222</td>
</tr>
<tr>
<td>2</td>
<td>Injection in vivo</td>
<td>“Nuclei fraction”</td>
<td>10,260 10,856 565</td>
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<td></td>
<td></td>
<td>“Supernatant fraction”</td>
<td>10,800 11,350 1,288</td>
</tr>
<tr>
<td>3</td>
<td>Perfusion in vitro</td>
<td>Total</td>
<td>11,373 11,966 1,243</td>
</tr>
<tr>
<td>4</td>
<td>Perfusion in vitro</td>
<td>Total</td>
<td>30,264 38,213 3,526</td>
</tr>
<tr>
<td>5</td>
<td>Perfusion in vitro</td>
<td>Total</td>
<td>13,190 17,600 869</td>
</tr>
</tbody>
</table>

* In this experiment glucose 1-phosphate and fructose-1,6-di-phosphate were also isolated. The specific activities were 985 for the former and 1,070 for the latter.

### Distribution of C14 from Glycerol in Various Hexoses

In Experiments 1 and 2, 0.5 mmole of glycerol-1,3-C14 (0.5 mc) were injected in the pudic artery supplying the udder during 8 minutes. In Experiment 3, an isolated cow's udder was perfused with blood containing 0.2 mmole of glycerol-1,3-C14 (0.5 mc) during 15 minutes. In Experiment 4, glycerol-1,3-C14 (0.5 mc) and glucose-2-C14 (0.25 mc) were perfused through the udder for 12 minutes. The esters were isolated from the udder as described in the text. In Experiment 2, the tissue was treated by the method of Dounce (15) to separate a fraction containing nuclei. The esters were then isolated from the “nuclei fraction” and “supernatant fraction” and from the total tissue.
Distribution of \(^{14}C\) in hexoses from nucleotides and phosphate esters isolated from mammary gland tissue after injection of glycerol-1,3-\(^{14}C\)

For comparison, values are given for glucose and galactose from lactose (12) from milk obtained from the injected side of the udder 1.6 hours after injection of glycerol-1,3-\(^{14}C\).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Total</th>
<th>Carbons of hexose</th>
<th>Recovery of (^{14}C) in degradation</th>
<th>Source</th>
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<tr>
<td></td>
<td>c.p.m./pmole</td>
<td>c.p.m./pmole C based on C-4 = 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>hexose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose of lactose</td>
<td>46.7</td>
<td>107.0</td>
<td>21.5</td>
<td>Reference (12)</td>
</tr>
<tr>
<td>Galactose of lactose</td>
<td>910.0</td>
<td>11.3</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>1,222</td>
<td>12.3</td>
<td>4.8</td>
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<tr>
<td>UDP-glucose</td>
<td>11,950</td>
<td>14.5</td>
<td>7.1</td>
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<tr>
<td>UDP-galactose</td>
<td>12,220</td>
<td>12.5</td>
<td>9.9</td>
<td></td>
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<tr>
<td>Glucose 1-P</td>
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<td>14.1</td>
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<td>Fructose-1,6-diphosphate</td>
<td>1,070</td>
<td>14.1</td>
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<td>Glucose-6-P</td>
<td>565</td>
<td>14.6</td>
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<tr>
<td>UDP-glucose</td>
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<tr>
<td>UDP-galactose</td>
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<td>4.0</td>
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</tbody>
</table>

* Average value of carbon atoms 1, 2, and 3.
* Calculated on the basis that all the \(^{14}C\) of carbon atoms 4 and 5 was in carbon 4.
* Average value of carbon atoms 2 and 3.
* Calculated that all the \(^{14}C\) of carbon atoms 5 and 6 was in carbon 6.

Distribution of \(^{14}C\) in hexoses from glucose-6-phosphate, nucleotides, and lactose isolated from mammary gland tissue after perfusion with glycerol-1,3-\(^{14}C\) and glucose-2-\(^{14}C\)

pool giving rise to galactose and not that giving rise to the glucose moiety. It appeared that this difficulty could be overcome by the simultaneous use of glycerol-1,3-\(^{14}C\) and glucose-2-\(^{14}C\).

Distribution of \(^{14}C\) from Glycerol-1,3-\(^{14}C\) and Glucose-2-\(^{14}C\) in Various Hexose—It was known from the investigations of Peeters that when glucose-2-\(^{14}C\) is perfused through the lactating mammary gland, the glucose of the milk lactose contains about twice as much \(^{14}C\) as the galactose and is labeled almost exclusively in C-2, whereas the galactose is labeled predominantly in C-2, but also in C-1 and C-3. Glycerol, on the other hand, labels the galactose predominantly in C-4 and C-6. Thus, if there were two pools of glucose-6-P, one arising via Reaction E (Fig. 1), which was a precursor of the glucose moiety and a second by Reaction D, which was a precursor of the galactose moiety, the glucose-6-P of the two pools should have different \(^{14}C\) patterns when both glycerol-1,3-\(^{14}C\) and glucose-2-\(^{14}C\) were substrates. In this experiment, the lactose was isolated from the tissue and the \(^{14}C\) patterns of the hexose moieties were determined. These moieties should depict the pattern of the two pools if such existed.

It was observed (Table IV) that the distribution of \(^{14}C\) in the glucose-6-P was not identical in this experiment with that of the UDP-glucose and UDP-galactose. C-2 of the glucose-6-P had a higher \(^{14}C\) concentration relative to C-4 than was found in the UDP hexoses (380 compared to 198 and 90). The distribution of \(^{14}C\) in UDP-hexoses was very similar to the galactose moiety of the lactose, but the glucose of the lactose differed from all the hexose esters and practically all of its \(^{14}C\) was in C-2. The hexose units of the lactose had a relatively low specific activity, but this was expected since the tissue contained milk that was formed before the injection of labeled compounds. Since there should be one pool of glucose-6-P labeled like the galactose and the glucose-6-P was labeled differently than either the glucose or galactose moieties of the lactose, it seems clear that there was more than one pool of glucose-6-P. Since the glucose moiety of lactose contained more \(^{14}C\) than the galactose moiety, the pool giving rise to the glucose moiety obviously should have had a higher specific activity than that giving rise to galactose. Thus, there must have been a pool of low activity glucose-6-P in the tissue which was not involved in lactose synthesis. The fact that the glucose-6-P had somewhat more \(^{14}C\) in C-2 relative to C-4 than did the galactose moiety, may be a consequence of the conversion of glucose-2-\(^{14}C\) to the pool of glucose-6-P with a lower turnover which is not involved in lactose synthesis. The almost exclusive labeling in C-2 of the glucose moiety of lactose when both glycerol-1,3-\(^{14}C\) and glucose-2-\(^{14}C\) are substrates is in accord with the view that free glucose is the precursor of the glucose moiety. Additional evidence favoring this mechanism has recently been obtained by Watkins and Hassid (31) with washed
particles obtained from homogenized mammary glands of guinea pigs. They have found that glucose-C\textsuperscript{14} is converted to lactose more rapidly than is glucose-C\textsuperscript{6}-P.

The simultaneous use of glucose-2-C\textsuperscript{14} and glycerol-1,3-C\textsuperscript{14} was of further interest because it offered the possibility of differentiating between the two types of exchange Reaction C and F which are indicated in Fig. 1. If the exchange was with fructose-6-P (Fig. 1, Reaction C), and glucose-2-C\textsuperscript{14} entered UDP-galactose via glucose-6-P through Reaction D (Fig. 1), then the ratio of C\textsuperscript{14} in C-2 and C-4 should be the same in the UDP-glucose and UDP-galactose. On the other hand, if the exchange was with UDP-galactose (Fig. 1, Reaction F), then the UDP-galactose might differ from the UDP-glucose. Table IV shows that the UDP-galactose had a higher concentration of C\textsuperscript{14} in C-4 than in C-2, whereas the situation was reversed in the UDP-glucose where C-2 had a greater activity than C-4. These results are suggestive that there may be exchange or synthesis of the different ratio may result from mixing of pools during the extraction from the tissue. However, the results are suggestive in view of the recent isolation of UDP-dihydroxyacetone by Smith et al. (14).

An important aspect of this work is the demonstration of the extreme difficulty of establishing mechanisms of metabolism either in vivo or in perfusion experiments. This has been true, even under conditions in which precursors were isolated from the tissue. The problem of study of pathways in vivo is a task which must be faced but it poses many obstacles.

SUMMARY

In two experiments, uridine nucleotide hexoses, glucose 6-phosphate, and in one case, glucose 1-phosphate and fructose-1,6-diphosphate were isolated from the tissue of the mammary gland of a cow immediately after injection of glycerol-1,3-C\textsuperscript{14} into the pudic artery and in a third experiment the compounds were isolated after glycerol-1,3-C\textsuperscript{14} was perfused through an isolated mammary gland. The specific activity of the glucose and galactose of the uridine diphosphate hexoses was 10-fold higher than that of the hexose phosphates. However, the pattern of labeling in the various carbons of all hexose derivatives isolated was the same. The uridine diphosphate galactose had a slightly greater specific activity than did uridine diphosphate glucose.

In a fourth experiment, a cow's mammary gland was perfused with glycerol-1,3-C\textsuperscript{14} and glucose-2-C\textsuperscript{14} and lactose was isolated in addition to the nucleotide hexoses and glucose 6-phosphate. The specific activity of glucose and galactose of the nucleotides was again 10-fold higher than that of glucose 6-phosphate and the uridine diphosphate galactose had a higher specific activity than uridine diphosphate glucose. The pattern of labeling of the galactose of lactose was similar to the uridine diphosphate hexoses and that of glucose of lactose was similar to that of the perfused glucose. The labeling pattern of glucose 6-phosphate indicated that it was a hybrid more closely resembling the uridine diphosphate hexoses than the injected glucose.

It is proposed that there are two or more pools of glucose 6-phosphate in the mammary gland and that one of these pools turns over slowly and thus does not acquire much C\textsuperscript{14} from the labeled substrates. This low activity pool apparently is not involved in lactose synthesis and may be a pool from a non-secretory tissue of the gland. It is proposed that uridine di-phosphate galactose is the galactosyl donor and that and free glucose, not glucose 1-phosphate, is the acceptor in the biogenesis of lactose by mammary gland. If glucose 1-phosphate were the precursor of the glucose moiety, it would be necessary to assume that it was present in a very small pool and thus did not significantly influence the C\textsuperscript{14} pattern of the combined pools of hexose phosphates of the tissue. The C\textsuperscript{14} pattern of the uridine diphosphate galactose has been found to differ somewhat from the uridine diphosphate glucose, and it is proposed that there may be a pathway by which C\textsuperscript{14} is introduced directly into uridine diphosphate galactose from triose phosphates.

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