Lipid-Linked Oligosaccharides containing Glucose in Lactating Rabbit Mammary Gland

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(Received 23 June 1978)

1. Microsomal fractions of lactating rabbit mammary gland incubated with UDP-glucose formed lipid-linked mono- and oligo-saccharides. The lipid-linked monosaccharide had chromatographic properties similar to those of dolichol phosphate mannose and yielded glucose on acid hydrolysis. 2. Incubation of the microsomal fraction with GDP-[U-14C]mannose yielded an oligosaccharide lipid of approximately seven monosaccharide units. Further incubation with UDP-glucose increased the size of the oligosaccharide by approximately two units. 3. Explants of lactating rabbit mammary gland incorporated [U-14C]glucose into both lipid-linked mono- and oligo-saccharides. The oligosaccharide lipid was of approx. 11 monosaccharide units. 4. Considerable redistribution of radioactive label occurred in the explant system, and radioactively labelled glucosamine and mannose, as well as glucose, were detected on acid hydrolysis of the oligosaccharide lipid. 5. Glucose was also detected in the acid hydrolysate of explant proteins. Radioactive glucosamine, galactosamine, galactose and mannose were also found in this fraction.

The role of dolichol-linked sugars in the formation of the inner-core oligosaccharides of glycoproteins containing an asparagine-linked oligosaccharide chain has been reviewed by Waechter & Lennarz (1976) and Hemming (1977). Although the first reports of dolichol phosphate glucose and a glucose-containing oligosaccharide lipid were made by Leloir’s group (Behrens & Leloir, 1970; Parodi et al., 1972; Behrens et al., 1973), much of the research in this area has concerned the synthesis of glucosamine- and mannose-containing lipid intermediates. However, M. J. Spiro et al. (1976a,b) and R. G. Spiro et al. (1976) reported the occurrence of glucose-containing oligosaccharide lipids when slices of bovine thyroid, kidney, thymus, liver and pancreas, and also hen oviduct were incubated with [14C]glucose. Herscovics et al. (1977a) have also detected glucose, in addition to mannose and glucosamine, by g.l.c. analysis of sugars released by acid hydrolysis of oligosaccharide lipids of calf pancreas microsomal fractions.

In a biosynthetic scheme proposed by Behrens (1974) it was suggested that two glucose units are added via dolichol phosphate glucose to the growing mannose- and glucosamine-containing oligosaccharide chain attached to dolichol before transfer of the completed oligosaccharide to protein. Lengthening of microsomal oligosaccharide chains by one or two glucose units has been demonstrated by incubating UDP-[U-14C]glucose with calf brain microsomal fractions (Scher et al., 1977) and a cell-free fibroblast preparation (Robbins et al., 1977).

The involvement of polyprenol-linked sugars as intermediates in glycoprotein synthesis in lactating rabbit mammary gland has been demonstrated in cell-free preparations (White, 1978) and explants (Speake & White, 1978). Estimation of the size of the oligosaccharide moiety released from the oligosaccharide lipids by treatment with mild acid suggested that the major microsomal oligosaccharide was approximately seven glucose units, whereas that of the explants was 10–12 units. The present paper describes the incorporation of glucose into lipid intermediates and proteins of explants of lactating rabbit mammary gland.

Materials and Methods

Animals

Primiparous lactating rabbits of the New Zealand White strain (albinos) were obtained from the Joint Animal Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. The animals were at least 6 months old at the time of mating. Explants were taken from mammary glands of rabbits in mid-lactation (10–15 days post partum).

Materials

Glucose-free Medium 199 (Morgan et al., 1950) was obtained from Wellcome Research Laboratories,
Beckenham BR3 3BS, Kent, U.K. Sheep prolactin (NIH-P-SII) (26.4±u./mg) was a gift from Dr. N. Bates, Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases, Bethesda, MD, U.S.A. Ox insulin was a gift from Mr. J. V. Birkinshaw, Boots Pure Drug Co., Nottingham, U.K., and an acid hydrolysate of amylose was kindly provided by Dr. J. Kennedy, Department of Chemistry, University of Birmingham, Birmingham, U.K. Bio-Rad Laboratories, Poole, Dorset, U.K. Bio-Gel P-6 was purchased from Bio-Rad Laboratories, St. Albans, Herts., U.K. All other chemicals and solvents were A.R. grade and obtained from commercial suppliers.

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin (fatty acid-poor) as a standard.

Preparation of mammary explants
Explants of lobuloalveolar tissue were prepared as described by Speake et al. (1975).

Preparation of microsomal fractions
Microsomal fractions were prepared from tissue homogenized in 250mm-potassium phosphate buffer, pH7.0, containing 5mm-2-mercaptoethanol and 1mm-EDTA as described by White (1978).

Chromatographic methods
Paper chromatography was performed on Whatman 3MM paper in the following solvent systems: A, ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.); B, ethyl acetate/pyridine/water (12:5:4, by vol.); C, isobutyric acid/water/aq. NH3 (sp.gr.0.88) (57:39:4, by vol.). T.l.c. was carried out on silica gel H in the following solvents: D, chloroform/methanol/formic acid/water (140:30:17:1, by vol.); E, chloroform/methanol/aq. NH3 (sp.gr. 0.88) (13:6:1, by vol.); F, chloroform/methanol/water (65:25:4, by vol.).

Treatments with acid
Mild and strong acid hydrolysates were carried out as described by Speake & White (1978).

Assay of the incorporation of [14C]glucose from UDP-[U-14C]glucose into endogenous acceptors of a lactating-rabbit mammary-gland microsomal fraction
The assay mixture contained UDP-[U-14C]glucose (1μM, 4×10^5d.p.m.), 10mm-MnCl2, 3mm-NaF, 1.8mm-MgATP, 2.5mm-EDTA, 10mm-Tris/maleate buffer, pH7.0, and microsomal protein (4mg) in a final volume of 100μl. The reaction was terminated by adding chloroform/methanol (2:1, v/v; 4ml) and lipid-linked mono- and oligo-saccharide and protein fractions were isolated as described by White & Waechter (1975).

Preparation of lipid-linked oligosaccharides from microsomal fractions
Six tubes containing the standard incubation mixture, except that 4μm-GDP-[U-14C]mannose (100000 d.p.m.) replaced UDP-[U-14C]glucose, were incubated for 30min at 37°C. Unlabelled UDP-glucose (final concentration 1μM) was then added to each tube and the mixture incubated for a further 10min. Lipid-linked oligosaccharides fractions were then isolated as described by White & Waechter (1975). In a separate experiment unlabelled GDP-mannose (4μM) was used in the first 30min incubation and UDP-[U-14C]glucose (1μM, 4×10^5d.p.m.) was added for the second incubation of 10min. Again, lipid-linked oligosaccharides fractions were isolated.

Measurement of the incorporation of α-[U-14C]glucose into endogenous acceptors of mammary explants
Groups of 20 explants were incubated for 2h at 37°C under O2/CO2 (19:1) in 1ml of Medium 199 (glucose-free) containing glycerol 10.86mm, insulin (5μg/ml), prolactin (1μg/ml) and cortisol (1μg/ml). The incubation medium also contained 20μCi of α-[U-14C]glucose (sp. radioactivity 292μCi/mmol). The explants were then washed with Medium 199 (3×1ml) and homogenized in chloroform/methanol (2:1, v/v; 3ml), and lipid-linked sugar and protein fractions were obtained as described above. The radioactivity in each fraction was determined as described by Speake & White (1978).

Comparison of chain length of oligosaccharides derived from explant and microsomal lipid-linked oligosaccharides
Oligosaccharides were prepared from lipid-linked oligosaccharides by mild acid hydrolysis and after evaporation of HCl were redissolved in 100mm-Tris/HCl buffer, pH8.5. They were then loaded on to a Bio-Gel P-6 column (80cm×1.5cm) prepared in the same buffer. The columns were eluted with loading buffer, and fractions (1ml) were collected. Xylene/ Triton-based scintillation fluid (10ml: White et al., 1971) was added to each fraction, which were then assayed for radioactivity.

An estimate of the size of the oligosaccharides was made by using the paper-chromatography system described by Lucas et al. (1975) and by Speake & White (1978), with an amylose hydrolysate as a standard.

Sephadex G-25 column chromatography
The residual protein left after lipid extractions was
heated at 90°C for 30 min in 10 mM-sodium phosphate buffer, pH 7.0 (1 ml), containing sodium dodecyl sulphate (7%, w/v). After centrifugation (11 000 g•min) to remove non-solubilized protein, the supernatant fraction was applied to a Sephadex G-25 column prepared in loading buffer. Elution was performed with the loading buffer and the eluate was monitored for protein by light absorption at 280 nm. Fractions (2.7 ml) were collected and 1 ml was assayed for radioactivity. Fractions containing protein were subjected to strong acid hydrolysis and the hydrolysate was analysed for component sugars by paper chromatography in solvent A.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the procedure of Weber & Osborn (1969) at an acrylamide concentration of 10% (w/v). Protein samples were heated at 100°C for 5 min with sodium dodecyl sulphate (final concn. 2%, w/v) and 2-mercaptoethanol (final concn. 2%, w/v). The samples (50-150 µg of protein) were cooled and applied to the gels. Protein standards were from the Dalton Mark VI TM kit [Sigma (London) Chemical Co., Poole, Dorset, U.K.]. This contained bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 43 000), pepsin (mol.wt. 34 700), trypsinogen (mol.wt. 24 000), β-lactoglobulin (mol.wt. 18 400) and lysozyme (mol.wt. 14 500).

Results

All experiments were carried out at least three times and those described are typical.

Incorporation of glucose from UDP-[U-14C]glucose into endogenous acceptors of mammary-gland microsomal fractions

There was a rapid incorporation of radioactive label into both mono- and oligosaccharide lipid fractions. Maximal incorporation into monosaccharide lipid occurred at 1 min, whereas the oligosaccharide lipid was maximally labelled at between 5 and 10 min (Fig. 1). Incorporation of radioactive label into protein was approximately linear for 15 min, but continued over the 2 h incubation period.

Analysis of chloroform/methanol (2:1, v/v)-soluble fractions

(i) From microsomal incubations. The chloroform/methanol (2:1, v/v) fraction bound to a silicic acid column in chloroform and was eluted with chloroform/methanol (2:1, v/v). It had similar Rf values to mannosylphosphodolichol on t.l.c. in acidic, basic and neutral solvents (D, E and F). On mild acid hydrolysis it released all the radioactivity in a single product that corresponded to glucose on paper chromatography in solvent B (Fig. 2a).

(ii) From explant incubations. Chromatography of the fraction soluble in chloroform/methanol (2:1, v/v) on a column of silicic acid indicated that approx. 8% of the radioactivity incorporated into this fraction was present as polar lipid. Approx. 20% of the radioactivity incorporated into the polar lipid fraction was present in products that were stable to mild alkaline hydrolysis. T.l.c. in solvent D of mild alkali-stable polar lipid indicated the presence of two radioactive compounds, one of which co-chromatographed with mannosylphosphodolichol. The second compound is probably a glycosphingolipid.

Mild acid hydrolysis of the polar lipid fraction liberated approx. 6% of the radioactivity in this
fraction into the aqueous phase. Paper chromatography in solvent A indicated that approx. 30% of the radioactivity released from the polar lipid fraction by mild acid hydrolysis co-migrated with glucose, and approx. 70% with glucosamine (Fig. 2b). A minor peak corresponding to the $R_F$ of mannose was also seen.

Properties of the oligosaccharides isolated from lipid-linked oligosaccharides

Oligosaccharides released by mild acid hydrolysis of the lipid-linked oligosaccharides isolated from explants and microsomal incubations were chromatographed on Bio-Gel P-6 (Fig. 3). The major oligosaccharide derived from hydrolysis of the oligosaccharide lipids synthesized by microsomal fractions incubated with GDP-[U-$^{14}$C]mannose eluted in fractions 101–111. Addition of UDP-glucose to a microsomal system that had been incubated for 30 min with GDP-[U-$^{14}$C]mannose increased the oligosaccharide chain length such that it was eluted in fractions 94–99 (Fig. 3a). However, the lengthened oligosaccharide was still shorter than that isolated from explants, which was eluted in fractions 79–84 (Fig. 3b). In an attempt to quantify the size of the oligosaccharides, they were chromatographed on paper in solvent C with an amylose hydrolysate as reference (Fig. 4). The oligosaccharide derived from GDP-mannose-incubated microsomal fraction corresponded to 6–7 glucose units (Fig. 4b), and further incubation of a microsomal fraction preincubated with unlabelled GDP-mannose for a further 15 min in the presence of UDP-[U-$^{14}$C]glucose increased the oligosaccharide chain length by one or two glucose units (Fig. 4c). However, as with the Bio-Gel P-6 column, this was still shorter than the oligosaccharide isolated from explant lipid-linked oligosaccharide (10–12 units, Fig. 4a). Lipid-linked oligosaccharides that had been labelled with [U-$^{14}$C]glucose in the
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Fig. 4. Paper chromatography of the oligosaccharides released from chloroform/methanol/water (10:10:3, by vol.) fractions synthesized by explants incubated with D-[U-14C]glucose (a), microsomal fractions incubated with GDP-[U-14C]mannose (b) and microsomal fractions incubated with UDP-[U-14C]glucose after preincubation with GDP-mannose (4 μM) (c).

The oligosaccharides were chromatographed on Whatman 3MM paper in solvent C for 48h with glucose oligomers of known chain length as standards. The chromatogram was cut in segments 1 cm in length and the radioactivity in each segment was determined by liquid-scintillation counting. The bars and corresponding numbers represent the position of migration of standard glucose oligomers, i.e. 1 represents the position of (Glc)1; 2 represents (Glc)2, etc.

explant system were subjected to strong acid hydrolysis. Paper chromatography in solvent A of the water-soluble products of acid hydrolysis is shown in Fig. 5. A peak of radioactivity is seen in glucose, but considerable redistribution of label is seen with other peaks corresponding to mannose and glucosamine and/or galactosamine. However, it would appear that glucose is present in the oligosaccharide chain. Glucose was the sole radioactive product obtained on strong acid hydrolysis of the microsomal lipid-linked oligosaccharides (results not shown).

Analysis of proteins

The residual protein fractions obtained after extracting explants for mono- and oligo-saccharide lipids were solubilized in sodium phosphate buffer, pH 7.0, containing sodium dodecyl sulphate (7%)

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Fig. 5. Paper chromatography of the products of strong acid hydrolysis of the chloroform/methanol/water (10:10:3, by vol.) fractions of explants incubated with D-[U-14C]glucose. Chromatography was carried out in solvent system A for 20h. The chromatogram was cut into segments 1 cm in length and the radioactivity in each segment was determined. The positions of migration of glucosamine, galactosamine, mannose and glucose are shown.

Fig. 6. Distribution of radioactivity after polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of explant protein labelled with D-[U-14C]glucose. The protein fractions of explants from mammary glands of lactating rabbits after incubation with D-[U-14C]glucose were solubilized in sodium dodecyl sulphate/2-mercaptoethanol and applied to a Sephadex G-25 column (25 cm × 3 cm). Samples of the protein eluted in the void volume were then analysed by polyacrylamide-gel electrophoresis. The gel was cut into slices 0.25 cm in length and the radioactivity in each slice was determined. The mobilities of tracking dye (T.D.) and protein standards (mol.wt. × 10^{-2}) are indicated by arrows.
hydrolysis of explant protein labelled from [U-14C]glucose in mammary gland microsomal preparations. The hydrolysates were applied to a column (25 cm x 3 cm) of Sephadex G-25 in the same solvent. All of the radioactivity applied to the column eluted with protein in the void volume. Analysis of this eluted protein by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate is shown in Fig. 6. Much radioactivity stayed at the top of the gel. The major labelled protein corresponds to casein. Other peaks occur at mol. wts. of approx. 20000, 70000 and 90000. The total protein was subjected to strong acid hydrolysis and the products were analysed for sugars by paper chromatography in solvent A (Fig. 7). [U-14C]Glucose was co-chromatographed as a standard. Besides the peak corresponding to glucose other peaks that corresponded to mannose, galactose and galactosamine and/or glucosamine were present.

Discussion

The results presented indicate that lactating rabbit mammary tissue is able to incorporate glucose into lipid-linked mono- and oligosaccharides and also protein in both microsomal and explant systems. Synthesis of the lipid-linked monosaccharide in the microsomal system was stimulated by the addition of dolichol phosphate (results not shown) and was chromatographically similar to dolichol phosphate mannose. A precursor–product relationship appeared to exist between the lipid-linked sugars in the cell-free system. In explant preparations, however, little (less than 0.5%) of the radioactivity in the chloroform/methanol (2:1, v/v) extract was present as polypreynl phosphate glucose. Most of the radioactivity in this fraction appeared in neutral lipid, presumably owing to metabolism of glucose to acetyl-CoA and subsequent fatty acid synthesis. Much redistribution of radiolabel into other sugars, glucosamine, galactosamine and mannose also occurred in the explant (Figs. 2b, 5 and 7). Despite this extensive redistribution of label, analysis of the sugars obtained by acid hydrolysis of explant oligosaccharide lipid and protein by paper chromatography indicated that glucose was a component of both these fractions. This is in agreement with the results of R. G. Spiro et al. (1976), who detected glucose in oligosaccharide lipids and proteins of slices of bovine thyroid that had been incubated with [U-14C]glucose.

The first reports of glucose-linked lipids in mamalian systems were by Leloir’s group, who demonstrated dolichol phosphate glucose and a glucose-containing dolichol phosphate oligosaccharide of approx. 20 monosaccharide units, which was able to donate the glucosyl oligosaccharide to endogenous protein in rat liver (Behrens & Leloir, 1970; Behrens et al., 1973; Parodi et al., 1972).

Herscovics et al. (1977b) described the formation of dolichophosphate glucose and a glucosyl oligosaccharide lipid in calf pancreas microsomal fraction and showed that the glucose on the oligosaccharide was α-linked at the non-reducing terminus, since it was labile to α-glucosidase. Transfer of [14C]glucose-labelled oligosaccharide from an oligosaccharide lipid to endogenous proteins has also been shown in rabbit reticulocyte membranes by Parodi & Martin-Barrientos (1977), and Parodi (1977) described the transfer of an oligosaccharide containing N-acetylglucosamine, mannose and glucose to endogenous proteins of baker’s yeast.

Previous work in this laboratory (Speake & White, 1978; White, 1978) has shown that the oligosaccharide lipid synthesized from microsomal fractions (7 units) is shorter than that synthesized in mammary explants (10–12 units). Addition of UDP-glucose to a mammary-gland microsomal system synthesizing mannose-containing oligosaccharide lipid increased the size of the oligosaccharide by approximately two monosaccharide units, as measured by paper chromatography (Fig. 4) and gel filtration of the oligosaccharide released on mild acid hydrolysis of the oligosaccharide lipid (Fig. 3). However, this oligosaccharide is still smaller than that released on mild acid hydrolysis of the explant oligosaccharide lipid. It would appear that glucose is a component of the oligosaccharide, but that further sugar residues are added to complete the chain. We have not yet been able to increase further the size of the microsomal oligosaccharide lipid by subsequent addition of nucleotide sugars to the incubation medium. Thus the microsomal system synthesizes an oligosaccharide of approximately seven monosaccharide units when incubated with GDP-mannose, and addition of UDP-glucose increases this to approximately nine monosaccharide units, but this is
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still smaller than the explant oligosaccharide labelled from [U-\(^{14}\)C]-glucose, which is approx. 11 monosaccharide units. Similar elongations of the carbohydrate chains of oligosaccharide lipids have been shown in cell-free fibroblast preparations (Robbins et al., 1977) and calf brain white-matter microsomal fractions (Scher et al., 1977). In both these systems addition of UDP-glucose to preparations synthesizing mannose- and N-acetylglucosamine-containing oligosaccharide lipids increased the size of the oligosaccharide by approximately two monosaccharide units such that the proportions of sugars in the oligosaccharide were glucosamine/mannose/glucose, 2:4:8:2.

Few observations have been made on whole-cell oligosaccharide lipids labelled from glucose. M. J. Spiro et al. (1976a,b) showed that slices of bovine thyroid, kidney, thymus and liver and also in oviduct incorporated glucose into oligosaccharide lipids of 14–15 monosaccharide units consisting of N-acetylglucosamine, mannos and glucose in the proportions 2:11:1–2, whereas pancreas slices synthesized a glucose-free oligosaccharide lipid of about ten units. Analysis of the carbohydrate composition of the endogenous oligosaccharide lipids of calf pancreas microsomal fraction by g.l.c. and reduction with Na\(\text{B}_3\)H\(_4\) gave N-acetyl-d-glucosamine/d-mannose/d-glucose proportions in the range 1:2.5:0.5 to 1:5:1.5, with N-acetylglucosamine at the reducing end (Herscovics et al., 1977a). The mammary explant oligosaccharide lipid labelled from glucose in the present study is the same size as that labelled from [\(^2\)-\(^3\)H]mannose and N-acetyl[\(^{14}\)C]glucosamine (Speake & White, 1978) and suggests that all three sugars are part of the same oligosaccharide chain.

It is thought that the dolichol phosphate-linked sugar pathway is utilized in the formation of the core oligosaccharides of glycoproteins containing asparagine-linked oligosaccharides (for review see Waechter & Lennarz, 1976). The finding of glucose in oligosaccharide lipids is unexpected, since this sugar is not commonly found in the core regions of such glycoproteins (Kornfeld & Kornfeld, 1976). Tabas et al. (1978) have suggested that synthesis of vesicular-stomatitis-virus G protein in Chinese-hamster ovary cell involves participation of an oligosaccharide lipid that has an oligosaccharide mol.wt. estimated at 2080 on Bio-Gel P-6 and contains glucose. However, once the oligosaccharide had been transferred to the peptide, processing of the oligosaccharide occurred involving the loss of monosaccharide residues from the non-reducing terminal to form a core unit. This core unit was subsequently glycosylated to complete the oligosaccharide chain found in glycoprotein. It is not known whether such a process occurs in the synthesis of mammalian glycoproteins containing asparagine-linked oligosaccharides, but M. J. Spiro et al. (1976a) have stated that if glycosylation of thyroglobulin occurs via the lipid-linked sugar pathway considerable modification of the oligosaccharide by mannosidase action would have to take place to convert the oligosaccharide transferred from lipid into either the A or B carbohydrate units of the completed protein. The profile of radioactively labelled protein on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 6) is similar to that obtained from [\(^2\)-\(^3\)H]mannose- and N-acetyl[\(^{14}\)C]glucosamine-labelled tissue (Speake & White, 1978), and despite considerable redistribution of label (Fig. 7) glucose does appear to be a component of some of the glycoproteins of the mammary gland, although this glucose may not have been added via the lipid-linked sugar pathway.

We thank the Medical Research Council for financial support of this project and for a research fellowship to B. K. S., and Miss Susan Hudspeth for excellent technical assistance.

References


