The Formation of Lipid-Linked Sugars as Intermediates in Glycoprotein Synthesis in Rabbit Mammary Gland

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1. The incorporation of D-[1-¹⁴C]mannose, D-[2-³H]mannose and *N*-acetyl-D-[1-¹⁴C]-glucosamine into glycoproteins and lipid-linked intermediates of mammary explants obtained from lactating rabbits was studied. The amount of radioactivity incorporated into lipid-linked intermediates was very low compared with the incorporation into protein. Most of the radioactivity incorporated into the chloroform/methanol-soluble fraction was present as neutral lipid. Radioactivity from D-[2-³H]mannose was incorporated mainly into the fatty acid moiety, whereas radioactivity from D-[1-¹⁴C]mannose and *N*-acetyl-D-[1-¹⁴C]glucosamine was present in the glycerol moiety of triacylglycerol. 2. The labelled lipid-linked intermediate that was soluble in chloroform/methanol/water (10:10:3, by vol.) was partially characterized and was found to exhibit properties characteristic of an oligosaccharide linked to lipid via a pyrophosphate bridge. It migrated largely as a single zone of radioactivity on t.l.c. and was eluted from a column of DEAE-cellulose acetate as a single peak by 50 mM-ammonium acetate. 3. The oligosaccharide moiety was released from the lipid by mild acid hydrolysis. The size of the oligosaccharide was estimated by paper chromatography to be 10 or 11 monosaccharide units. 4. D-[1-¹⁴C]Mannose was incorporated largely into glycopeptides with molecular weights in the range 40000–80000, as determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Label from *N*-acetyl-D-[1-¹⁴C]glucosamine was incorporated into a glycopeptide with an electrophoretic mobility identical with that of rabbit casein (mol.wt. 32000) as well as into glycopeptides of higher molecular weight. 5. Approx. 50% of the total radioactivity in the protein labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine was present as galactosamine, a component of the carbohydrate portion of rabbit casein. No labelled galactosamine was present in the lipid-linked oligosaccharide labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine. It thus appears that the lipid-linked oligosaccharide is not involved in the glycosylation of casein.

Work by a number of investigators has indicated that polyprenols function as glycosyl carriers in the synthesis of the carbohydrate moiety of certain glycoproteins, and this has been comprehensively reviewed by Waechter & Lennarz (1976) and Hemming (1974, 1977). In particular, the inner 'core' region of a large class of glycoproteins is thought to be assembled initially as a dolichol-linked oligosaccharide. This oligosaccharide is then transferred to protein.

Mannose and *N*-acetylglucosamine have been identified as major components of the oligosaccharides isolated from a number of tissues, e.g. rat liver (Behrens *et al.*, 1973), rat myeloma (Hsu *et al.*, 1974), hen oviduct (Lucas *et al.*, 1975), bovine aorta (Forsee & Elbein, 1975), pig liver (Oliver & Hemming, 1975) and *Saccharomyces cerevisiae* (Lehle & Tanner, 1975), although glucose (Behrens *et al.*, 1971; Parodi *et al.*, 1973; M. J. Spiro *et al.*, 1976*a,b*) and xylose (Waechter *et al.*, 1974) have also been shown to be present in some oligosaccharide-lipids.

Much of the evidence for polyprenol-linked intermediates in glycoprotein synthesis has been obtained by using microsomal fractions prepared from the particular tissue under investigation. Both dolichol phosphate (Dallner *et al.*, 1972) and associated glycosyl transferases (Adamany & Spiro, 1975; Heifetz & Elbein, 1977) appear to be located in this fraction. Relatively few studies have been made on undisturbed tissue. Struck & Lennarz (1976) have reported a lipid-linked glycosyl-carrier system in isolated hen oviduct cells, and R. G. Spiro *et al.* (1976) and M. J. Spiro *et al.* (1976*a,b*) have isolated oligosaccharide-lipids from slices of calf kidney, pancreas, thymus, liver and thyroid, and also from hen oviduct after incubation with various radioactively labelled substrates. Pulse-chase experiments suggested that, in the thyroid, the oligosaccharide-lipid donated oligosaccharide to protein.

The present paper describes the incorporation of labelled sugars into protein and into lipid-linked oligosaccharide intermediates by explants of lactating

rabbit mammary tissue. Some properties of the lipid-linked oligosaccharide and of the glycosylated proteins are presented.

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. The experiments described used explants from mammary glands obtained from 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 i.u./mg) was a gift from the Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases, Bethesda, MD, U.S.A. Ox insulin was a gift from Mr. V. J. Birkinshaw, Boots Pure Drug Co., Nottingham, U.K., and an acid hydrolysate of amylose was kindly provided by Dr. John Kennedy, Department of Chemistry, University of Birmingham, Birmingham, U.K. D-[1-¹⁴C]Mannose, N-acetyl-D-[1-¹⁴C]glucosamine and D-[2-³H]mannose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cortisol 21-acetate was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. DEAE-cellulose (DE-52) was purchased from H. Reeve Angel and Co., London E.C.4, U.K. T.l.c. plates precoated with Avicel F were obtained from Anachem, Luton, Beds., U.K. Rabbit casein was a gift from Miss K. Al-Sarraj of this Department. All other chemicals and solvents were A.R. grade and obtained from commercial suppliers.

Preparation of mammary explants

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

Measurement of the incorporation of D-[1-¹⁴C]mannose, N-acetyl-D-[1-¹⁴C]glucosamine and D-[2-³H]mannose into endogenous acceptors of mammary explants

Duplicate groups of 20 explants were incubated for various periods of time at 37°C under O₂/CO₂ (19:1) in 1 ml of Medium 199 (glucose-free) containing glycerol (1 mg/ml), insulin (5 µg/ml), prolactin (1 µg/ml) and cortisol (1 µg/ml). The incubation medium also contained 1.0 µCi of D-[1-¹⁴C]mannose

(sp. radioactivity 58 mCi/mmol), 1.0 µCi of N-acetyl-D-[1-¹⁴C]glucosamine (sp. radioactivity 52 mCi/mmol) or 10 µCi of D-[2-³H]mannose (sp. radioactivity 2 Ci/mmol). Only a single radioactive substrate was added to each incubation vessel. For characterization purposes, a highly labelled product was prepared by including larger amounts of radioactivity in the incubation: 75 µCi of [¹⁴C]mannose, 75 µCi of N-acetyl[¹⁴C]glucosamine or 125 µCi of [³H]mannose.

At the end of the incubation period, each group of explants was washed with Medium 199 and homogenized in chloroform/methanol (2:1, v/v; 3 ml). Three fractions were obtained as described by Waechter *et al.* (1973), i.e. (i) soluble in chloroform/methanol (2:1, v/v), (ii) soluble in chloroform/methanol/water (10:10:3, by vol.) and (iii) an insoluble protein pellet.

The radioactivity incorporated into dolichol-linked monosaccharide was routinely determined as the radioactivity released into an aqueous phase by mild acid hydrolysis of the fraction soluble in chloroform/methanol (2:1, v/v).

For measurement of the radioactivity incorporated into dolichol-linked oligosaccharide, the fraction soluble in chloroform/methanol/water (10:10:3, by vol.) was chromatographed on a strip of Whatman 3MM paper (2 cm × 10 cm) with water as the solvent. The radioactivity remaining at the origin was eluted from the paper with chloroform/methanol/water (10:10:3, by vol.; 5 ml) and determined by liquid-scintillation counting as described by White & Waechter (1975).

The lipid-free protein pellet was dissolved in 0.3 ml of 90% (v/v) formic acid and the radioactivity was determined (Speake *et al.*, 1976).

In some incubations the reaction was terminated by homogenizing the explants in 1 ml of 0.25 M-sodium phosphate buffer, pH 7.0. The homogenate protein was precipitated with trichloroacetic acid (final concn. 5%, w/v). The precipitate was washed five times by resuspension in 1 ml of 0.25 M-sodium phosphate buffer, pH 7.0, followed by reprecipitation with trichloroacetic acid (final concn. 5%, w/v). The precipitate was washed finally with 2 ml of 95% (v/v) ethanol, dissolved in 0.3 ml of 90% (v/v) formic acid and the radioactivity was determined by liquid-scintillation counting (Speake *et al.*, 1976).

The values obtained for the incorporation of radioactivity into explant protein, measured in the lipid-free insoluble pellet and in the pellet that has been precipitated with trichloroacetic acid, were essentially identical.

Chromatographic methods

Descending paper chromatography was carried out on Whatman 3MM paper in the following solvent

systems: A, propan-2-ol/acetic acid/water (3:1:1, by vol.); B, ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.); C, isobutyric acid/water/aq. NH_3 (sp.gr. 0.880) (57:39:4, by vol.).

Ascending paper chromatography was performed on sodium tetraborate-treated Whatman 3MM paper in solvent D, butan-1-ol/pyridine/water (4:6:3, by vol.).

T.l.c. was carried out on silica gel H in the following solvent systems: E, chloroform/methanol/acetic acid/water (25:15:4:2, by vol.); F, hexane/ethyl ether/acetic acid (80:20:1, by vol.).

T.l.c. on Avicel was performed in solvent system C.

Alkaline AgNO_3 (Trevelyan *et al.*, 1959) was used for the detection of sugars. Radioactive zones were detected by liquid-scintillation counting of 1 cm strips of the chromatogram (White & Waechter, 1975).

Chromatography of the fraction soluble in chloroform/methanol (2:1, v/v) was carried out on columns (15 cm $\times$ 1 cm) of silicic acid. Eluents were chloroform (75 ml), acetone (50 ml), chloroform/methanol (1:2, v/v; 50 ml) and methanol (50 ml).

Chromatography of the dolichol-linked oligosaccharide was carried out on a column (15 cm $\times$ 1 cm) of DEAE-cellulose acetate with chloroform/methanol/water (10:10:3, by vol.) as the solvent. The column was eluted sequentially with 50 ml of chloroform/methanol/water (10:10:3, by vol.), 50 ml of 5 mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.) and 50 ml of 5 mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.). Fractions (2 ml) were collected.

The oligosaccharide obtained after mild acid hydrolysis of the dolichol-linked oligosaccharide was chromatographed on a column (15 cm $\times$ 1 cm) of of Sephadex G-25 in 0.1 M-sodium phosphate buffer, pH 6.4. Fractions (1 ml) were collected.

Chromatography of protein fractions was performed on a column (20 cm $\times$ 2.5 cm) of Sephadex G-25 in 10 mM-sodium phosphate buffer, pH 6.4, containing sodium dodecyl sulphate (7%, w/v). Protein samples were boiled for 5 min in 1 ml of 10 mM-sodium phosphate buffer, pH 6.4, containing sodium dodecyl sulphate (7%, w/v), before chromatography. Fractions (2 ml) were collected.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Weber & Osborn (1969). The acrylamide concentration was 10% (w/v). Protein samples were boiled for 5 min with sodium dodecyl sulphate (final concn. 2%, w/v) and 2-mercaptoethanol (final concn. 2%, w/v). The samples were allowed to cool and applied to the polyacrylamide gels. Between 50 and 150 μg of protein was applied to each gel. Protein was determined by the method of Lowry

et al. (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was also carried out with several proteins of known subunit molecular weight. The proteins used were ovalbumin (43000), bovine serum albumin (68000), rabbit casein (33000) and lysozyme (14500).

Treatments with acid

Mild acid hydrolysis of dolichol-linked monosaccharides and of dolichol-linked oligosaccharides was carried out as described by White & Waechter (1975). The sample was incubated in 0.1 M-HCl in aq. 50% (v/v) propan-1-ol (1 ml) at 50°C for 30 min. The resulting hydrolysate was dried under N_2 to remove HCl.

Strong acid hydrolysis of oligosaccharide and protein was performed in 4 M-HCl (2 ml) at 100°C for 2–4 h. The hydrolysate was dried under N_2 .

Treatment with alkali

Mild alkaline hydrolysis of the fraction soluble in chloroform/methanol (2:1, v/v) was carried out as described by White & Waechter (1975).

Acetylation

N-Acetylation of hexosamines derived from strong acid hydrolyses of oligosaccharides and protein was carried out in acetic anhydride/methanol (1:5, v/v; 0.6 ml) as described by Clamp *et al.* (1972). After 6 h at room temperature (20°C) the solution was evaporated to dryness under N_2 . Methanol (1 ml) was then added and the evaporation repeated. The addition of methanol and evaporation were repeated a further five times. The residue was dissolved in water (100 μl) before paper chromatography in solvent D.

Results

Incorporation of radioactivity from D-[2- ^3H]mannose and N-acetyl-D-[1- ^{14}C]glucosamine into lipid and protein fractions

The time-course of incorporation of D-[2- ^3H]mannose and N-acetyl-D-[1- ^{14}C]glucosamine into endogenous acceptors of mammary explants is shown in Fig. 1. There was rapid incorporation of both precursors into protein. This incorporation was linear with time during the 3 h time course.

The incorporation of radioactivity into the dolichol-linked oligosaccharide fraction was very low compared with the incorporation of label into protein, the amount of radioactivity present as dolichol-linked oligosaccharide after a 3 h incubation

Fig. 1. Variation with time of the incorporation of (a) $D-[2\text{-}^3H]mannose$ and (b) $N\text{-acetyl-}D\text{-}[1\text{-}^{14}C]glucosamine$ into protein (○) and dolichol-linked oligosaccharide (●) fractions of mammary explants

Duplicate groups of 20 explants were incubated for the times indicated at $37^\circ C$ under O_2/CO_2 (19:1) in 1 ml of Medium 199 (glucose-free) containing either $10 \mu Ci$ of $D-[2\text{-}^3H]mannose$ or $1.0 \mu Ci$ of $N\text{-acetyl-}D\text{-}[1\text{-}^{14}C]glucosamine$. At the end of the incubation period, each group of explants was homogenized in chloroform/methanol (2:1, v/v; 3 ml). Dolichol-linked monosaccharide, dolichol-linked oligosaccharide and protein fractions were prepared as described in the Materials and Methods section, and the radioactivity in each fraction was determined. The incorporation of radioactivity into dolichol-linked monosaccharides was very low and is not shown.

being approx. 5% of that present in protein. The incorporation of $D-[2\text{-}^3H]mannose$ into dolichol-linked oligosaccharide was linear with time for about 30 min and then reached a plateau. In contrast, the incorporation of $N\text{-acetyl-}D\text{-}[1\text{-}^{14}C]glucosamine$ into the dolichol-linked oligosaccharide did not reach a maximum until after about 2 h of incubation.

Only a trace amount of radioactivity was incorporated into the dolichol-linked monosaccharide

fraction. The amount of radioactivity present as dolichol-linked monosaccharide after a 3 h incubation was approx. 0.25% of that present in protein.

Characterization of the fraction soluble in chloroform/methanol (2:1, v/v)

Considerable incorporation of radioactivity into the fraction soluble in chloroform/methanol (2:1, v/v) did, however, take place. The extent of this incorporation ranged from 5 to 20% of the incorporation into protein. Chromatography of this fraction on a column of silicic acid indicated that 80–90% of this radioactivity was incorporated into the neutral-lipid fraction. T.l.c. of the neutral-lipid fraction in solvent F before and after mild alkaline hydrolysis revealed that label from $D-[2\text{-}^3H]mannose$ was incorporated mainly into the fatty acid portion of triacylglycerols, whereas radioactivity derived from $D-[1\text{-}^{14}C]mannose$ and $N\text{-acetyl-}D\text{-}[1\text{-}^{14}C]glucosamine$ was present mainly in the glycerol moiety of triacylglycerols.

Mild acid hydrolysis of the polar-lipid fraction labelled from $D-[2\text{-}^3H]mannose$ liberated about 60% of the radioactivity present in the fraction into an aqueous phase. T.l.c., in solvent E, of that portion of the polar-lipid fraction that was stable to mild alkaline hydrolysis indicated the presence of a single radioactive compound that co-chromatographed with authentic mannosylphosphoryldolichol prepared from a rat parotid microsomal fraction. Mild acid hydrolysis of the polar-lipid fractions labelled from either $D-[1\text{-}^{14}C]mannose$ or $N\text{-acetyl-}D\text{-}[1\text{-}^{14}C]glucosamine$ liberated approx. 20% of the radioactivity present in the fractions into an aqueous phase (Table 1). Thus a greater proportion of the total radioactivity present in the fraction soluble in chloroform/methanol (2:1, v/v) was found to exhibit properties characteristic of a dolichol-linked monosaccharide when $D-[2\text{-}^3H]mannose$, as opposed to ^{14}C -labelled sugars, was used as substrate.

Properties of the lipid-linked oligosaccharide

Analysis by t.l.c. of the fraction soluble in chloroform/methanol/water (10:10:3, by vol.) extracted from 2 h incubations with either $D-[1\text{-}^{14}C]mannose$ or $N\text{-acetyl-}D\text{-}[1\text{-}^{14}C]glucosamine$ as substrate was performed on Avicel in solvent system C, as described by Chambers & Elbein (1975). Approx. 75% of the applied radioactivity migrated as a single peak of R_F 0.78, as depicted in Fig. 2. A minor radioactive peak that migrated with R_F 0.57 was also observed. Approx. 5% of the applied radioactivity migrated to the solvent front, as did mannosylphosphoryldolichol. There was essentially no chromatic difference between the fraction soluble in chloroform/methanol/water (10:10:3, by vol.)

Table 1. *Distribution of radioactivity in the fraction soluble in chloroform/methanol (2:1, v/v)*

Groups of 20 mammary explants were incubated with D-[1-¹⁴C]mannose (75 μ Ci), N-acetyl-D-[1-¹⁴C]glucosamine (75 μ Ci) or D-[2-³H]mannose (125 μ Ci) for 2 h at 37°C in 1 ml of glucose-free Medium 199 under O₂/CO₂ (19:1). The fraction soluble in chloroform/methanol (2:1, v/v) was prepared as described in the Materials and Methods section. Neutral-lipid and polar-lipid fractions were isolated by chromatography of the fraction soluble in chloroform/methanol (2:1, v/v) on a column of silicic acid as described in the Materials and Methods section. Mild acid hydrolysis of the polar-lipid fraction was performed in 0.1 M-HCl in aq. 50% (v/v) propan-1-ol (1 ml) at 50°C for 30 min. The resulting hydrolysate was dried under N₂. Chloroform/methanol (2:1, v/v; 4 ml) and water (1 ml) were added to the sample, and the radioactivity in the resultant aqueous phase was determined.

Radioactive precursor	Total radioactivity in the fraction soluble in chloroform/methanol (d.p.m.)	Percentage distribution of radioactivity		
		Neutral lipid	Mild-acid-stable polar lipid	Mild-acid-labile polar lipid
D-[2- ³ H]Mannose	64 $\times 10^4$	81	8	11
D-[1- ¹⁴ C]Mannose	26 $\times 10^3$	90	8	2
N-Acetyl-D-[1- ¹⁴ C]glucosamine	19 $\times 10^3$	86	12	2

Fig. 2. *T.L.C. on Avicel of the fraction soluble in chloroform/methanol/water (10:10:3, by vol.) extracted from explants after incubation with D-[1-¹⁴C]mannose (●) and N-acetyl-D-[1-¹⁴C]glucosamine (○)*

The fraction soluble in chloroform/methanol/water (10:10:3, by vol.) was prepared as described in the Materials and Methods section and was purified by paper chromatography with water as the solvent before t.l.c. on Avicel in solvent system C. The chromatogram was cut into segments 1 cm in length and the radioactivity in each segment was determined. The arrow indicates the position of mannosylphosphoryldolichol.

labelled from D-[1-¹⁴C]mannose and that labelled from N-acetyl-D-[1-¹⁴C]glucosamine, suggesting that mannose and N-acetylglucosamine are largely incorporated into a single lipid-linked oligosaccharide.

The lipid-linked oligosaccharide fraction labelled from D-[1-¹⁴C]mannose was applied to a column of

DEAE-cellulose acetate. Less than 7% of the total radioactivity applied to the column was eluted with chloroform/methanol/water (10:10:3, by vol.). A further 13% of the radioactivity was eluted with 5 mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.). However, elution with 50 mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.) removed over 80% of the total radioactivity in a single peak (Fig. 3). In contrast, mannosylphosphoryldolichol was eluted from the column by chloroform/methanol/water (10:10:3, by vol.), indicating that the dolichol-linked oligosaccharide has a higher negative charge.

Mild acid hydrolysis of the lipid-linked oligosaccharide led to the release of the radioactivity into an aqueous phase. The kinetics of the hydrolysis indicate that the linkage between the oligosaccharide and the lipid is cleaved with a half-life of about 12 min (results not shown).

Properties of the oligosaccharide isolated from the lipid-linked oligosaccharide

Oligosaccharides labelled from D-[1-¹⁴C]mannose, N-acetyl-D-[1-¹⁴C]glucosamine and D-[2-³H]mannose were isolated after mild acid hydrolysis of the respective lipid-linked oligosaccharides.

When the oligosaccharides were chromatographed on a column of Sephadex G-25 in 0.1 M-sodium phosphate buffer, pH 6.4, all the radioactivity was eluted in the void volume.

The size of the oligosaccharides labelled from D-[1-¹⁴C]mannose and N-acetyl-D-[1-¹⁴C]glucosamine was determined by paper chromatography in solvent C. Glucose oligomers, prepared by partial acid hydrolysis of amylose, were included as standards. The number of monosaccharide units in the oligosaccharide was determined from a plot of the log (distance migrated) against the number of glucose residues in the standards (Lucas *et al.*, 1975). The

Fig. 3. DEAE-cellulose acetate chromatography of the fraction soluble in chloroform/methanol/water (10:10:3, by vol.) obtained after incubation of mammary explants with D-[1- 14 C]mannose

The column was eluted sequentially with 50ml of chloroform/methanol/water (10:10:3, by vol.), 50ml of 5mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.) and 50ml of 50mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.). The point of addition of each successive eluent is indicated by the arrows. Fractions (2ml) were collected.

results presented in Fig. 4 indicate the presence of a single 14 C-labelled component corresponding to an oligosaccharide consisting of 10 or 11 glucose residues. The size of the oligosaccharide labelled from D-[1- 14 C]mannose was apparently identical with that labelled from N-acetyl-D-[1- 14 C]glucosamine.

Oligosaccharides labelled from N-acetyl-D-[1- 14 C]glucosamine and from D-[2- 3 H]mannose were subjected to hydrolysis in strong acid. Paper chromatography in solvent systems A and B of the hydrolysate derived from the oligosaccharide labelled from N-acetyl-D-[1- 14 C]glucosamine indicated the presence of a single radioactive product, which co-chromatographed with unlabelled glucosamine (Fig. 5b). Chromatography of the D-[2- 3 H]mannose-labelled hydrolysate in the same solvent systems showed that approx. 65% of the total radioactivity was present as mannose. The remaining 35% of the total radioactivity was present in an unidentified product (Fig. 5a).

Characterization of the glycoproteins labelled from D-[1- 14 C]mannose, N-acetyl-D-[1- 14 C]glucosamine and D-[2- 3 H]mannose

Chromatography of the protein fractions on a column of Sephadex G-25 in 10mM-sodium phosphate

Fig. 4. Determination of the molecular size of the oligosaccharides labelled with D-[1- 14 C]mannose (●) and N-acetyl-D-[1- 14 C]glucosamine (○)

The oligosaccharide samples were chromatographed on Whatman 3MM paper in solvent C for 48 h along with glucose oligomers of known chain length. The chromatogram was cut into 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.

buffer, pH 6.4, containing 7% (w/v) sodium dodecyl sulphate showed that 95% of the applied radioactivity was eluted with the protein peak in the void volume. None of the radioactivity applied to the column was eluted in the position of free mannose or N-acetylglucosamine.

The proteins labelled from D-[1- 14 C]mannose and from N-acetyl-D-[1- 14 C]glucosamine were compared by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The distribution of radioactivity in the gels is shown in Fig. 6. D-[1- 14 C]Mannose was incorporated predominantly into peptides with molecular weights greater than 40000 on incubation with explants of lactating mammary tissue (Fig. 6a). In contrast, radioactivity from N-acetyl-D-[1- 14 C]glucosamine was incorporated by explants of lactating tissue into a peptide of electrophoretic mobility identical with that of rabbit casein (mol.wt. 32000), as well as into peptides of a higher molecular weight (Fig. 6b). Mammary explants obtained from mid-pregnant rabbits, however, were found to incorporate radioactivity from N-acetyl-D-[1- 14 C]glucosamine exclusively into peptides of molecular weights greater than that of rabbit casein (Fig. 6c). Apparently the peptide with the same mobility as rabbit casein is glycosylated only by mammary tissue that is fully differentiated.

Fig. 5. Paper chromatography of the products of strong acid hydrolysis of the oligosaccharide labelled from (a) D-[2-³H]mannose and (b) N-acetyl-D-[1-¹⁴C]glucosamine

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 standard mannose and glucosamine are shown.

The nature of the radioactive residues present in the protein fraction was investigated after hydrolysis of the protein in strong acid. Paper chromatography, in solvent systems A and B, of the protein hydrolysate labelled from D-[2-³H]mannose, indicated that approx. 60% of the radioactivity was present as mannose. The remainder of the applied radioactivity migrated with a mobility identical with that of the unidentified compound that was observed to be present in the dolichol-linked oligosaccharide. It appears therefore that the same unknown compound is present in both the dolichol-linked oligosaccharide and protein fractions, and in approximately the same proportion. Analysis of the hydrolysate of the protein labelled from D-[1-¹⁴C]mannose showed that considerable redistribution of label from mannose to glucose had taken place. Approx. 40% of the total radioactivity was present in

mannose, 30% in glucose and 30% in the same unknown compound as described above. Chromatography, in solvent systems A and B, of the protein hydrolysate labelled from N-acetyl-D-[1-¹⁴C]glucosamine indicated the presence of a single radioactive peak that co-chromatographed with both glucosamine and galactosamine. Re-acetylation of the protein hydrolysate, followed by paper chromatography in solvent system D (Fig. 7), indicated that approx. 50% of the total radioactivity was present as N-acetylglucosamine and 50% was present as N-acetylgalactosamine.

Discussion

Studies with particulate enzyme preparations from a variety of tissues have demonstrated the widespread involvement of lipid-linked intermediates in the glycosylation of proteins [see Waechter & Lennarz (1976) and Hemming (1977) for reviews]. A major problem with such investigations has been the limited amounts of endogenous proteins in these preparations that are able to serve as acceptors for oligosaccharides transferred from lipid carriers. Consequently the incorporation of labelled sugars into protein by these preparations is extremely low in comparison with the incorporation into dolichol-linked intermediates (Waechter *et al.*, 1973; Hsu *et al.*, 1974; Behrens *et al.*, 1973; Oliver & Hemming, 1975; White & Waechter, 1975; Adamany & Spiro, 1975). It has been reported that thyroid slices are able to incorporate labelled sugars into protein far more efficiently than a thyroid particulate enzyme preparation (M. J. Spiro *et al.*, 1976a). On the other hand, thyroid slices, in contrast with thyroid particulate systems, were found to incorporate an extremely low amount of radioactivity into dolichol-linked monosaccharides.

The present paper describes the incorporation of mannose and N-acetylglucosamine into endogenous acceptors of rabbit mammary-gland explants. Both sugars were rapidly incorporated into a protein fraction. The incorporation of labelled sugar into dolichol-linked oligosaccharide was very low in comparison with the incorporation into protein. The amount of radioactivity incorporated into dolichol-linked monosaccharides was even lower. These results are in sharp contrast with those obtained with a microsomal fraction prepared from lactating rabbit mammary glands (White, 1977), which incorporates mannose from GDP-mannose into protein only to a very limited extent, in comparison with the incorporation into dolichol-linked intermediates.

Two possible explanations may be proposed for the striking qualitative and quantitative differences in the capacities of undisrupted cells and particulate-enzyme preparations to incorporate labelled sugars into endogenous acceptors. First, the proportion of

the total sugar residues incorporated into glycoproteins of mammary explants via lipid-linked intermediates may be quite small. In this case the vast majority of sugar residues would be transferred directly from the nucleotide sugar to protein. This explanation does not, however, preclude the possibility that the core oligosaccharide of many mammary glycoproteins is transferred to the protein acceptor via lipid-linked intermediates, with the more distal sugar residues then being added, one at a time, to the growing protein-linked oligosaccharide chain.

In this situation, to explain the relatively low incorporation into lipid-linked intermediates, it would be necessary to envisage the number of sugar residues present in the core oligosaccharide to be very small in comparison with the total number that are eventually incorporated into the completed glycoprotein.

An alternative explanation would assume that the amount of protein that can serve as an acceptor of oligosaccharide transferred from lipid is much greater in undisrupted cells than in microsomal preparations. In cells that are actively synthesizing protein, a continuous supply of newly synthesized polypeptides

Fig. 7. Paper chromatography of the hydrolysate of the protein labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine. The hydrolysate was re-acetylated before ascending chromatography in solvent system D 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 standard *N*-acetylgalactosamine and *N*-acetylglucosamine are shown.

Fig. 6. Distribution of radioactivity after polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of explant protein labelled with D-[1-¹⁴C]mannose and *N*-acetyl-D-[1-¹⁴C]glucosamine.

The protein fractions of explants from the mammary glands of lactating rabbits after incubation with D-[1-¹⁴C]mannose (a) and *N*-acetyl-D-[1-¹⁴C]glucosamine (b) were solubilized in sodium dodecyl sulphate/2-mercaptoethanol and analysed on 10% acrylamide gels containing sodium dodecyl sulphate. The protein fraction obtained from explants taken from the mammary gland of a rabbit in day 11 of pregnancy after incubation with *N*-acetyl-D-[1-¹⁴C]glucosamine (c) was treated in a similar fashion. The gel was cut into slices 0.25 cm in length and the radioactivity in each slice was determined. The mobilities of the tracking dye (T.D.), rabbit casein and protein standards (mol.wt. × 10⁻³) are indicated by arrows.

will be available for glycosylation. In contrast, microsomal preparations will contain a much smaller pool of potential terminal acceptors for the lipid-linked oligosaccharides. In support of this idea, an inhibition of protein synthesis in thyroid slices by puromycin completely prevented the transfer of oligosaccharide from lipid-linked intermediate to protein (M. J. Spiro *et al.*, 1976a). Furthermore, the results of Kiely *et al.* (1976) indicate that glucosamine and mannose are incorporated by oviduct into incomplete ovalbumin polypeptide chains that are still associated with a ribosomal fraction. It thus seems likely that newly synthesized or nascent polypeptide chains are important potential substrates for glycosylation. The low amounts of radioactivity incorporated into lipid-linked intermediates by mammary explants may therefore be explained if the saccharide portion of these intermediates is rapidly transferred to protein. In contrast, the high incorporation of radioactivity into lipid-linked intermediates by mammary microsomal fractions may represent an accumulation of saccharide units in these intermediates due to the limiting amounts of acceptor protein present. It would then follow that the pool of dolichol phosphate, in cells that are actively synthesizing acceptor protein, will be far from saturated with saccharide units.

A major problem encountered when undisrupted cells are incubated with labelled sugars is the extent of redistribution of label that occurs. In particular, mammary explants obtained from lactating rabbits have been shown to synthesize large amounts of triacylglycerol (Strong & Dils, 1972). In the present study, a large proportion of the radioactivity derived from ^{14}C -labelled sugars was incorporated into triacylglycerol. Consequently, dolichol-linked monosaccharides accounted for only a very small proportion of the radioactivity present in the fraction soluble in chloroform/methanol (2:1, v/v). Explants were incubated with D-[2- ^3H]mannose to try to decrease the redistribution of label. The radioactive hydrogen atom of D-[2- ^3H]mannose is removed from mannose 6-phosphate by the mannose phosphate isomerase (D-mannose 6-phosphate ketol-isomerase, EC 5.3.1.8) reaction. The incorporation of radioactivity into glycolytic intermediates and consequently into triacylglycerols should therefore be prevented. Large amounts of radioactivity were, however, incorporated from D-[2- ^3H]mannose into triacylglycerols by mammary explants. Saponification of the triacylglycerol fraction, and subsequent t.l.c. in solvent F, indicated that the radioactivity was present in the fatty acid portion of the triacylglycerol. Presumably, the conversion of D-[2- ^3H]mannose 6-phosphate into fructose 6-phosphate, catalysed in the cell by mannose phosphate isomerase, produces an intracellular pool of $^3\text{H}_2\text{O}$ of a high radioactivity, and radioactivity is incorporated from this pool into

fatty acids during the reductive steps of biosynthesis.

The properties of the radioactively labelled component that was extracted from mammary explants by chloroform/methanol/water (10:10:3, by vol.) are consistent with the hypothesis that it represents an oligosaccharide linked via a pyrophosphate bridge to a lipid. The formation of lipid-linked oligosaccharides by cell-free systems of a number of tissues has been reviewed by Waechter & Lennarz (1976) and Hemming (1977). Microsomal preparations of rabbit mammary gland have also been shown to synthesize a lipid-linked oligosaccharide (White, 1977).

The lipid-linked oligosaccharide isolated from mammary explants migrated largely as a single peak of radioactivity on t.l.c. Similarly, the oligosaccharide released from the lipid by mild acid hydrolysis migrated largely as a single radioactive peak during paper chromatography. Although not conclusive, the results suggest that both mannose and *N*-acetylglucosamine are incorporated into the same dolichol-linked oligosaccharide.

The size of the saccharide portion of this intermediate corresponded to an oligosaccharide of 10 or 11 glucose units, as determined by paper chromatography, although its exclusion on Sephadex G-25 chromatography suggests a molecular weight of at least 2200, which would correspond to an oligosaccharide chain length of 12–13 units. By using similar methods, the dolichol-linked oligosaccharide synthesized by a mammary microsomal system was estimated to be of only seven or eight monosaccharide units (White, 1977). M. J. Spiro *et al.* (1976a,c) have shown that the lipid-bound oligosaccharide synthesized by thyroid slices is considerably longer than that synthesized by a thyroid cell-free system. In this instance an accurate estimation of the oligosaccharide size was made by Bio-Gel P-6 chromatography calibrated with several natural hetero-oligosaccharides. The lipid-bound oligosaccharides synthesized by slices from bovine kidney and thymus and hen oviduct were shown to consist of 13–15 monosaccharide residues by the same technique (M. J. Spiro *et al.*, 1976b). Lipid-linked oligosaccharides synthesized by cell-free preparations of a rat myeloma (Hsu *et al.*, 1974) and hen oviduct (Lucas *et al.*, 1975) contain only seven to nine residues, as determined by a radiochemical method and paper chromatography respectively. On the other hand, liver microsomal fractions synthesize a glucose-containing, lipid-linked oligosaccharide consisting of approx. 20 monosaccharide units (Parodi *et al.*, 1972). It has been suggested (M. J. Spiro *et al.*, 1976a) that the difference in the size of the lipid-linked oligosaccharide synthesized by undisrupted cells and cell-free systems is due to the lack of a glucose donor in the latter system. This suggestion is supported by the results of Robbins *et al.* (1977) with cell-free fibroblast preparations. Cell-free systems would

consequently be unable to synthesize the complete oligosaccharide.

In spite of the large amount of recent research on the participation of lipid-linked intermediates in glycoprotein synthesis, little is known about the nature of the proteins that are glycosylated via these intermediates. The endogenous proteins that serve as acceptors for oligosaccharides transferred from lipid carriers in myeloma microsomal fractions (Hsu *et al.*, 1974), rat liver microsomal fractions (Behrens *et al.*, 1973) and hen oviduct membranes (Lucas *et al.*, 1975) appear to be tightly bound to the membranes. Evidence also suggests that lipid-linked intermediates are involved in the glycosylation of some secretory proteins as well as membrane-associated proteins. The glycosylation of a kappa-type immunoglobulin light chain by a myeloma system (Eagon *et al.*, 1975) and of ovalbumin by hen oviduct (Struck & Lennarz, 1977) both require the participation of lipid-linked intermediates.

The lactating mammary gland synthesizes glycoproteins, both for export and to replenish membrane glycoproteins that are lost during the secretory process. Casein purified from rabbit milk is a glycoprotein containing galactose, galactosamine and sialic acid (K. Al-Sarraj, D. A. White & R. J. Mayer, unpublished work). Another major secretory protein, α -lactalbumin, purified from rat milk, has been identified as a glycoprotein containing mannose and *N*-acetylglucosamine (Brown *et al.*, 1977). A glycosylated form of α -lactalbumin has also been detected in bovine milk (Barman, 1970).

Explants of lactating rabbit mammary gland incorporate D-[1-¹⁴C]mannose largely into polypeptides of molecular weights between 40000 and 80000, as determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. In contrast, radioactivity from *N*-acetyl-D-[1-¹⁴C]-glucosamine was incorporated by explants of lactating rabbit mammary gland into a polypeptide of the same electrophoretic mobility as rabbit casein (mol. wt. 32000), as well as into polypeptides of higher molecular weight. Approx. 50% of the total radioactivity in protein labelled from *N*-acetyl-D-[1-¹⁴C]-glucosamine was present as galactosamine (Fig. 7). Since rabbit casein contains galactosamine, it is conceivable that a proportion of the radioactivity incorporated from *N*-acetyl-D-[1-¹⁴C]-glucosamine into protein is incorporated into casein. No labelled galactosamine was detected in the dolichol-linked oligosaccharide fraction, implying that the glycosylation of casein does not utilize dolichol-linked intermediates. It should be stressed that the labelled polypeptide with an electrophoretic mobility identical with that of rabbit casein has not been conclusively identified as casein. It is at least apparent, however, that the polypeptide in question is only synthesized (or glycosylated) by fully differen-

tiated mammary tissue, since mammary explants obtained from mid-pregnant rabbits did not incorporate label from *N*-acetyl-D-[1-¹⁴C]-glucosamine into this polypeptide (Fig. 6c). This observation to some extent supports the supposition that the aforementioned polypeptide is casein, since the rate of casein synthesis in mammary explants taken from mid-pregnant rabbits is extremely low (Speake *et al.*, 1976).

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