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The Transfer of Mannose to Dolichol Diphosphate Oligosaccharides in Pig Liver Endoplasmic Reticulum

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The transfer, catalysed by pig liver microsomal preparations, of mannose, from GDP-mannose, to lipid-linked oligosaccharides and the properties of the products are described. Solubility, hydrolytic and chromatographic data suggest that they are dolichol diphosphate derivatives. The presence of two *N*-acetyl groups in at least part of the heterogenous oligosaccharide portion was tentatively deduced. Reduction with borohydride of the oligosaccharide showed that the newly added mannose residues were not at its reducing end. Periodate oxidation suggested that 60% of these were at the non-reducing terminus and that 40% were positioned internally. T.l.c. showed the presence of seven oligosaccharide fractions with chromatographic mobilities corresponding to glucose oligomers with 7–13 residues. The molar proportions of the oligosaccharide fractions in the mixture were determined by borotritiide reduction and the number of mannose residues added to each oligosaccharide fraction during the incubation was calculated. Two of the oligosaccharide fractions had received on average one, or slightly more than one, mannose residue per chain during the incubation; four of the other fractions were each shown to be a mixture, 20–25% of which had received one mannose residue during the incubation and 75–80% of which had not been mannosylated during the incubation. This supported other evidence for the presence of endogenous lipid-linked oligosaccharides in the microsomal preparation which had been formed before the incubation *in vitro*. Evidence for the possibility of two pools of dolichol monophosphate mannose, one being more closely associated with mannosyl transfer to dolichol diphosphate oligosaccharides than the other, is also discussed.

Microsomal fractions of rat liver catalyse the transfer of mannose from GDP-[^{14}C]mannose to dolichol phosphate mannose and to a mixture of lipid-linked oligosaccharides, believed to be dolichol diphosphate oligosaccharides (Behrens *et al.*, 1973). The formation of a lipid-linked oligosaccharide can also be catalysed by microsomal preparations of mouse myeloma (Hsu *et al.*, 1974). In both reports evidence was presented for a role for these compounds as intermediates in the transfer of mannose from GDP-mannose and dolichol phosphate mannose to protein.

Pig liver endoplasmic reticulum possesses an active mannosyl transferase using GDP-mannose as donor and dolichol phosphate as acceptor (Richards & Hemming, 1972). Glycoproteins and glycolipids often exhibit species and tissue specificity and it was decided to determine if pig liver produced dolichol diphosphate oligosaccharide and if this contained a single oligosaccharide moiety or a mixture of oligosaccharides. The present paper provides evidence for the formation of dolichol diphosphate oligosaccharides in pig liver similar to those formed in rat liver. Some properties of the oligosaccharide portion are described which provide further insight into the properties of the mannosyl transferase systems in pig liver.

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Methods and Materials

Methods

Pig liver microsomal preparation. This was prepared in the manner described by Richards & Hemming (1972). The final preparation contained approx. 80mg of protein/ml when assayed by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as a standard.

Incubation conditions and recovery of radioactive products. The standard incubation medium contained $MnCl_2$ (8.8mM), EDTA (2.2mM), Tris–HCl buffer (26.4mM, pH7.1), Triton X-100 (0.089%), GDP-[U-^{14}C]mannose (1.56μM, 0.05μCi) unless indicated otherwise, microsomal preparation (150μl) and water to 225μl. Incubations were at 37°C for 60min (unless otherwise stated) in a 15ml centrifuge tube. In order to study the variation of the formation of ^{14}C-labelled products with the period of incuba-

tion, incubation mixtures were scaled up tenfold and 220μl was removed at the appropriate time-intervals for assay.

Incubations were terminated by mixing with water (1ml) and heating at 100°C for 90s. The pellet produced by centrifugation in a bench centrifuge (1000g,10min) was resuspended in water (1ml) and the centrifugation repeated. Water (1ml) was again added, followed by chloroform–methanol (2:1, v/v, 2ml) and the shaken mixture was centrifuged as before. The lower chloroform layer was removed by Pasteur pipette and the insoluble interphase and upper phase was extracted a further twice by adding chloroform–methanol (2:1, v/v, 2ml) and repeating the mixing, centrifugation and removal of the lower layer. The upper layer was then removed by Pasteur pipette and the insoluble interphase was used as the source of the dolichol diphosphate oligosaccharides (Behrens *et al.*, 1971; see below). The lower layers were combined and the volume was increased to 10ml by the addition of chloroform–methanol (2:1, v/v). Water (5ml) was then added and the shaken mixture was centrifuged as above. The lower layer was washed once more in this way and was then evaporated to dryness under N_2. The ^{14}C-labelled lipid in this extract was taken to be dolichol monophosphate [^{14}C]mannose (Richards & Hemming, 1972; Evans & Hemming, 1973).

Chloroform–methanol (1:1, v/v, 0.5ml) was then added to the insoluble interphase remaining after thorough extraction as described above to soak up residual water, followed by chloroform–methanol–water (10:10:3, by vol., 3ml). The suspension was mixed and centrifuged as above. The solution was removed by Pasteur pipette and the insoluble pellet was extracted a further twice with the same solvent mixture. The combined extracts were evaporated to dryness under N_2 and the residue was termed dolichol diphosphate oligosaccharides. This extraction procedure was based on that of Behrens *et al.* (1971).

Before radioassay, the insoluble pellet [presumed to be protein (see Richards & Hemming, 1972)] was resuspended in 5% (w/v) trichloroacetic acid (2ml) and centrifuged as before. Further washing of the pellet with trichloroacetic acid and water (2×2ml) was carried out before it was dried in a freeze-drier and dissolved in methanolic 1M-hyamine hydroxide (1ml).

Chromatographic methods. Chromatography of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides on columns (10cm×1.5cm diam.) of silicic acid was essentially as described by Barr & Hemming (1972). Eluents were chloroform (100ml), acetone (50ml), chloroform–methanol (1:1, v/v, 50ml) and methanol (50ml). Chromatography on columns (15cm×2cm diam.) of DEAE-cellulose acetate was as described by Barr & Hemming (1972) except that chloroform–methanol–water (10:10:3, by vol.) was used as solvent and a linear gradient of ammonium acetate (0–40mM in 300ml) was used as eluent. Ammonium acetate was removed from eluates by washing with water (0.2vol.) taking care to recover the resulting interphase with the lower chloroform layer.

The water-soluble product obtained after mild acid treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides was chromatographed on a column (40cm×1cm diam.) of Sephadex G-50 (fine grade) in 0.1M sodium phosphate buffer, pH6.2, at a flow rate of 6ml/h. Fractions (1ml) were collected and bacitracin (mol.wt. 1420) and adrenocorticotropic hormone (mol.wt. 4500) were used as standards.

T.l.c. utilized the adsorbents Kieselgel G (E. Merck A.G., Darmstadt, Germany) for systems A, B and C; Kieselguhr G (E. Merck A.G.)–0.1M-sodium phosphate buffer, pH5, for system F; Kieselgel G–Kieselguhr G (3:1, w/w) for system G and cellulose CC41 (Whatman from W. &. R. Balston Ltd., Maidstone, Kent, U.K.) for systems D and E. Developing solvents for these systems were as follows.

A, chloroform–methanol–water (65:25:4, by vol.); B, propan-1-ol–water (7:3, v/v); C, di-isobutyl ketone–acetic acid–water (20:15:2, by vol.); D, ethyl acetate–butan-1-ol–acetic acid–water (6:8:5:8, by vol.); E, ethyl acetate–pyridine–water (10:4:1, by vol.); F, butan-1-ol–acetone–0.1M-sodium phosphate buffer, pH5, [4:5:1, by vol. (Stahl, 1965)]; G, propan-1-ol–nitromethane–water [5:2:3, by vol. (Huber *et al.*, 1968)].

Chromatograms and electrophoretograms (see below), were stained with either the anisaldehyde spray reagent (Stahl, 1965), which on heating gave a dark-blue colour with mono- and oligo-saccharides, or the aniline–diphenylamine spray reagent (Dawson *et al.*, 1969).

Paper electrophoresis. Cellulose paper (3MM, Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; 20cm×10cm) was used in a Shandon U77 electrophoresis tank (Shandon Southern Instruments, Surrey, U.K.) at 20V/cm for 200min.

Treatments with acid. Mild treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides with acid was as described by Richards & Hemming (1972). Strong treatment with acid (Spiro, 1966) involved heating at 108°C for 20h with 1M-H_2SO_4. The solution was then neutralized with $Ba(OH)_2$ (saturated solution) and the resulting clear supernatant was freeze-dried.

Treatments with alkali. Mild treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides with alkali was as described by Richards & Hemming (1972) except that the butanol was omitted from the extracting solvent and the interphase was included with the chloroform

layer. Strong treatment of the fraction with alkali containing dolichol diphosphate ^{14}C-labelled oligosaccharides was essentially as described by Behrens *et al.* (1971). NaOH (1M, 0.2ml) was added to the fraction dissolved in propan-1-ol (2ml) in a test tube and the mixture was heated at 64°C for 40min. Ethyl formate (1M in methanol, 0.2ml) was then added, followed by chloroform (4ml) and water (4ml). The two resultant layers were separated and evaporated to dryness, the upper layer with a freeze-drier and the lower layer, plus interphase, under N_2.

Strong treatment with alkali of the water-soluble product produced by mild acid treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides was achieved by solution in 1M-KOH (2ml) and heating under reflux at 100°C (Parodi *et al.*, 1972). A sample (1ml) was removed after 60min and the remaining portion was heated for a further 60min. Both samples were neutralized with $HClO_4$ and the supernatant solution was removed from the precipitated salt and freeze-dried.

Acetylation. The product from strong alkali treatment of the water-soluble product resulting from mild acid treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides was dissolved in water (1ml) and acetylated (Roseman & Daffner, 1956) by the addition of acetic anhydride (0.1ml) followed by $NaHCO_3$ (satd. solution, 0.1ml). After 10min at room temperature the mixture was boiled for 3min, cooled, neutralized with 1M-Na_2CO_3 (0.5ml) and freeze-dried.

Treatment with phosphatase. The water-soluble product of strong treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides with alkali was subjected to phosphatase treatment as described by Linhardt & Walker (1963). Calf intestinal phosphatase (0.02mg, BDH Chemicals Ltd., Poole, Dorset, U.K.) was added to the sample dissolved in NaOH–1M-glycine buffer, pH10.5 (1ml). Incubation at 37°C for 60min was terminated by the addition of 10%, w/v, trichloroacetic acid (1ml). The supernatant solution was removed from the precipitate and freeze-dried.

Periodate oxidation. The method used was that of Verhue & Hers (1966). The water-soluble product from mild acid treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides was dissolved in water (0.3ml) and 0.46M-sodium periodate (freshly prepared, 0.2ml) was added with shaking. After a period of 48h (unless otherwise stated) at 0°C excess of periodate was destroyed with 10M-ethylene glycol (0.02ml) and 10min later formic acid was removed by extracting with diethyl ether (3×5ml). This was neutralized with NaOH and evaporated to dryness before radioassay. The remaining aqueous solution was either freeze-dried or subjected to $NaBH_4$ reduction and acid hydrolysis (Spiro, 1964).

Borohydride reduction and acid hydrolysis. The products of periodate oxidation were desalted by passing the solution through a column (5cm×1cm diam.) of Amberlite MB-3 resin (BDH Chemicals Ltd.) and eluting with water (20ml). The volume was decreased to approximately 0.2ml in a freeze-dryer and to this was added 0.1M-$NaBH_4$ dissolved in 0.1M-sodium borate buffer, pH8.1 (2ml). After a period of 60min at 30°C the solution was neutralized with acetic acid and desalted as before. The solution was decreased to a small volume (approx. 0.2ml) in a freeze-dryer and treated with 0.05M-H_2SO_4 (2ml) at 80°C for 60min to release short-chain polyols. The mixture was neutralized with NaOH and again desalted as before. The products were then chromatographed on a column (5cm×1cm diam.) of charcoal (BDH Chemicals Ltd.)–Celite (BDH Chemicals Ltd.) (1:1, w/w). Water (20ml) was passed through the column to elute polyols and ethanol–water (3:7, v/v, 20ml) to remove any remaining oligosaccharide. Fractions were evaporated to dryness and assayed for radioactivity (see below).

$NaBH_4$ reduction of the water-soluble product resulting from mild treatment with acid of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides was achieved in the same way as described above. However, the acid treatment was as described for strong treatment with acid (Spiro, 1966) as described above.

Reduction with NaB^3H_4 of the water-soluble product from mild treatment with acid of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides was by the method described by McLean *et al.* (1973). A solution in 0.1M-KOH (10μl) was mixed with NaB^3H_4 (600μCi, 4μmol) dissolved in 0.1M-KOH (20μl). After 48h at room temperature 1M-HCl (300μl) was added and the solution was evaporated to dryness under N_2. Water (300μl) was then added and the evaporation repeated. The addition of water and evaporation was repeated a further five times and the products were then chromatographed on a column of charcoal–Celite as described above. This removed acid-stable ^3H-labelled contaminants, present in the NaB^3H_4, from the products of the reduction which were eluted with ethanol–water (3:7, v/v).

[1-^{14}C]Mannose (31.8Ci/mol) was treated with NaB^3H_4 in the same way, but in the final chromatography the product was eluted with water (see above).

Preparation of glucose oligomers. Glucose oligomers were prepared for t.l.c. standards by partial acid hydrolysis of amylose [Sigma (London) Chemical Co., Ltd., London S.W.6, U.K.] as described by Thoma & French (1957). After neutralizing the H_2SO_4 with solid $BaCO_3$ the clear solution was removed from the precipitate and freeze-dried to a small volume before desalting as described above under 'Borohydride reduction and acid hydrolysis'.

Radioassay and radiodetection. Radioactivity was measured by liquid-scintillation counting in either an LS-200 (Beckman Instruments Inc., U.S.A.) or an ABAC SL40 (Intertechnique, Plaisir, France) instrument as described by Richards & Hemming (1972). The presence of radioactivity on t.l.c. plates and on paper electrophoretograms was detected by radioscanning (Richards & Hemming, 1972) and by radioautography (Barr & Hemming, 1972).

Chemicals and solvents

Radiochemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). The purchase of all other chemicals and solvents and the purification of solvents were as described by Richards & Hemming (1972).

Results

1. *Formation of the dolichol diphosphate ^{14}C-labelled oligosaccharides*

The variation with time of the incorporation of ^{14}C into the dolichol diphosphate ^{14}C-labelled oligosaccharides, into dolichol monophosphate [^{14}C]-mannose and into protein is illustrated in Fig. 1. The incorporation into protein was fairly rapid over the first 15min, but then increased only gradually. The maximum incorporation of ^{14}C was 2–3% of the added GDP-[U-^{14}C]mannose.

T.l.c. in system A showed that the lipid extracted by chloroform–methanol–water (10:10:3, by vol.) from the products of a 15min incubation contained two ^{14}C-labelled lipids of R_F 0.05 and 0.30 corresponding to dolichol diphosphate oligosaccharides and dolichol monophosphate mannose respectively. The distribution of ^{14}C between these two components varied with time. Dolichol monophosphate [^{14}C]mannose accounted for 75, 50, 40 and 0% of the ^{14}C extracted by chloroform–methanol–water (10:10:3, by vol.) after incubation periods of 3, 10, 20 and 40min respectively. Although this distribution of ^{14}C varied slightly between enzyme preparations dolichol monophosphate [^{14}C]mannose was never detected in extracts by this solvent of 40min (or longer) incubations. The results for dolichol monophosphate [^{14}C]mannose in Fig. 1 (and subsequent values) take account of that extracted by chloroform–methanol–water (10:10:3, by vol.) and assayed after t.l.c. as well as that extracted by chloroform–methanol (2:1, v/v). Radioassay of dolichol diphosphate ^{14}C-labelled oligosaccharides also involved assay after t.l.c. examination. It was clear that [^{14}C]mannose was incorporated into the dolichol diphosphate oligosaccharide at a rate which was initially at least double that into protein, but which reached a plateau between 40 and 100min. The maximum amount of

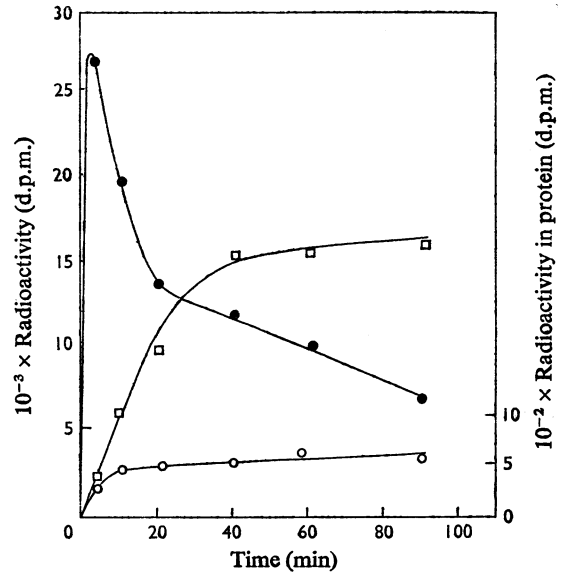

Fig. 1. *Variation with time of the incorporation of* [^{14}C]-*mannose into dolichol monophosphate mannose* (●), *dolichol diphosphate oligosaccharides* (□) *and protein* (○) *fractions of the pig liver microsomal preparation during incubation with GDP-*[*U-*^{14}*C*]*mannose* (0.025 *μCi*, 0.39 *nmol*)

Radioassay of dolichol monophosphate [^{14}C[mannose was the sum of that recovered by extraction with chloroform–methanol (2:1, v/v) and that recovered by subsequent extraction with chloroform–methanol–water (10:10:3, by vol.) and assayed after t.l.c. in system A. Radioassay of dolichol diphosphate ^{14}C-labelled oligosaccharides followed t.l.c. examination in system A of the lipid recovered by extraction with chloroform–methanol–water (10:10:3, by vol.). Further details are given in the Methods and Materials and in the Results sections.

^{14}C recovered in this lipid was approx. 25% of the added GDP-[U-^{14}C]mannose. This degree of activity was usually maintained in pig liver microsomal preparations that had been stored at −20°C for up to 1 month, but further storage resulted in gradual loss of activity, incorporation averaging 5% after 3 months storage.

The transfer of ^{14}C to dolichol monophosphate mannose followed a similar time-course to that described by Richards & Hemming (1972).

2. *Properties of the dolichol diphosphate ^{14}C-labelled oligosaccharides*

(*a*) The efficiency of several solvents in extracting ^{14}C from the particulate interphase resulting from extraction of 60min incubation products with

Table 1. *Relative efficiencies of extraction by different solvents of radioactivity from the particulate material remaining after extraction with chloroform–methanol (2:1, v/v) of pig liver endoplasmic reticulum after incubation with GDP-[U-^{14}C]mannose*

The ^{14}C extracted has been expressed as a percentage of that extracted by chloroform–methanol–water (10:10:3, by vol.).

Extracting solvent	% extracted
Chloroform–methanol–water (10:10:1, by vol.)	28.4
Chloroform–methanol–water (20:10:1, by vol.)	15.4
Butan-1-ol	7.0
Water	1.3
Chloroform–methanol–water (10:10:3, by vol.) after precipitation of the particulate material by 5% (w/v) trichloroacetic acid	65.0

chloroform–methanol (2:1, v/v) is shown in Table 1. This shows that chloroform–methanol–water (10:10:3, by vol.) was the most successful solvent. It is also clear that there was some loss of extractable ^{14}C after treatment with trichloroacetic acid, possibly due to the acid lability of dolichol diphosphate oligosaccharides.

(*b*) Analyses by t.l.c. of the dolichol diphosphate ^{14}C-labelled oligosaccharides extracted from 60min incubations was performed with systems A, B and C. Radioscanning of the developed chromatograms indicated broad peaks with R_F values of 0.05, 0.25 and 0.05 respectively. Radioautography showed six distinct, but very close radioactive areas corresponding to each of the broad peaks on the radioscans. Dolichol monophosphate [^{14}C]mannose gave R_F values of 0.3, 0.6 and 0.4 in these three systems.

(*c*) The fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides from 60min incubations was chromatographed on columns of silicic acid and most (97%) of the ^{14}C recovered was eluted as polar material by chloroform–methanol (1:1, v/v) although this sometimes accounted for less than 60% of that applied to the column. When chromatographed on DEAE-cellulose acetate the elution pattern was as shown in Fig. 2. The radioactivity emerged from the column at an ammonium acetate concentration of 12–13mM. Recovery was usually over 90%. Dolichol monophosphate [^{14}C]mannose was eluted by 3–4mM-ammonium acetate.

(*d*) After mild alkali treatment of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharide, at least 82% of the radioactive lipid remained unchanged as judged by solubility and chromatographic properties. Conversely, only 6% of the ^{14}C remained unchanged after mild acid treatment, the major product being water-soluble. In t.l.c systems D and E this radioactive product had an R_F value of 0.1 whereas mannose-1-phosphate, mannose, α-methyl mannoside, the dolichol diphosphate ^{14}C-labelled oligosaccharide and dolichol monophosphate [^{14}C]mannose had R_F values of 0.15, 0.35, 0.49, 0.67 and 0.84 respectively in system D and 0, 0.49, 0.82, 0 and 0.89 respectively in system E. Electrophoresis of the water-soluble ^{14}C-labelled product in 0.1M sodium borate buffer, pH10, confirmed that the liberated ^{14}C ($R_{mannose}$ 0.56) was different from mannose, mannose-1-phospahte ($R_{mannose}$ 1.75), dolichol monophosphate mannose which remained at the origin and the lipid extracted by chloroform–methanol–water (10:10:3, by vol.) which also remained at the origin. However, strong acid treatment of the water-soluble product released all of the radioactivity as [^{14}C]mannose as judged by t.l.c. in systems D and E and by electrophoresis in the borate system.

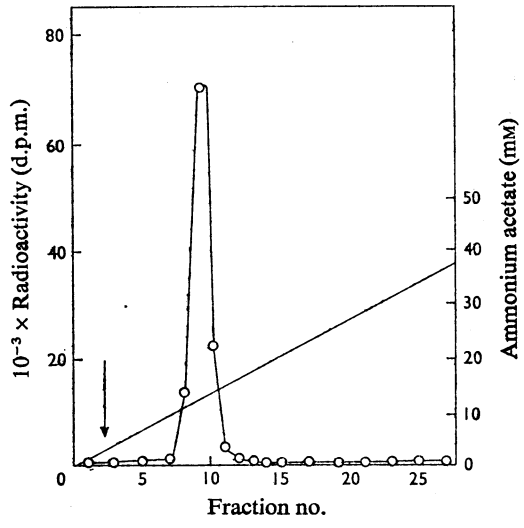

Fig. 2. *Chromatography of the fraction containing dolichol diphosphate oligosaccharides on a column of DEAE-cellulose acetate with a gradient of ammonium acetate in chloroform–methanol–water* (10:10:3, *by vol.*) *as eluent*

Fractions (10ml) were collected. Further details are given in the Materials and Methods section. Dolichol monophosphate mannose is normally eluted at the arrow.

(*e*) Strong treatment of the dolichol diphosphate ^{14}C-labelled oligosaccharide with alkali rendered 95% of the ^{14}C water-soluble. Electrophoresis in pyridine acetate buffer (pH6.5) showed the product to be negatively charged with a mobility $R_{mannose\ 1\text{-}phosphate}$ of 0.6. The negative charge was removed by incubation with calf intestinal phosphatase.

3. *Further properties of the water-soluble product from mild treatment with acid of the dolichol diphosphate ^{14}C-labelled oligosaccharides*

(*a*) Although the ^{14}C-labelled material was neutral to electrophoresis in NaOH–0.1 M-glycine buffer, pH 8.8, it had a small positive charge (the same mobility, usually 2 cm, as *N*-acetylglucosamine) when electrophoresed in 1.5 M-formic acid–2 M-acetic acid, pH 2. Strong alkali treatment for 60 min resulted in a mixture that on electrophoresis (pH 2) separated into three radioactive areas, A, B and C with mobilities relative to *N*-acetylglucosamine of 1.0, 2.0 and 3.0 respectively and accounting for 50, 40 and 10% of the ^{14}C radioactivity respectively. After alkali treatment for 120 min areas A, B and C accounted for 50, 25 and 25% of the ^{14}C respectively. Acetylation of the alkali-treated mixture followed by electrophoresis (pH 2) resulted in the presence of only one radioactive area, A. When authentic glucosamine was treated to the same acetylation procedure it electrophoresed as *N*-acetylglucosamine. Exposure of authentic *N*-acetylglucosamine to the strong treatment with alkali for 60 min gave a product with an identical mobility, on electrophoresis, to authentic glucosamine, which was 3.6 times the mobility of *N*-acetylglucosamine.

(*b*) When the water-soluble product was treated with $NaBH_4$ followed by strong acid hydrolysis the ^{14}C chromatographed in t.l.c. system F as mannose (R_F 0.53). Similar treatment of [^{14}C]mannose yielded only [^{14}C]mannitol (R_F 0.32).

(*c*) The results of periodate oxidation on the water-soluble product were compared with those of periodate oxidation on methyl [U-^{14}C]mannoside (prepared by acid methanolysis of a sample of the GDP-[U-^{14}C]mannose used in incubations). Table 2 shows the recovery of ^{14}C as formic acid from the water-soluble product as a percentage of the recovery of ^{14}C as formic acid from an equivalent amount of methyl [U-^{14}C]mannoside. It can be seen that periodate oxidation was complete at 48 h by which time the amount of ^{14}C released as formic acid from the water-soluble product was approximately 60% of that released from the methyl [U-^{14}C]mannoside.

Table 2. *Release of [^{14}C]formic acid by periodate treatment of the water-soluble material released by mild treatment of the dolichol diphosphate ^{14}C-labelled oligosaccharide with acid*

The recovery of [^{14}C]formic acid from each nCi of ^{14}C-labelled water-soluble material has been expressed as a percentage of the recovery of [^{14}C]formic acid from each nCi of methyl [U-^{14}C]mannoside during the same series of periodate treatments.

Duration of periodate treatment (h)	Sample	Recovery of [^{14}C]formic acid (%)	
48	1	63	
48	2	69	64 average
48	3	60	
72	4	61	
72	5	57	59 average
72	6	59	

After removal of the formic acid the remaining oxidation products were treated with $NaBH_4$ and acid before chromatography on a charcoal–Celite column. Most (96%) of the ^{14}C remaining was eluted with water in the polyol fraction (see the Methods and Materials section) confirming that all of the [^{14}C]mannose residues of the water-soluble product had been attacked by the periodate.

(*d*) Gel chromatography of the water-soluble product on a column of Sephadex G-50 demonstrated the presence of five distinct peaks in the molecular weight range 1500–3500.

(*e*) The successful separation from each other of oligosaccharides containing up to 19 glucose units was achieved in the t.l.c. system G on plates 40 cm long with a single development of 24 h rather than with four developments on 20 cm-long plates recommended by Huber *et al.* (1968). The latter method caused bunching of the short-chain compounds.

The radioactive water-soluble portion released from the dolichol diphosphate ^{14}C-labelled oligosaccharides was analysed by t.l.c. in system G in the same way with a marker sample of the mixture of glucose oligomers alongside. After radioautography the glucose oligomers were stained. Radioactivity was associated mainly with seven separate components (A–G) corresponding in mobility to oligomers of $(glucose)_7$ to $(glucose)_{13}$ and with values for $R_{mannose}$ of 0.72, 0.68, 0.64, 0.60, 0.55, 0.51 and 0.47 respectively. Traces of radioactive material had a mobility corresponding to mannose (same R_F value as glucose) and to methyl mannoside running just ahead of mannose. Omission of methanol from the hydrolytic mixture (see the Methods and Materials section) resulted in the disappearance of methyl [^{14}C]mannoside from the products but did not affect the distribution of radioactivity between the components corresponding to $(glucose)_7$ to $(glucose)_{13}$.

Samples of the fraction containing dolichol diphosphate ^{14}C-labelled oligosaccharides recovered after incubations of 5, 10, 20 and 60 min and not purified were treated with acid and the water-soluble material liberated was in each case subjected to t.l.c. in system G with marker samples of glucose oligomers alongside. After radioautography radioactive areas were removed for radioassay. The 60 min sample gave percentage distribution of ^{14}C of 1, 12, 13, 22, 18, 16, 11 and 6 corresponding to mannose and oligosaccharides A–G. Samples from the shorter periods of incubation gave a higher proportion of the monomer in keeping with the higher proportion of dolichol

Table 3. *Result of NaB^3H_4 reduction of the ^{14}C-labelled oligosaccharide mixture released by mild acid treatment of the dolichol diphosphate ^{14}C-labelled oligosaccharides*

The incorporation of mannose is based on the ^{14}C incorporated from GDP-[U-^{14}C]mannose (83 Ci/mol). The incorporation of $^3H+^1H$ is based on the ^3H incorporated from NaB^3H_4 and on the $^3H/^{14}C$ ratio of [^3H] and [^{14}C]mannitol produced by reduction of [^{14}C]mannose (31.8 Ci/mol) with the same NaB^3H_4. Chromatography was in t.l.c. system G. Oligosaccharides A–G were separated by t.l.c. in system G. Further details are given in the Methods and Materials and the Results sections.

Oligosaccharide	Mannose incorporated during incubation (pmol) X	$^3H+^1H$ incorporated (pmol) Y	Percentage distribution [^{14}C]Mannose	Percentage distribution ^3H	Ratio X/Y
A	35	152	9.6	15.5	0.22
B	52	230	14.2	23.0	0.22
C	70	70	19.2	7.1	1.00
D	65	46	17.0	4.7	1.40
E	60	248	16.0	25.0	0.27
F	60	118	16.0	12.0	0.50
G	24	122	6.8	12.1	0.20

monophosphate [^{14}C]mannose extracted with the dolichol diphosphate ^{14}C-labelled oligosaccharides (see the Results section 1). The relative proportions of ^{14}C-labelled oligosaccharides A–G were not changed markedly by differences in periods of incubation.

(*f*) A sample of the water-soluble portion released from dolichol diphosphate ^{14}C-labelled oligosaccharides of a 60 min incubation was treated with NaB^3H_4. After t.l.c. in system G with a sample of glucose oligomers alongside, a radioautogram was prepared and the areas labelled with ^{14}C were removed for radioassay (^{14}C and ^3H). The areas of the chromatogram corresponding to glucose oligomers 1–6 and which contained no ^{14}C were also removed for ^3H assay. The borohydride treatment caused negligible change in the chromatographic mobilities of the ^{14}C-labelled oligosaccharides A–G. The amount of ^3H taken up per pmol of mannose was determined in an identical reduction of [^{14}C]-mannose of known specific radioactivity. Knowing this value, and the specific radioactivity of the GDP-[^{14}C]mannose used in the incubation, the amount of mannose (pmol) and of $^1H + ^3H$ (pmol) incorporated into these oligosaccharides was calculated. That the conditions used for NaB^3H_4 reduction ensured complete reduction was confirmed by t.l.c. in system F of the products of [^{14}C]mannose reduction. The ^{14}C chromatographed as mannitol (see also the Results section 3*b*).

Table 3 shows that in this experiment the distribution of ^{14}C was similar to that found in Results section 3(*e*). The molar distribution of the oligosaccharides in the mixture is shown by the distribution of ^3H, assuming one reducing terminal per oligosaccharide. The ratio of pmol of mannose/pmol of ^3H (plus ^1H) taken up (i.e. the average number of mannose residues incorporated during the incubation per reducing terminus in each oligosaccharide chain) is also given in Table 3.

It is clear that oligosaccharides B and E were present at higher molar concentrations than the others and that both contained, on average, only one newly incorporated mannose residue in every four or five oligosaccharide chains. On the other hand, the oligosaccharides C and D were present at lower molar concentrations than were the others and contained on average 1 and 1.4 respectively newly incorporated mannose residues in each oligosaccharide chain.

The portion of the chromatogram corresponding to glucose oligomers 1–6 had incorporated a considerable amount of $^3H+^1H$ from NaB^3H_4 (equivalent to 3.5 nmol) spread evenly over this range of chain lengths. Negligible quantities of ^{14}C were recovered in this region.

Discussion

The solubility and chromatographic characteristics of the lipid extracted by chloroform–methanol–water (10:10:3, by vol.) confirm that the material is very similar to that formed in other tissues (Behrens *et al.*, 1973). In particular the pig liver material appears to consist of a mixture of oligosaccharides linked glycosidically via a pyrophosphate bridge to a lipid, probably dolichol, as discussed above.

The effect of strong treatment with alkali on the oligosaccharides released by mild treatment with dilute acid is tentative evidence in favour of the presence of *N*-acetyl groups in at least 50% of the component molecules. Possibly at least two such groups are present in some molecules for the second new product (C), the amount of which increases with time, has double the positive charge of the first new product (B) and both can be converted into the original neutral material (A) on *N*-acetylation. The failure of $NaBH_4$ (followed by acid hydrolysis) to

generate [^{14}C]mannitol from the ^{14}C-labelled oligosaccharide showed that the [^{14}C]mannose had not been incorporated at the reducing end of the oligosaccharide. The results obtained by periodate oxidation suggest that 60% of the [^{14}C]mannose is at the non-reducing end of the chain and 40% is positioned internally.

The heterogeneity of the dolichol diphosphate ^{14}C-labelled oligosaccharides was demonstrated by gel chromatography of the released oligosaccharide portion. T.l.c. of the oligosaccharide portion provided more detail indicating a mixture of seven oligosaccharides with chromatographic mobilities equivalent to glucosans of seven to thirteen units each. In view of the periodate results, the distribution of ^{14}C did not necessarily reflect the relative molar proportions of each oligosaccharide. The results of borotritiide reduction of the oligosaccharide mixture showed in fact that the degree of incorporation of mannose during the incubation appeared not to be related to the relative molar amounts of each oligosaccharide unit in the dolichol diphosphate oligosaccharide. The minor components of the mixture (C and D) incorporated, on average, more than one new mannose residue per oligosaccharide unit, whereas the major components of the mixture (B and E) consisted of a mixture of molecules chain-lengthened during the incubation and of others not chain-lengthened. A majority of the oligosaccharide chains were of the latter type. This demonstrates clearly that the microsomal preparation contained endogenous dolichol diphosphate oligosaccharide and that these compounds were not formed only during the incubation *in vitro*. It is also of interest that several smaller oligosaccharide units (released by acid from the dolichol diphosphate oligosaccharides) escaped addition of mannose residues completely during the incubation.

Although the incorporation of [^{14}C]mannose into the dolichol diphosphate oligosaccharide mixture rose steadily during the first 40min of the incubation the distribution of ^{14}C among the oligosaccharide portion varied very little throughout this period. This suggests either the action of a mannosyl transferase of fairly loose specificity to the size of the oligosaccharide portion of the acceptor, or a series of mannosyl transferases each specific to the size of the oligosaccharide portion and all of much the same degree of activity.

After 40min of incubation the incorporation of [^{14}C]mannose reached a plateau. However, even at 60min several oligosaccharide chains were without newly added mannose (Table 3) and this suggests that the levelling off of incorporation was not due to a shortage of acceptor but was due either to a lack of mannosyl donor or to a loss of transferase activity. Exogenous dolichol monophosphate mannose will donate mannose to the dolichol diphosphate oligosaccharide (Oliver & Hemming, 1975). GDP-mannose is also a possible direct donor (Behrens *et al.*, 1973). By 60min of incubation approximately 20% of the exogenous GDP-[^{14}C]mannose remains (Richards & Hemming, 1972). At this stage there is also still ample dolichol monophosphate mannose (Fig. 1). It is therefore possible that the fall in the rate of transfer of mannose is due to an inactivation of the mannosyl transferase(s).

However, the alternative explanation of a decreased availability of donor should not be ruled out entirely. Although dolichol monophosphate [^{14}C]mannose was present in the preparation at 60min it may not have been readily available to the transferase. There is evidence (see the Results section) that two pools of dolichol monophosphate mannose exist, one of which is readily extracted by chloroform–methanol (2:1, v/v) and the other not so, but extractable along with dolichol diphosphate oligosaccharide by chloroform–methanol–water (10:10:3, by vol.).

The latter pool was more readily depleted than the former during the formation of the oligosaccharide derivative. It was completely depleted by 40min (see the Results section) when the recovery of [^{14}C]mannose in dolichol diphosphate oligosaccharide (Fig. 1) levelled off. This situation is consistent with this second pool of dolichol monophosphate mannose being closely associated with the mannosyl transferase complex using dolichol diphosphate oligosaccharide as acceptor and may explain the difficulty of extracting it with chloroform–methanol (2:1, v/v). Possibly the more readily extracted material is less closely associated with the transferase and is not so readily available to it.

Thus the dolichol monophosphate [^{14}C]mannose present in microsomal preparations after 60min incubation may not be easily available to the transferase catalysing the mannosylation of dolichol diphosphate ^{14}C-labelled oligosaccharide. In effect there could be a shortage of substrate for this enzyme.

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