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The Formation of Mono-*N*-acetylhexosamine Derivatives of Dolichol Diphosphate by Pig Liver Microsomal Fractions

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Incubation of pig liver microsomal preparations with UDP-*N*-[U-^{14}C]acetylglucosamine yields a ^{14}C-labelled lipid. The requirement for Mn^{2+}, the pH optimum, time-dependence and the reversibility by UMP of the transferase are reported. Evidence is presented in favour of the lipid being a mixture of dolichol diphosphate *N*-[^{14}C]acetylglucosamine and dolichol diphosphate *N*-[^{14}C]acetylmannosamine. Available data suggest that the epimerization takes place while the hexosamine is bound in this lipid-soluble form. The *N*-acetylmannosamine appeared not to be released into the medium. The subfractionation of the microsomal fraction to separate transferase activity from membrane-bound β-*N*-acetylglucosaminidase activity is also reported.

The role of dolichol monophosphate as an acceptor for glycosyl transfer in microsomal preparations has been reviewed several times (e.g. Behrens, 1974; Hemming, 1974). Studies have concerned a variety of mammalian, avian, yeast and fungal systems and the results have usually been interpreted in relation to protein glycosylation. The first report of the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to form an acid-labile alkali-stable lipid was by Molnar's group (Tetas *et al.*, 1970; Molnar *et al.*, 1971) using rat and rabbit liver preparations. Subsequently it was shown that in rat liver the endogenous lipid acceptor was probably dolichol monophosphate and the product was dolichol diphosphate *N*-acetylglucosamine (Behrens *et al.*, 1971). The same product has been identified in calf pancreas, (Ghalambor *et al.*, 1974). This laboratory has been involved primarily with the pig liver system and has reported on mannosyl transfer to dolichol phosphate (Richards & Hemming, 1972; Evans & Hemming, 1973). We now describe the transfer of *N*-acetylglucosamine and its subsequent modification.

Materials and Methods

Pig liver microsomal preparations

A fraction of 'total microsomes' was prepared essentially as described by Richards & Hemming (1972). This procedure involved centrifuging a homogenate of pig liver in 0.05M-Tris–maleate buffer (pH7.1) containing 0.25M-sucrose at 10000*g* and 2°C for 20min, followed by centrifugation of the supernatant at 105000*g* for 90min. The '105000*g* pellet' was washed with NaCl (see below) and was then resuspended in 0.05M-Tris–maleate buffer (pH7.4) at a concentration of 35–40mg of protein/ml and stored at −20°C.

Fractionation of the microsomal preparation was based on the method described by Ehrenreich *et al.* (1973). The '10000*g* supernatant' described above (20ml) was layered over 1.15M-sucrose (20ml in 0.05M-Tris–maleate buffer, pH7.1) which was layered over 1.30M-sucrose (20ml in 0.05M-Tris–maleate buffer, pH7.1) in a 65ml centrifuge tube. This was then centrifuged at 63500*g* and 2°C for 210min in a swing-out rotor (3×65ml, no. 59591) in an MSE 65 centrifuge. The pellet was called microsomal fraction 1, the band between 1.30M- and 1.15M-sucrose being called microsomal fraction 2 and that between 1.15M- and 0.25M-sucrose being called microsomal fraction 3. The two bands were recovered by aspiration and they were then resuspended in 0.4M-NaCl in 0.05M-Tris–maleate buffer (pH7.4) and centrifuged at 105000*g* for 90min. The pellet of microsomal fraction 1 was also washed by resuspension in 0.4M-NaCl in 0.05M-Tris–maleate buffer and centrifuging in the same way. Finally, each pellet was resuspended in 0.05M-Tris–maleate buffer (pH7.4) at a concentration of 5–10mg of protein/ml.

Protein was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Incubation conditions and recovery of lipid

Incubations were performed over 30min in small centrifuge tubes at 30°C, with shaking in medium containing $MnCl_2$ (8mM), EDTA (1mM), 2-mercapto-

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ethanol (8mM), 0.05M-Tris–maleate (pH7.4), microsomal fraction (200μl), UDP-*N*-[U-^{14}C]acetylglucosamine (0.1μCi, 286mCi/mmol) and water to 250μl. Termination of incubations was by shaking with chloroform–methanol–4mM-$MgCl_2$ (3:2:1, by vol.) (Leloir *et al.*, 1973). The lower layer was removed and the extraction repeated. The combined lower layers were washed twice with Folch upper phase (Folch *et al.*, 1957) and were then evaporated to dryness under N_2.

When dolichol phosphate (10nmol) was added to incubations it was first evaporated to dryness with EDTA (2.5nmol) and $MnCl_2$ (20nmol) and then dissolved in medium of the final concentrations described above plus deoxycholate (0.2%).

Chromatographic methods

Chromatography on DEAE-cellulose acetate was as described by Barr & Hemming (1972). The lipid from a standard incubation was chromatographed on a column (6cm×0.6cm diam.) by using 20ml of each eluent. T.l.c. systems were as follows. A, silica gel G: chloroform–methanol–water (65:25:4, by vol.); B, silica gel G: di-isobutyl ketone–acetic acid–water (20:15:2, by vol.); C, cellulose (Whatman CC41) containing $ZnSO_4$: butan-1-ol–pyridine–water (6:4:3, by vol.); D, cellulose (Whatman CC41) containing $ZnSO_4$: butan-1-ol–pyridine–water (4:3:4, by vol.); E, cellulose (Whatman CC41) containing sodium borate: ethyl acetate–pyridine–water (2:1:2, by vol.). The adsorbent for systems C and D was prepared by pre-development of the plate with 0.1M-$ZnSO_4$ followed by heating at 110°C for 10min. Adsorbent for system E was prepared similarly but by using 0.2M-sodium borate (pH8.0) for pre-development.

Mild treatment of lipid with HCl and NaOH

These treatments were as described by Richards & Hemming (1972).

Radioassays

Radioactivity was measured as described by Richards & Hemming (1972) but with an Intertechnique ABAC SL40 liquid-scintillation counter. Radioscanning and radioautography of t.l.c. plates were also as described by Richards & Hemming (1972). Values for distribution of radioactivity on t.l.c. plates were based on scintillation counting of the radioactive areas after removal and suspension in appropriate solutions of scintillators (Richards & Hemming, 1972).

Assay of β-N-acetylglucosaminidase activity

This method was based on that described by Touster *et al.* (1970) with *p*-nitrophenyl *N*-acetylglucosaminide (2mM) as substrate, but at pH7.4, by using the standard incubation medium used for following transferase activity (see above). Incubation was for 60min at 30°C. After termination of the reaction by addition of 0.1M-NaOH–glycine buffer (pH9.5) the particulate matter was centrifuged down (105000*g*, 90min) and the extinction of the clear supernatant fluid at 400nm was measured with a sample prepared in exactly the same way but omitting the incubation step as control.

Chemicals and solvents

Di-*N*-acetylchitobiose was a gift from Dr. P. W. Kent of the University of Durham. UDP-*N*-[U-^{14}C]-acetylglucosamine was supplied in aq. 2% (v/v) ethanol from The Radiochemical Centre (Amersham, Bucks., U.K.) and was stored at −20°C. All other materials were obtained and purified as described by Richards & Hemming (1972).

Results

Microsomal fractionation

Table 1 shows that all of the microsomal fractions contained *N*-acetylglucosaminyltransferase activity but that it was concentrated primarily in microsomal fraction 1. This fraction also lacked detectable β-*N*-acetylglucosaminidase activity, whereas this enzyme was present in fraction 3. Consequently most studies were performed on fraction 1.

Electron microscopy indicated that the membranes of each fraction were vesiculated. Fraction 1 contained ribosomes attached to the vesicles, whereas fraction 2 and 3 were smooth.

Radiochemical purity of the lipid

The ^{14}C-labelled lipid recovered from incubations of UDP-*N*-[U-^{14}C]acetylglucosamine with microsomal fraction 1 and with the total microsomal fraction was chromatographed on DEAE-cellulose acetate. Microsomal fraction 1 invariably yielded a

Table 1. *Distribution of UDP-N-acetylglucosamine–lipid N-acetylglucosaminyltransferase activity and of β-N-acetylglucosaminidase activity in pig liver microsomal fractions*

The various microsomal fractions are explained in the Materials and Methods section. β-*N*-Acetylglucosaminidase was measured with *p*-nitrophenyl β-*N*-acetylglucosamine as substrate at pH7.4.

Microsomal fraction	Transferase activity (d.p.m./30min per mg of protein)	β-*N*-Acetylglucosaminidase activity (nmol/30min per mg of protein)
Total	4040	5.3
1	12190	—
2	6500	—
3	3500	32.0

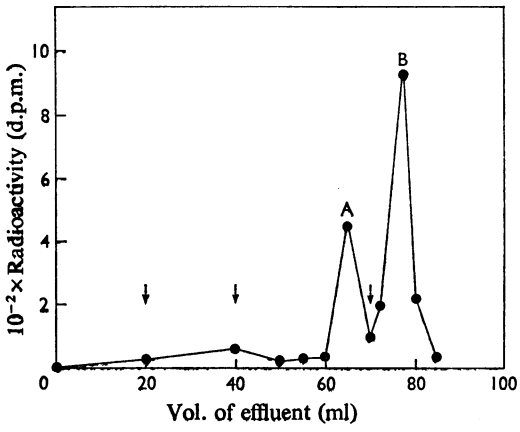


Fig. 1. *Separation of dolichol phosphate [^{14}C]mannose (A) from N-[^{14}C]acetylglucosamine lipid (B) by chromatography on DEAE-cellulose acetate*

Details are given in the Materials and Methods section. Solvent changes were to methanol at 20ml, to 0.01M-ammonium acetate at 40ml and to 0.05M-ammonium acetate at 70ml.

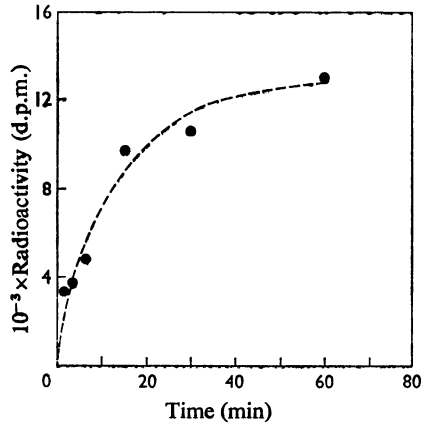


Fig. 2. *Variation with time of the incorporation of N-[^{14}C]acetylglucosamine into lipid when UDP-N-[U-^{14}C]-acetylglucosamine was incubated with microsomal fraction* 1

Details are given in the Materials and Methods section.

^{14}C-labelled lipid, at least 90% of which was retained on the anion exchanger from solution in chloroform–methanol (2:1, v/v), methanol and 0.01M-ammonium acetate in methanol, but which was eluted as a single peak of ^{14}C by 0.05M-ammonium acetate in chloroform–methanol (2:1, v/v) (see Fig. 1).

The ^{14}C-labelled lipid of the total microsomal fraction usually yielded two components on chromatography on DEAE-cellulose acetate. A minor component (usually approx. 20% of the total ^{14}C-labelled lipid) could be eluted by chloroform–methanol (2:1, v/v), whereas the major component (usually 80% of the total ^{14}C-labelled lipid) was eluted by 0.05M-ammonium acetate.

The major component had the same R_F value (approx. 0.30) in the t.l.c. system A as the ^{14}C-labelled product from microsomal fraction 1, whereas the minor component had a higher R_F value (approx. 0.40). For this reason ^{14}C-labelled lipid from incubations with the total microsomal fraction was always purified by chromatography on DEAE-cellulose acetate, and results refer to the ^{14}C-labelled lipid eluted by 0.05M-ammonium acetate. The minor component eluted by chloroform–methanol was not studied further. Studies on the ^{14}C-labelled lipid produced by microsomal fraction 1 were on the total ^{14}C-labelled lipid extracted. Frequent checks on this lipid by chromatography on DEAE-cellulose acetate confirmed that always over 90% of the ^{14}C was eluted by 0.05M-ammonium acetate.

Formation of ^{14}C-labelled lipid

Fig. 2 shows that the formation of ^{14}C-labelled lipid was time-dependent. By 1h the incorporation was maximal and had reached a plateau. At 15 and 30min the incorporation was approx. 75 and 90% of maximum respectively. Most routine incubations were for 30min.

The variation of yield of ^{14}C-labelled lipid with the quantity of microsomal preparation is shown in Fig. 3. When the total microsomal fraction was used incorporation rose linearly and then reached a plateau at 6mg of protein per incubation mixture (0.250ml). The incorporation into microsomal fraction 1 continued to rise in a fairly linear manner over the whole range tested (0–8mg of protein per incubation). Usually incubations with microsomal fraction 1 were conducted at a protein concentration of 1–2mg/incubation and with total microsomal fraction at 7–8mg/incubation.

The formation of ^{14}C-labelled lipid was dependent on the presence of bivalent metal ions. Fig. 4 shows that the transferase in microsomal fraction 1 was saturated at Mn^{2+} concentrations of 4mM and above, whereas the transferase activity rose linearly with increasing concentrations of Mg^{2+} over the range tested (0–12mM). At 10mM-Mg^{2+} the transferase activity was equal to that at 4mM-Mn^{2+}. Routine incubations contained 8mM-Mn^{2+}. The transferase exhibited an optimum activity at a pH of 7.4, but there was also considerable activity at other pH values between 4.0 and 8.5. All routine incubations were performed at pH 7.4.

The presence of UMP (50nmol) in the incubation medium from the beginning of the incubation caused a 50% fall in the incorporation of ^{14}C into lipid. Similar concentrations of UDP were without

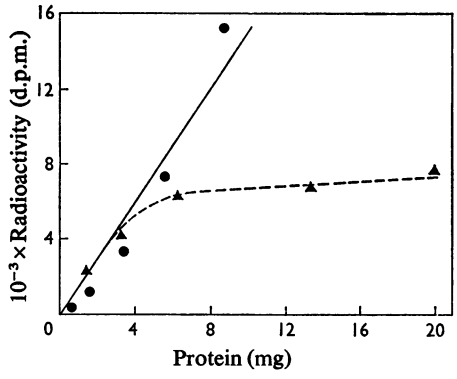


Fig. 3. *Variation with protein concentration of the incorporation of N-[^{14}C]acetylglucosamine into lipid when UDP-N-[U-^{14}C]acetylglucosamine was incubated with microsomal fraction* 1 (●) *and with the total microsomal fraction* (▲)

Details are given in the Materials and Methods section.

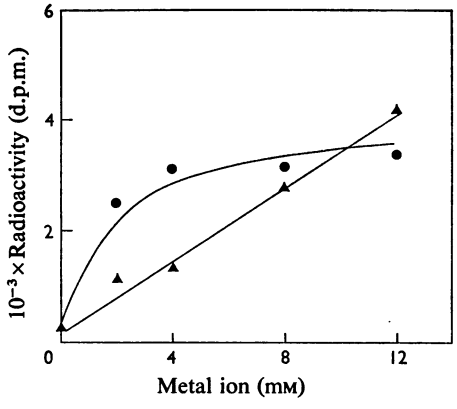


Fig. 4. *Variation with bivalent metal ion concentration of the incorporation of N-[^{14}C]acetylglucosamine into lipid when UDP-[U-^{14}C]acetylglucosamine was incubated with microsomal fraction* 1

Details are given in the Materials and Methods section. ●, Mn^{2+}; ▲, Mg^{2+}.

significant effect. The results in Table 2 suggest that UMP does in fact reverse the transfer of *N*-[^{14}C]-acetylglucosamine from UDP-*N*-[^{14}C]acetylglucosamine to lipid. After 30min incubation with UDP-*N*-[^{14}C]acetylglucosamine, UMP (100nmol) was added and the incubation was continued for a further 30min. The recovery of ^{14}C in the lipid fraction was only 15% of that in the control incubations for 30 and 60min in the absence of UMP. Thus the addition of UMP to a system containing ^{14}C-labelled lipid caused a fall of 85% in the quantity of ^{14}C associated with lipid 30min later. The presence of the same quantity of UMP from the beginning of the incubation resulted in a fall in ^{14}C recovered by 53%. UDP caused a fall of 15% in recovery of ^{14}C.

The presence of exogenous dolichol monophosphate (10nmol) in an incubation medium caused a twofold increase in the recovery of ^{14}C associated with lipid (from 5850d.p.m. to 11700 d.p.m.) compared with a control incubation. The control contained deoxycholate (0.2%) but was otherwise standard. The presence of deoxycholate made little difference to standard incubations. The chromatographic properties, on DEAE-cellulose acetate and in t.l.c. system A, of this ^{14}C-labelled lipid were identical with those of the ^{14}C-labelled lipid formed from endogenous acceptors in the control incubations.

The transferase activity to lipid was stable during storage of microsomal fractions at −20°C for several weeks. However, when microsomal fractions were stored at 4°C transferase activity decayed quite rapidly, for example reaching 50% of normal after 16h.

Properties of the ^{14}C-labelled lipid

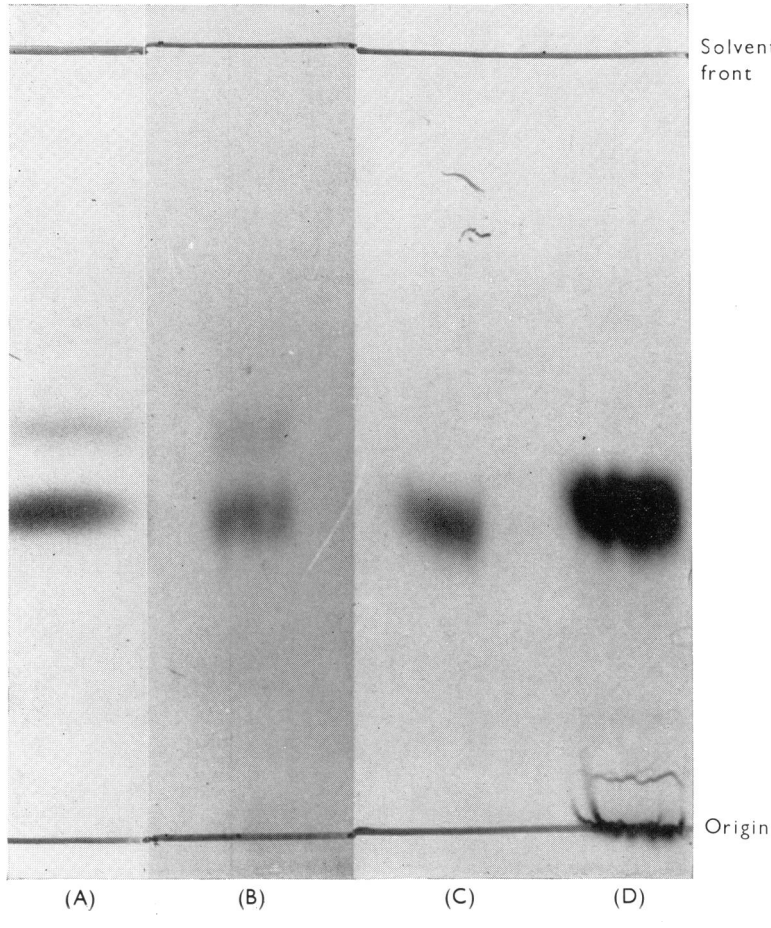
The ^{14}C-labelled lipid was eluted from a column of DEAE-cellulose acetate in the manner expected of a prenol diphosphate sugar, i.e. by 0.05M-ammonium acetate. Fig. 1 illustrates this point and shows also that the ^{14}C-labelled lipid product could be separated from admixed dolichol monophosphate [^{14}C]mannose, which was eluted by 0.01M-ammonium acetate.

The ^{14}C-labelled lipid chromatographed as a single ^{14}C-labelled entity in t.l.c. systems A and B, with R_F values of approx. 0.30 and 0.26 respectively. The chromatographic properties of the ^{14}C-labelled lipid were essentially the same after 6, 30 and 60min of incubation, although at 60min traces of a slightly less

Table 2. *Effect of adding UMP and UDP* (100*nmol each*) *on the incorporation of ^{14}C from UDP-N-[^{14}C]acetylglucosamine into lipid during an incubation with microsomal fraction* 1

Further details of incubation conditions are given in the Materials and Methods section.

Addition	Time of addition (min)	Duration of incubation (min)	Incorporation of ^{14}C into lipid (d.p.m.)
—	—	30	4800
—	—	60	4690
UMP	0	60	2510
UMP	30	60	590
UDP	0	60	4020
UDP	30	60	3970



EXPLANATION OF PLATE 1

Radioautogram after t.l.c. in system C of the water-soluble ^{14}C-labelled moiety of (A) the acid hydrolysate of ^{14}C-labelled lipid formed by incubating UDP-N-[U-^{14}C]acetylglucosamine with a 1-month-old microsomal fraction 1, (B) the same formed with a 2-month-old microsomal fraction 1, (C) N-[^{14}C]acetylglucosamine, (D) the incubation medium corresponding to (A) after a 30 min incubation

Details are given in the Materials and Methods section.

mobile component were just apparent. These properties were not changed by alteration of other incubation parameters mentioned under 'Formation of ^{14}C-labelled lipid'.

Mild treatment of the ^{14}C-labelled lipid with dilute NaOH resulted in 90% of the ^{14}C remaining lipid-soluble and no noticeable change in chromatographic properties. On the other hand mild treatment with dilute HCl rendered 96% of the ^{14}C water-soluble.

T.l.c. in system C of the water-soluble ^{14}C-labelled product of mild treatment with acid yielded two radioactive areas (Plate 1, A and B). The major component (approx. 70% of the total ^{14}C) had R_F 0.40 and the minor component R_F 0.48. In this system authentic compounds chromatographed alongside had the following R_F values; glucosamine 0.05, di-*N*-acetylchitobiose 0.30, mannose 0.30, *N*-acetylgalactosamine 0.35, *N*-acetylglucosamine 0.40 (Plate 1, C), *N*-acetylmannosamine 0.48, 1-methoxy-*N*-acetylglucosamine 0.60. In t.l.c. system D the major component had R_F 0.45 and the minor component R_F 0.51, values identical with those of *N*-acetylglucosamine and *N*-acetylmannosamine. These two radioactive areas also had the same R_F values as *N*-acetylglucosamine (0.30) and *N*-acetylmannosamine (0.40) in t.l.c. system E.

The amount of ^{14}C with R_F 0.48 in system C as a percentage of the total associated with lipid remained fairly constant, varying generally between 29 and 33% with enzyme preparations less than 2 weeks old. In particular, addition of dolichol phosphate or UMP to the incubation made negligible difference to the proportion of radioactivity chromatographing as *N*-acetylmannosamine after acid hydrolysis of the lipid. The only parameter that appeared to affect this proportion was the age of the stored microsomal preparation. Thus after storage of the microsomal preparations at −20°C for 5 months *N*-acetylmannosamine accounted for only 8% of the mixture formed.

It was established that exposure of *N*-[^{14}C]acetylglucosamine to the hydrolytic and chromatographic treatment of the lipid-linked *N*-[^{14}C]acetylglucosamine did not result in any change of chromatographic properties in t.l.c. system C and in particular did not give rise to material chromatographing as *N*-acetylmannosamine.

Absence of N-acetylmannosamine from the aqueous fraction after incubations

A fraction of the upper phase resulting from lipid extraction after incubation for 15, 30 and 60min was subjected to t.l.c. in system C. This yielded radioactivity on the chromatograms corresponding in R_F values to *N*-acetylglucosamine (0.40) and to UDP-*N*-acetylglucosamine (0.0) (Plate 1, D). Mild treatment with acid (0.01M, 10min) of the upper phase before t.l.c. resulted in all of the radioactivity travelling with the same R_F value as *N*-acetylglucosamine. There was no evidence for the presence of *N*-[^{14}C]acetylmannosamine on any of these chromatograms. A similar study after reversal of the transfer reaction by incubation with UMP also failed to reveal the presence of *N*-[^{14}C]-acetylmannosamine.

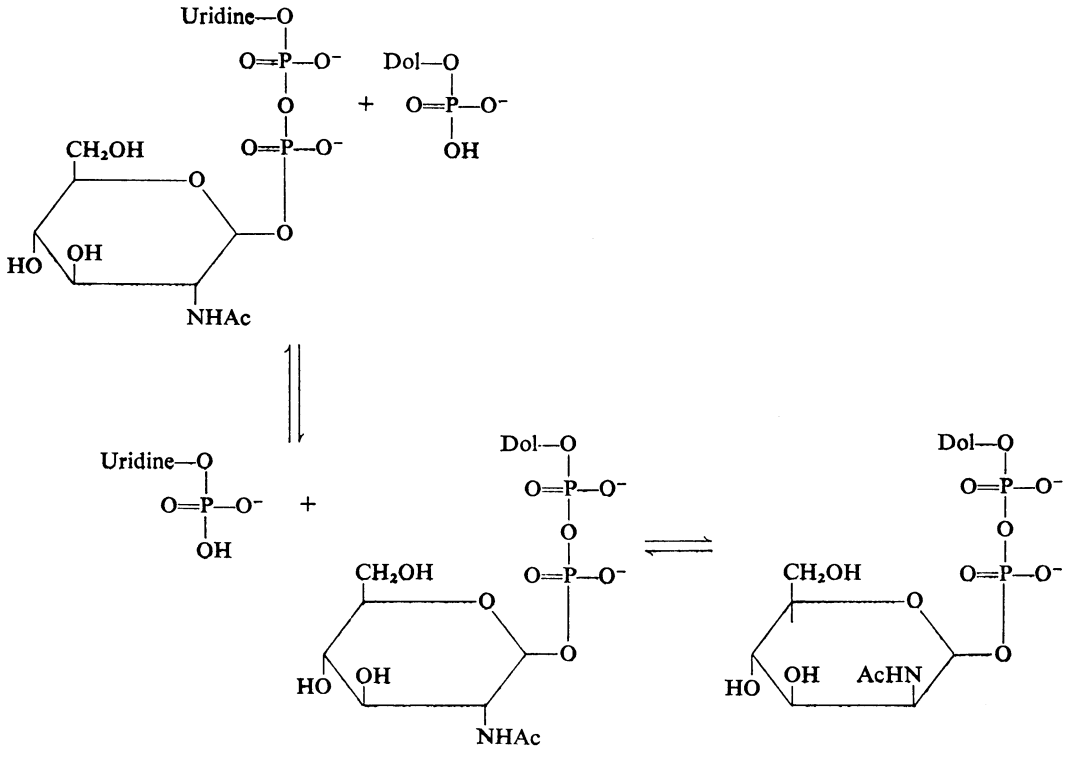
Discussion

Since the pig liver microsomal preparation contained β-*N*-acetylglucosaminidase activity that could not readily be washed from the membranes with 0.4M-NaCl and was active at pH7.4, it became necessary to separate the membrane-bound *N*-acetylglucosaminyltransferase from the membrane-bound β-*N*-acetylglucosaminidase (especially as the latter would be functional under the incubation conditions used to study the activity of the former enzyme; Table 1). There was a possibility that the hydrolase would attack the products of transferase activity and complicate the study of these products. Table 1 shows that it was possible to obtain a membrane fraction, rich in rough endoplasmic reticulum (see under 'Microsomal fractionation'), that contained transferase activity of relatively high specific activity and that lacked β-*N*-acetylglucosaminidase activity at pH7.4. The total microsomal fraction contained both activities.

The incubation conditions were selected initially on the basis of work on rat liver in the literature (Behrens *et al.*, 1971). The optimum pH of 7.4 was confirmed for pig liver, as was the requirement for bivalent metal ions. However, it was shown that Mn^{2+} worked more efficiently than Mg^{2+} in the pig liver system (Fig. 4), in contrast with the preference for Mg^{2+} by the rat liver system.

While attached to the lipid the [^{14}C]hexosamine chromatographed as a single substance both on ion-exchange columns and in two t.l.c. systems. In both types of chromatography mobility of the ^{14}C was typical of dolichol phosphate sugars. The presence of a sugar 1-phosphate bond in the molecule is suggested by the release of sugar when exposed to mild treatment with acid. The fact that the [^{14}C]-sugar remained lipid-soluble and with unchanged chromatographic properties after mild treatment with alkali is consistent with this.

The mobility of the ^{14}C-labelled lipid on DEAE-cellulose acetate is in favour of it being a dolichol diphosphate sugar. The radioactivity ran just behind the region for dolichol monophosphate sugars, consistent with an extra negative charge on the ^{14}C-labelled lipid and in the same position as that described for synthetic dolichol diphosphate *N*-acetylglucosamine by Ghalambor *et al.* (1974). Further evidence in favour of the ^{14}C-labelled lipid being a dolichol diphosphate sugar is the apparent incorporation of exogenous dolichol phosphate into



Scheme 1. *Scheme proposed to explain the data reported in this paper*

Abbreviation: Dol, dolichol.

the compound and also the reversal of the transfer reaction by UMP but not by UDP, showing that sugar phosphate was being transferred from the nucleotide diphosphate sugar.

Studies on the sugar portion showed that 70% of the product was still in the form of *N*-acetylglucosamine (co-chromatography in three t.l.c. systems), but that 30% had been converted into *N*-acetylmannosamine (co-chromatography in three t.l.c. systems). The discovery of the latter was surprising, for its attachment to dolichol diphosphate has not been described before. Careful checks of the isolation and hydrolytic procedures showed that it was almost certainly a product of the incubation and not of these other procedures. The increase in the ratio of *N*-acetylglucosamine to *N*-acetylmannosamine which follows an increased period of storage of the microsomal fraction also favoured a microsomal source for the epimerization activity.

The absence of free *N*-acetylmannosamine at the end of incubations, even after mild treatment of the water-soluble extract with acid, indicates the absence of UDP-*N*-acetylmannosamine or *N*-acetylmannosamine 1-phosphate from the medium. This suggests that the 2-epimerization of glucosamine to mannosamine did not occur while the hexosamine was attached to the nucleotide diphosphate and also shows that dolichol diphosphate *N*-acetylmannosamine did not release either *N*-acetylmannosamine or *N*-acetylmannosamine 1-phosphate into the medium during the incubation. The absence of *N*-acetylmannosamine attached to nucleotide diphosphate after reversal of the transferase reaction by UMP indicates the absence of an *N*-acetylmannosamine phosphate–dolichol phosphate transferase. On the other hand, after this reversal of the reaction the ratio of *N*-acetylmannosamine to *N*-acetylglucosamine appeared to be unchanged, indicating the reversible nature of the epimerization step(s).

These data suggest the system shown in Scheme 1. Spivak & Roseman (1966) and Salo & Fletcher (1970) have described in detail the properties of an enzyme preparation from rat liver that is capable of

catalysing the formation of free *N*-acetylmannosamine from UDP-*N*-acetylglucosamine. A lag period and two pH optima (6.7 and 7.7) prompted Spivak & Roseman (1966) to suggest two discrete steps. They also observed activation of the overall activity in the presence of Mn^{2+} ions and an inhibition in the presence of Mg^{2+} ions. The activity was found to be unstable on storage. On the basis of detailed studies using non-radioactive UDP-*N*-acetylglucosamine and UDP-*N*-acetylmannosamine and ^3H_2O, Salo & Fletcher (1970) proposed a scheme in which the reversible epimerization occurs while the sugar is bound to the enzyme. Their data excluded the possibility that UDP-*N*-acetylmannosamine might be an intermediate in the process.

In these respects there appears to be a close similarity between the system described by Spivak & Roseman (1966) and by Salo & Fletcher (1970) and that reported in the present paper. However, the two systems cannot be the same for the following reasons.

First, the system reported by Salo & Fletcher (1970) releases UDP, not UMP, from UDP-*N*-acetylglucosamine, and the *N*-acetylhexosamine, not the *N*-acetylhexosamine phosphate, is transferred to the intermediate. Secondly, this system appears to be water-soluble, whereas the pig liver system described in the present paper is membrane bound. Thirdly, the water-soluble system releases free *N*-acetylmannosamine into the medium, whereas the membrane system does not.

The formation of dolichol diphosphate *N*-acetylmannosamine was also observed by the present authors in studies with rat liver microsomal preparations. In this case the ratio of *N*-acetylmannosamine to *N*-acetylglucosamine was 1:9. The presence of the *N*-acetylmannosamine derivative was not described in previous reports on the formation of dolichol diphosphate *N*-acetylglucosamine by rat liver microsomal fractions (Leloir *et al.*, 1973). Spivak & Roseman (1966) reported that Mn^{2+} ions activate and Mg^{2+} ions inactivate the water-soluble 2-epimerase activity. Leloir's group used Mg^{2+} ions in their transferase studies (Leloir *et al.*, 1973), and possibly the membrane-bound epimerase was also suppressed.

The significance of this lipid-bound epimerization in mammalian tissues is not clear. There have been no reports of the presence of *N*-acetylmannosamine in mammalian polysaccharides, glycoproteins or glycolipids. The formation of *N*-acetylmannosamine 6-phosphate en route to sialic acids presumably requires the release of the sugar. There is good evidence that under appropriate conditions dolichol diphosphate *N*-acetylglucosamine will accept a second molecule of *N*-acetylglucosamine and that subsequent mannosylation of the resulting di-*N*-acetylchitobiose derivative can then occur in hen oviduct and rat liver (Levy *et al.*, 1974) and in pig liver (G. Palamarczyk & F. W. Hemming, unpublished work). It seems probable that this sequence of glycosylations is part of the overall process involved in the glycosylation of some proteins.

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