EVIDENCE FOR XYLOSYL LIPIDS AS INTERMEDIATES IN XYLOSYL TRANSFERS IN HEN OVIDUCT MEMBRANES

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Received November 16, 1973

SUMMARY: Hen oviduct membranes catalyze the transfer of xylose-[^{14}C] from UDP-xylose-[^{14}C] into a xylosyl lipid; a product tentatively identified as an oligosaccharide lipid; and glycoprotein(s). The synthesis of the xylosyl lipid is stimulated by exogenous dolichol phosphate. This finding, along with studies on the chemical properties of the xylosyl lipid, suggests that it has the structure xylosyl phosphoryl polyisoprenol. When exogenous, partially purified [^{14}C]-xylosyl lipid is incubated with the membrane preparation, label is found in the oligosaccharide lipid and in glycoprotein(s).

In an earlier report we provided evidence for the formation of mannosyl phosphoryl polyisoprenol by membranous enzyme preparations from bovine thyroid and hen oviduct (1). In oviduct, the mannolipid participates in the transfer of mannose to glycoprotein and a mannosylated product which is soluble in $CHCl_3$-CH_3OH-H_2O (10:10:3). The work of Leloir and his coworkers (2,3) indicates that an oligosaccharide lipid with similar solubility properties mediates the transfer of glucose from glucosyl phosphoryl dolichol to glucoprotein(s) in liver. The possibility that other sugars might be transferred into glycoprotein via lipophilic sugar carriers has prompted us to test UDP-xylose for its ability to serve as a substrate for xylosyl transferases utilizing lipid acceptors.

MATERIALS AND METHODS: UDP-xylose-[^{14}C] (176 mCi/mM), UDP-glucose-[^{14}C] (238 mCi/mM) and GDP-mannose-[^{14}C] (165 mCi/mM) were purchased from New England Nuclear Corp. Ammonyx was provided by Dr. W. Finnerty, University of Georgia. All other chemicals were obtained from commercial sources.

For preparation of membranes, magnum sections of oviducts of freshly killed laying hens were excised, finely minced and added to 2 volumes of 50 mM Tris·HCl, pH 7.0 containing 5% sucrose, 0.9% NaCl and 1 mM EDTA. The tissue was homogenized by 12 strokes of a Dounce homogenizer (Vitro) using pestle B, and then centrifuged at 600 x g for 15 min. The resulting supernate was centrifuged at 6,500 x g for 15 min. The pellet resulting from centrifugation of the 6,500 x g supernate at 39,000 x g for 15 min, was resuspended in the homogenization buffer at a protein concentration of 30-50 mg/ml.

To prepare labeled xylosyl lipid a reaction mixture containing 215 mg

membrane protein, 10 mM dithiothreitol, 10 mM $MnCl_2$ and 5 μM UDP-xylose-[^{14}C] in a total volume of 5 ml was incubated at 37° for 30 min. A single radioactive lipid product was extracted and purified by the scheme previously described for mannosyl phosphoryl polyisoprenol through the second DEAE-cellulose chromatography step (1).

Chromatographic procedures and solvent designations were the same as previously reported (1).

RESULTS: The time course for the labeling of endogenous acceptors by UDP-xylose-[^{14}C] is shown in Fig. 1. Labeled xylose appears very rapidly in the

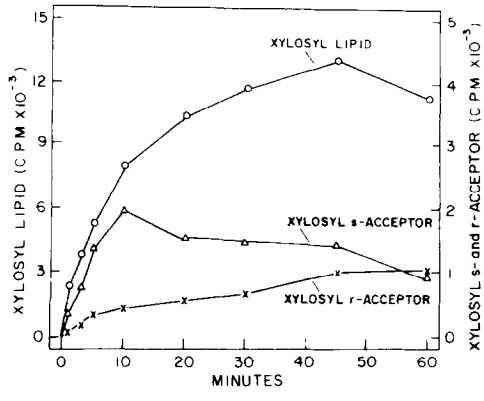

Figure 1. Kinetics of transfer of [^{14}C]-xylose from UDP-xylose-[^{14}C] into endogenous acceptors. Each assay tube contained membrane protein (2 mg), 2.5 μM UDP-xylose-[^{14}C] and 10 mM $MnCl_2$ in a total volume of 0.20 ml. At the indicated time 4 ml of $CHCl_3$-CH_3OH (2:1) was added and the assay procedure described previously was followed (1). The xylosyl lipid was extracted with $CHCl_3$-CH_3OH (2:1); the labeled product recovered in $CHCl_3$-CH_3OH-H_2O (10:10:3) was designated as xylosyl s-acceptor; the radioactivity remaining in the residue is designated as xylosyl r-acceptor.

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xylosyl lipid and less rapidly in the oligosaccharide lipid, designated as xylosyl s-acceptor. Label accumulated slowly in xylosyl r-acceptor, the xylose-containing glycoprotein(s). The relative incorporation rates for the three labeled products are in the necessary order to allow for a precursor product relationship between the xylosyl lipid and the other two xylosylated endogenous acceptors.

The transfer of labeled xylose from UDP-xylose-[^{14}C] into xylosyl lipid (Fig. 2A) and the other two xylosylated products required a divalent metal ion, Mn^{++} being the most effective. EDTA (10 mM) was strongly inhibitory.

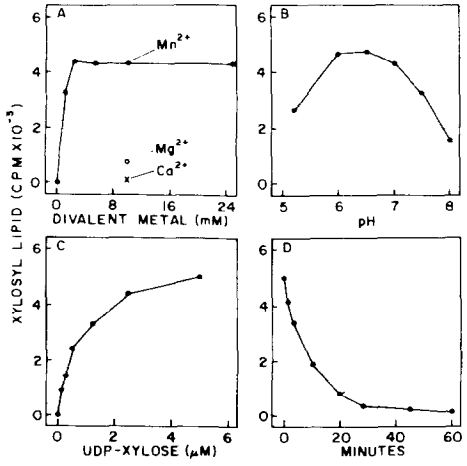

Figure 2. A) Dependence of xylosyl lipid formation from UDP-xylose-[^{14}C] on divalent metal ion concentration. Assay mixtures contained 2 mg protein in homogenization buffer, 2.5 μM UDP-xylose-[^{14}C] and divalent metal ion as indicated in a total volume of 0.20 ml. The assay mixtures were incubated at 37° for three minutes and the radioactive glycosyl lipid was measured as previously described (1).

B) pH profile for xylosyl lipid formation from UDP-xylose-[^{14}C]. Assay mixtures contained 2 mg protein, 10 mM $MnCl_2$, 2.5 μM UDP-xylose-[^{14}C] and 50 mM Tris-maleate at indicated pH in a total volume of 0.20 ml.

C) Dependence of xylosyl lipid formation on UDP-xylose concentration. Assay mixtures contained 2 mg protein in homogenization buffer, 10 mM $MnCl_2$, UDP-xylose-[^{14}C] as indicated in a total volume of 0.20 ml.

D) Kinetics of mild acid hydrolysis of xylosyl lipid. Xylosyl lipid was treated with 0.1N HCl in 50% 1-propanol at 50°. The release of water-soluble xylose-[^{14}C] was assayed as previously described (1).

The formation of labeled lipid was also inhibited by the presence of UDP. The optimal pH for lipid synthesis is between 6.0-7.0 (Fig. 2B). From the dependence of the formation of labeled lipid on UDP-xylose concentration shown in Fig. 2C an apparent K_m for UDP-xylose of 0.55 μM was calculated.

The labeled xylosyl lipid is stable to mild alkaline methanolysis (0.1N KOH in CH_3OH-toluene (3:1) at 0° for 60 min.). When treated with mild acid a single radioactive water-soluble product is released ($t_{1/2}$ = 6 min.) which has been identified as free D-xylose by co-chromatography in three paper chromatography systems (Fig. 2D). When the xylosyl lipid was chromatographed on EDTA-treated SG-81 paper and developed in solvent systems A, B and C, a single radioactive component with a R_f slightly greater than that of mannosyl phosphoryl polyisoprenol was evident. The xylosyl and mannosyl lipids had the same chromatographic mobility on DEAE-cellulose chromatography. As shown in Table I, the synthesis of the xylosyl lipid is

TABLE I

Effect of exogenous dolichol phosphate on the transfer of labeled sugars from their nucleotide derivatives into glycolipid.

Sugar Nucleotide	Dolichol Phosphate	Glycolipid Formed (pmoles)
GDP-Mannose	-	28.9
GDP-Mannose	+	119.6
UDP-Xylose	-	2.6
UDP-Xylose	+	8.6
UDP-Glucose*	-	41.2
UDP-Glucose*	+	97.2

Assay mixtures contained enzyme (1.2 mg protein) suspended in the homogenization buffer, 2.5 μM labeled sugar nucleotide, 0.025% Triton X-100, 10 mM $MnCl_2$ (*$CaCl_2$ replaced $MnCl_2$), 10 mM dithiothreitol and dolichol phosphate (12 nmoles) where indicated in a total volume of 0.20 ml. The reaction tubes were incubated at 37° for three minutes. The formation of labeled glycolipids was assayed as described in the legend to Figure 1.

stimulated by exogenous dolichol phosphate, as is the synthesis of mannosyl phosphoryl polyisoprenol (1). In experiments not shown, glucosyl lipid synthesis was observed when UDP-glucose and Ca^{++} (instead of Mn^{++}) were added to the membrane preparation. Synthesis of this glucosyl lipid also was stimulated by addition of exogenous dolichol phosphate (Table 1). In each case the chromatographic mobility of the labeled product was the same whether or not exogenous dolichol phosphate was present.

Since the xylosyl lipid formed by hen oviduct membranes was similar chemically and chromatographically to the mannosyl phosphoryl polyisoprenol, which has been shown to mediate mannosylation reactions in oviduct membranes (1), exogenous partially purified [^{14}C]-xylosyl lipid was tested as a xylose donor. The results shown in Fig. 3 reveal that hen oviduct membranes catalyze the transfer of [^{14}C]-xylose from exogenous xylosyl lipid into a lipid-linked oligosaccharide (xylosyl s-acceptor) and into a xylose-containing glycoprotein(s) (xylosyl r-acceptor). The rapid appearance of radioactive xylose in the oligosaccharide lipid and the slow accumulation of label in the glycoprotein fraction is consistent with a precursor product relationship.

The incorporation of labeled xylose into both products did not require a metal ion and was not inhibited by EDTA. This result argues against the possibility that [^{14}C]-xylose is transferred from the lipid to endogenous UDP, with UDP-xylose-[^{14}C] then serving as the direct xylose donor, since as mentioned above, the transfer of [^{14}C]-xylose from its nucleotide form is

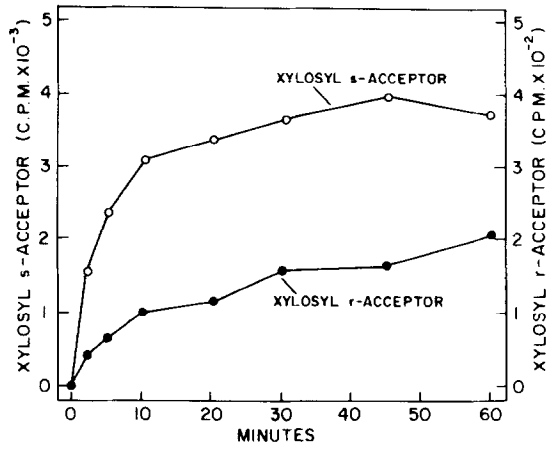

Figure 3. Kinetics of transfer of [^{14}C]-xylose from exogenous, partially purified [^{14}C]-xylosyl lipid into xylosyl s-acceptor and xylosyl r-acceptor. Each reaction mixture contained 0.20 ml (8 mg) of protein and 0.05 ml of [^{14}C]-xylosyl lipid (40,000 cpm) ultrasonically dispersed in 4% Ammonyx. The assay mixture was incubated at 37° for the indicated period of time and the reaction terminated by the addition of 5.0 ml of $CHCl_3$-CH_3OH (2:1). The labeled endogenous acceptors were assayed as previously outlined (1).

strongly inhibited by EDTA. The transfer of xylose from exogenous xylosyl lipid into endogenous acceptors was optimal at a concentration of Ammonyx between 0.4-1.0%.

The xylosyl s-acceptor formed from [^{14}C]-xylosyl lipid is chromatographically identical to the labeled product formed from UDP-xylose-[^{14}C]. Its mobility (R_f = 0.75, solvent system E) is similar to the mannose-containing oligosaccharide lipid (R_f = 0.70) reported previously (1). Following acid hydrolysis in 0.1N HCl in 80% aqueous tetrahydrofuran ($t_{1/2}$ = 20 min.) a water-soluble product is released which has a R_f of 0.15 in paper chromatography system E; upon gel filtration on Sephadex G-25 the water-soluble product elutes with a Ve/Vo of 1.15. These findings are consistent with it being an oligosaccharide. All of the radioactivity is released as free xylose under conditions of strong acid hydrolysis (2N HCl, 100°, 2 hr.) whether or not the product is treated with sodium borohydride prior to hydrolysis. This result indicates that the labeled xylose residue is not at the reducing end of the oligosaccharide chain.

Following strong acid hydrolysis all of the radioactivity in the xylosylated glycoprotein(s) (xylosyl r-acceptor) co-chromatographed with standard D-xylose on Whatman 3 MM when developed with solvent mixtures E, F or H. Upon incubation in 0.1N NaOH at 37° for 40 hours 60% of the radioactivity in

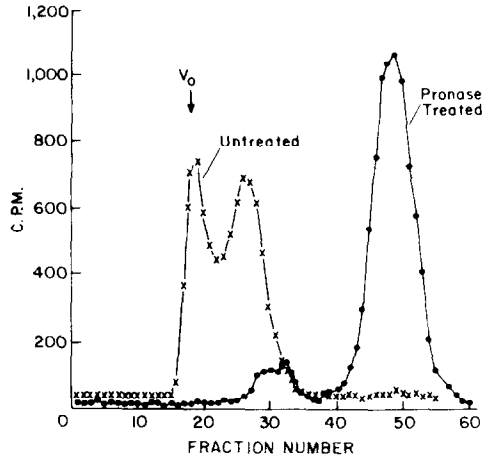

Figure 4. Gel filtration of [^{14}C]-xylosyl r-acceptor on Sephadex G-150 before and after pronase treatment. The procedure previously described for [^{14}C]-mannosylated glycoproteins was followed (1).

the xylosyl r-acceptor became soluble in trichloracetic acid. When xylosyl r-acceptor was solubilized and chromatographed on Sephadex G-150 two peaks were obtained (Fig. 4). However, following digestion with pronase essentially all of the radioactivity was recovered as small molecular weight material in the total inclusion volume. Thus, it appears that at least two xylose-containing glycoproteins are formed by hen oviduct membranes. A similar digestion experiment was performed with testicular hyaluronidase, but no significant degradation was observed.

DISCUSSION: We have shown that, in addition to mannose, hen oviduct membranes catalyze the transfer of xylose to dolichol phosphate; an endogenous acceptor, which is probably an oligosaccharide lipid; and glycoprotein(s). The time course of incorporation of xylose into the acceptors is consistent with the reaction scheme proposed by Leloir and coworkers (3) for glucoprotein biosynthesis in rat liver, and by us (1) for mannoprotein synthesis in oviduct. Thus, it appears possible that for some classes of glycoproteins the oligosaccharide chains are assembled at the lipid level and then transferred *en bloc* to the protein acceptor site.

The importance of xylose in linking the oligosaccharide chains to the polypeptide of proteoglycans, and an earlier report on the synthesis of xylose-containing glycoproteins in hen oviduct (4), led us to study the xylosyl transferases and their products in some detail. The xylosyl lipid formed has the chemical and chromatographic properties expected for a

xylosyl phosphoryl polyisoprenol and its biosynthesis is stimulated by exogenous dolichol phosphate. Direct evidence has shown that label from the xylosyl lipid is transferred to a large molecular weight fraction and a product that is soluble in $CHCl_3$-CH_3OH-H_2O. The latter product has solubility properties similar to the glucosylated oligosaccharide lipid found in rat liver (2,3). Upon treatment of the oviduct oligosaccharide lipid with mild acid, a xylose-containing compound is released which has the chromatographic properties of an oligosaccharide. Reduction of the oligosaccharide, followed by strong acid hydrolysis yields xylose, not xylitol, indicating that the xylosyl residue is not at the reducing end of the oligosaccharide.

The large molecular weight fraction, which contains at least two xylosylated proteins, is completely degraded by pronase. The possibility that classes of xylose-containing glycoproteins other than proteoglycans are present in oviduct membranes, as may be the case in erythrocyte membranes (5), is being investigated.

In preliminary experiments (data not shown) we have surveyed other sugar nucleotides as glycosyl donors in the synthesis of glycosyl lipid, oligosaccharide lipid and glycoprotein(s). In contrast to the results with the nucleotide derivatives of mannose, xylose, and glucose, studies with the nucleotides of N-acetyl galactosamine, fucose, sialic acid, and galactose reveal that little or no glycolipid and oligosaccharide lipid is formed, although incorporation into glycoprotein is observed. In the absence of other sugar nucleotides N-acetyl glucosamine is transferred from UDP-N-acetyl glucosamine into lipids and glycoproteins, but not into oligosaccharide lipid (c.f. 6). These findings suggest that in oviduct membranes, some sugars are transferred directly into glycoprotein from their nucleotide derivatives while others are transferred via lipid carriers. Whether or not the lipid-mediated steps involve the transfer of mono- and/or oligosaccharides to protein acceptors remains to be established.

ACKNOWLEDGMENT: We are indebted to Dr. C. Warren, Harvard Medical School for dolichol phosphate. This work was supported by N.S.F. Grant GB 31121. C.J.W. and J.J.L. were supported by postdoctoral fellowships from the National Institutes of Health. The expert technical assistance of Mrs. Betty J. Earles is gratefully acknowledged.

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