Dietary Effects on the Formation of Dolichyl Monophosphate Mannose by Microsomal Preparations of Rat Adipose Tissue

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Microsomal preparations from rat adipose tissue catalyse the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to an endogenous acceptor forming a [^{14}C]mannosyl lipid. The mannosyl lipid co-chromatographs with hen oviduct dolichyl monophosphate β -mannose on three solvent systems. It is stable to mild alkaline hydrolysis, but strong alkaline treatment yields a compound that co-migrates with mannose 1-phosphate. The mannosyl lipid is labile to mild acid hydrolysis, yielding [^{14}C]mannose. Formation of the compound is reversible by GDP, but not GMP, and is stimulated by exogenous dolichyl phosphate.

The kinetics of transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to form dolichyl monophosphate mannose were studied by using preparations derived from rats fed on one of four diets: G (high glucose), L (high lard), F (fructose) or GC (high glucose, 0.9% cholesterol). The K_m and V_{max} values for transfer from GDP-mannose were virtually indistinguishable in the four preparations.

In the absence of exogenous dolichyl phosphate, the largest amount of transfer of [^{14}C]mannose into the mannosyl lipid was observed with preparations from fructose-fed animals. Preparations from glucose-fed animals showed about 60% as much transfer, whereas membranes from rats fed the other diets showed intermediate values between the fructose- and glucose-fed animals. The inclusion of cholesterol in the glucose diet elicited an increase in transfer of mannose.

Under conditions of saturating exogenous dolichyl phosphate, preparations from lard-fed animals have 1.5 times as much enzyme activity as do preparations from animals fed the other three diets.

Several plasma-membrane-associated functions are modified in response to dietary manipulations. Adipose tissue preparations from animals fed a high-lard diet have a decreased hormone-stimulated lipolysis and decreased hormone-stimulated adenylate cyclase (Gorman *et al.*, 1973), a decreased insulin response and binding and decreased concanavalin A response (Ip *et al.*, 1976) and decreased glucose transport (Ip *et al.*, 1977) compared with rats fed a high-glucose diet. Furthermore, one or more of these changes are also observed in liver and intestinal epithelial cells (Sun *et al.*, 1977; Raheja *et al.*, 1975). The lipid compositions of liver plasma membranes from animals fed either a high-glucose or a high-lard diet are also different (Sun *et al.*, 1979). Recently, the response to noradrenaline in fat-pads was shown to be abolished by feeding a diet high in saturated fatty acids (Awad & Zepp, 1979).

The differences in response to concanavalin A sug-

gest that cell-surface glycoconjugates may be modified depending on whether the animal has been fed a high-lard or a high-glucose diet. In fact, Henriquez *et al.* (1979) have demonstrated that liver plasma-membrane glycoproteins from rats fed a high-lard diet have between 30–40% less sialic acid, hexosamine, fucose and mannose than do liver plasma-membrane glycoproteins from high-glucose-fed rats.

The glycosylation of proteins has been the subject of intensive investigation. Glycoproteins containing *N*-asparagine-linked oligosaccharides have a core region consisting of mannose and *N*-acetylglucosamine units (Kornfeld & Kornfeld, 1976). Sugars linked to dolichyl mono- and pyro-phosphate have been shown to be involved in the assembly and transfer of this core region (Behrens, 1974; Lucas & Waechter, 1976; Waechter & Lennarz, 1976; Hemming, 1977*a,b*).

The differences in response of isolated adipocytes

to concanavalin A and the differences of carbohydrate composition of liver plasma-membrane glycoprotein when animals are fed different diets suggest that differences in the biosynthesis of plasma-membrane glycoproteins can be induced by diet. For this reason, as well as the fact that the adipocyte plasma membrane has been characterized with respect to protein and glycoprotein species present (Trosper & Levy, 1974; Kawai & Spiro, 1977a,b), we have undertaken this study of lipid intermediates in adipose tissue. Furthermore, dolichol-linked saccharides have not previously been described in adipose tissue.

In addition to the high-glucose and high-lard diets, animals fed a fructose diet and a cholesterol diet were also included in the studies presented here. Sucrose is known to have effects on adipose tissue insulin sensitivity resembling those of fat feeding (Vrana & Fabry, 1973) and in a recent report (Mills & Adamany, 1978) inhibitors of cholesterol biosynthesis were shown to diminish dolichol-linked saccharide synthesis in smooth-muscle cells.

After the completion of this work, Hemming and his collaborators (Coolbear & Hemming, 1979; Tavares *et al.*, 1979) reported that the inclusion of 1% cholesterol in the diet causes an increase in the formation of dolichol from its precursors and increases in the activity catalysing the formation of dolichyl monophosphate mannose and dolichyl monophosphate glucose.

Experimental

Materials and routine methods

GDP-[^{14}C]mannose (sp. radioactivity 246 mCi/mmol) was purchased from New England Nuclear Corporation. Dolichyl phosphate (grade III) was purchased from Sigma Chemical Company. Hydromix liquid scintillation fluid was purchased from Yorktown Research, South Hackensack, NJ, U.S.A. Hydromix was diluted with an equal volume of toluene before use. Whatman SG-81 paper was obtained from H. Reeve Angel, Clifton, NJ, U.S.A. Other chemicals were reagent grade and from commercial sources unless otherwise noted.

Male Sprague-Dawley rats (150 g) were purchased from Taconic Farms, Germantown, NY, U.S.A., and fed Purina rat chow for 1 week, after which they were put on one of several diets. The high-glucose diet and high-lard diet have been previously described (Ip *et al.*, 1976; Tepperman *et al.*, 1978). Both diets contained 32% of the calories as casein and the remainder as either glucose or lard. Adequate amounts of vitamins, minerals and cellulose were added. In addition to the previously described glucose and lard diets, a fructose and a glucose-plus-cholesterol diet were fed to some groups of animals. The fructose diet was identical with the glu-

cose diet except that an equal amount of fructose was substituted for glucose. The glucose-plus-cholesterol diet was identical with the glucose diet except for the inclusion of 8.6 g of cholesterol/kg of diet. Rats were fed these diets for 1 week during which time food and water were available *ad libitum*.

Protein concentration was determined by the method of Lowry *et al.* (1951) and lipid phosphorus by the method of Bartlett (1959). Liquid-scintillation counting was done with Hydromix fluid using a Searle Analytic Delta 300 counter. Radiochromatograms were scanned with a Packard model 7201 chromatogram scanner.

Statistical evaluations were made by Student's *t* test with a difference between groups of $P < 0.05$ being considered significant.

Preparation of membrane fractions

Epididymal fat-pads were minced in 4 vol. (ml/g of tissue) of a buffer containing 10 mM-Tris/HCl (pH 7.4)/0.25 M-sucrose/1 mM-EDTA. Subsequent steps were carried out at 4°C. The minced tissue was homogenized by 12 passes of a Potter-Elvehjem homogenizer at 1325 rev./min. The crude homogenate was centrifuged at 750 g for 15 min. The infranant fraction was removed and centrifuged at 35 000 g for 15 min. The resulting supernatant fraction was centrifuged at 100 000 g for 1 h. The supernate was discarded and the pellet was suspended in the buffer to a protein concentration of 25–30 mg/ml.

Assay for transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to form dolichyl monophosphate [^{14}C]mannose

The assay has previously been described (Lucas & Levin, 1977). Each reaction mixture contained 10 μl of MnCl_2 (0.1 M), 50 μl of GDP-[^{14}C]mannose (3.3 μM , sp. radioactivity 440 c.p.m./pmol) and 50 μl of buffer containing less than 0.25 mg of microsomal protein. Incubations were for indicated times at 37°C and terminated by the addition of 2 ml of chloroform/methanol (2:1, v/v). When exogenous dolichyl phosphate was included as an acceptor 10 μl of the buffer was replaced by a suspension of dolichyl phosphate dispersed by sonication in 0.5% Ammonyx.

[^{14}C]Mannosyl lipid was prepared for chemical characterization by using microsomal preparations of fat-pads from chow-fed rats weighing approx. 250 g. Incubations were as described, except that 2 mg of membrane protein was used and the incubations were for 5 min.

Chromatography

Whatman SG-81 paper was prepared by the method of Steiner & Lester (1972) before use. Solvent systems used were: (A) chloroform/methanol/

water (65:25:4, by vol.); (B) di-isobutyl ketone/acetic acid/water (60:45:6, by vol.); (C) chloroform/methanol/conc. NH_3 (36:13:3, by vol.); Paper chromatography on Whatman 3MM employed the following solvent systems: (D) isobutyric acid/ NH_3 /water (57:4:39, by vol.); (E) ethyl acetate/pyridine/water (12:5:4, by vol.); Authentic mannose was identified by an aniline/diphenylamine reagent and mannose 1-phosphate was identified by a molybdate/ HClO_4 reagent (Dawson *et al.*, 1969).

Results

Characterization of [^{14}C]mannosyl lipid

Microsomal preparations of rat epididymal fat catalyse the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to a chloroform/methanol (2:1, v/v)-soluble compound. Chromatography of the [^{14}C]mannosyl lipid on SG-81 paper in three solvent systems (Fig. 1) reveals that it co-migrates with hen oviduct dolichyl monophosphate β -mannose.

The [^{14}C]mannosyl lipid is stable to treatment with mild alkali [0.1 M-KOH in methanol/toluene (3:1, v/v)] at 0°C for 60 min, conditions that deacylate phosphatidylglycerols. Strong alkaline hydrolysis (0.1 M-NaOH in 90% ethanol at 80°C for 10 min) releases a ^{14}C -labelled compound, which co-chromatographed in solvent system D with mannose 1-phosphate (Fig. 2a).

Mild acid treatment (0.1 M-HCl in 50% propanol at 50°C) releases a water-soluble compound with $t_{1/2} = 11$ min (Fig. 3). The ^{14}C -labelled water-soluble

product co-chromatographs with authentic mannose on solvent system E (Fig. 2b).

Formation of the [^{14}C]mannosyl lipid is rever-

Fig. 2. Hydrolytic products of [^{14}C]mannosyl lipid (a) [^{14}C]Mannosyl lipid was subjected to strong alkaline hydrolysis as described in the text. The water-soluble product (3000 c.p.m.) was mixed with a sample of mannose 1-phosphate and chromatographed in solvent system D. The hatched area indicates the location of mannose 1-phosphate. (b) [^{14}C]Mannosyl lipid was subjected to mild acid hydrolysis for 1 h as described in the text. The water-soluble product (3000 c.p.m.) was mixed with a sample of mannose and chromatographed in solvent system E. The hatched area indicates the location of mannose.

Fig. 1. Silica gel paper chromatography of [^{14}C]mannosyl lipid

Approx. 3000 c.p.m. of [^{14}C]mannosyl lipid were chromatographed with 3000 c.p.m. of hen oviduct dolichyl monophosphate [^{14}C]mannose. (1) Adipose mannosyl lipid; (2) oviduct mannosyl lipid; (3) mixture of adipose and oviduct mannosyl lipids. Chromatographs were run in solvent systems A, B, and C (a, b and c respectively) and exposed to Kodak No-Screen X-ray film.

Fig. 3. Time course of mild acid hydrolysis. $[^{14}\text{C}]$ Mannosyl lipid (5000 c.p.m.) was dried in a conical test tube and 250 μl of propanol and 250 μl of 0.2M-HCl were added and incubated at 50°C. At the indicated times 50 μl portions were withdrawn and added to 4 ml of chloroform/methanol (2:1, v/v) and 1 ml of 5 mM-NaOH. The radioactivity in both the aqueous and organic phases was counted to assess hydrolysis of the mannosyl lipid.

Table 1. Metal-ion dependence

Standard assay conditions were used, except that MnCl_2 was replaced by the additions shown.

Addition	Radioactivity incorporated (c.p.m.)
Water	0
EDTA (10mM)	15
MnCl_2 (2mM)	1695
MnCl_2 (10mM)	1777
CoCl_2 (2mM)	1970
CoCl_2 (10mM)	2177
CaCl_2 (10mM)	320
MgCl_2 (10mM)	1215

sible by the addition of GDP, but not by GMP (results not shown).

As will later be shown in the text, the formation of the chloroform/methanol-soluble compound is stimulated by the addition of exogenous dolichyl phosphate.

The sum of the chemical and chromatographic data indicates that the $[^{14}\text{C}]$ mannosyl lipid is dolichyl monophosphate β -mannose. The same compound is also formed by microsomal preparations from isolated adipocytes prepared as described by Rodbell (1964).

Characteristics of enzymic formation of dolichyl monophosphate mannose

The formation of dolichyl monophosphate $[^{14}\text{C}]$ -mannose exhibited a pH optimum at pH 7.4. The transfer of mannose requires a bivalent cation and the dependency of the reaction on bivalent cations is shown in Table 1. The order of effectiveness is $\text{Co}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+} \gg \text{Ca}^{2+}$.

The dependency of $[^{14}\text{C}]$ mannose transfer on membrane protein is shown in Fig. 4. The reaction was linear up to 0.3 mg of protein. Therefore, the kinetic experiments in the following section were performed with less than 0.25 mg of membrane protein per assay.

Effects of diets on dolichyl monophosphate mannose formation

The time course for the formation of dolichyl monophosphate $[^{14}\text{C}]$ mannose using membrane preparations from rats fed different diets is shown in Fig. 5. Inspection of the curves indicates that pre-

Fig. 4. Dependency of mannosyl lipid formation on protein

Transfer of $[^{14}\text{C}]$ mannose from GDP- $[^{14}\text{C}]$ mannose to endogenous acceptor was assayed as described in the Experimental section, except that the amount of membrane protein added was varied.

Fig. 5. Effect of diets on time course of mannosyl lipid formation

Microsomal preparations from rats fed different diets were assayed for [¹⁴C]mannosyl lipid formation as described in the Experimental section. Fat-pads from groups of 12 rats were pooled and membranes were prepared as described. The means $\pm$ S.D. are shown for glucose diet ($\blacktriangle$; four groups), lard diet ($\bigcirc$; four groups), glucose-plus-cholesterol diet ($\blacksquare$; three groups), rat chow diet ($\square$; two groups) and fructose diet ($\bullet$; three groups).

Fig. 6. Double-reciprocal plot of data in Fig. 5
The inverses of both time and mannosyl lipid formation from Fig. 5. were calculated and plotted as a double-reciprocal plot. The straight lines were fitted by a linear regression analysis. The linear correlation coefficients are 0.98–1.0.

parations from rats fed on the fructose diet exhibit the greatest transfer of [¹⁴C]mannose from GDP-[¹⁴C]mannose into dolichyl monophosphate mannose, whereas preparations from rats fed on the glucose diet have the least transfer. The curves can be fit to a straight line by taking the reciprocals of both the c.p.m. of [¹⁴C]mannose incorporated and time (Fig. 6). Analysis of the curves by such double-reciprocal plots to determine the maximal amount of mannose transferred also shows that preparations from fructose-fed animals have the greatest incorporation, although chow-fed and glucose-plus-cholesterol-fed rat preparations have approx. 75% as much transferred. Preparations from glucose-fed animals have 45% as much and preparations from lard-fed rats have 55% as much [¹⁴C]mannose transferred. [The difference in the 30 min time point between the lard- and glucose-fed animals was statistically significant ($P < 0.05$).] It is also noteworthy that preparations from glucose-plus-cholesterol-fed animals have significantly greater transfer of mannose than do the preparations from the glucose-fed animals.

The differences in the amount of dolichyl monophosphate [¹⁴C]mannose formed reflect differences in the amounts of endogenous dolichyl phosphate in the membranes. Studies with several other tissues (Lucas & Levin, 1977; Harford *et al.*, 1977; Lucas & Nevar, 1978; Lucas, 1979) have led to the suggestion that changes in endogenous dolichyl phosphate concentrations rather than changes in mannosyltransferase in protein glycosylation regulate mannosyl transfer. To determine if there are any changes in the mannosyltransferase(s) in response to diet, the enzyme(s) were further kinetically characterized.

Table 2 shows the apparent K_m and V_{max} values for GDP-[¹⁴C]mannose obtained from double-reciprocal (Lineweaver–Burk) plots for the membrane preparations from rats fed the various diets. The data in Table 2 show virtually no differences with respect to GDP-mannose dependency in the preparations from the rats fed the various diets.

Fig. 7 shows the dependence of [¹⁴C]mannose transfer on exogenous dolichyl phosphate. Saturating concentrations of dolichyl phosphate are about

Table 2. Apparent kinetic constants

Preparations from rats fed the indicated diets were assayed for 2 min as described in the Experimental section, except that various amounts of GDP-[¹⁴C]mannose were added

Diet	K_m (μ M)	V_{max} (c.p.m./mg)
Glucose	0.27	10650
Lard	0.25	10970
Fructose	0.23	11080
Glucose and cholesterol	0.22	11730

Fig. 7. Dependence of mannosyl lipid formation on exogenous dolichyl phosphate

Preparations from glucose-fed (▲) and lard-fed (○) rats were assayed as described in the Experimental section with various amounts of exogenously added dolichyl phosphate. Data are expressed as the ratio of stimulation with dolichyl phosphate versus nmol of dolichyl phosphate in the incubation mixture.

Fig. 8. Initial velocity of mannosyl lipid function. Preparations from rats fed different diets were assayed for up to 50s as described in the Experimental section with 10nmol of dolichyl phosphate added. Results are shown for glucose diet (▲; three groups), lard diet (○; four groups), glucose-plus-cholesterol diet (□; two groups) and fructose diet (●; three groups).

10nmol per incubation. This amount was used in the following experiment.

The relative amount of enzyme in the various preparations can be assessed by assaying the enzyme at saturating concentrations of GDP-[^{14}C]mannose and dolichyl phosphate. Fig. 8 shows that under such conditions the initial velocity of [^{14}C]mannose transfer in preparations from lard-fed animals is about 1.5 times the rate of transfer in the preparation from rats fed the other three diets. The 30s and 50s time points are statistically different ($P < 0.05$) when the preparations from lard-fed animals are compared with glucose-fed animals.

Discussion

The chemical and chromatographic data shown in Figs. 1–3 indicate that membrane preparations from adipose tissue can form a compound with the properties of dolichyl monophosphate β -mannose. This is the first evidence for a dolichol-linked saccharide in adipose tissue. Although dolichyl monophosphate mannose is an intermediate in the transfer of mannosyl units from GDP-mannose to protein acceptors in other systems (Behrens, 1974; Lucas & Waechter, 1976; Waechter & Lennarz, 1976; Hemming, 1977a,b), we have not observed transfer of mannose from dolichyl monophosphate mannose into an oligosaccharide-lipid or protein acceptor. The adipocyte does not produce large quantities of either secretory or membrane glycoproteins, and the absence of further transfer of the mannose residue from dolichyl monophosphate mannose may reflect very low concentrations (or absence) of additional endogenous acceptors. On the other hand, the absence of further transfer may be the result of inappropriate assay conditions. These possibilities require further investigation.

In other membrane systems studied, the concentration of endogenous dolichyl phosphate is rate limiting and modulation of dolichyl phosphate concentration has been postulated as a possible regulatory mechanism in the glycosylation of protein via the lipid intermediate pathway (Lucas & Levin, 1977; Harford *et al.*, 1977; Hemming, 1977b; Lucas & Nevar, 1978; Lucas, 1979). In the light of the observation that the carbohydrate composition of liver plasma-membrane glycoproteins is different when animals are fed different diets (Henriquez *et al.*, 1979), we undertook a study of the kinetics of mannosyl transfer to ascertain whether the apparent differences in protein glycosylation could be attributed to modulation of dolichyl phosphate concentrations or to differences in the mannosyltransferase(s).

The amount of dolichyl monophosphate mannose formed during incubations is a function of the endogenous dolichyl phosphate concentration. The time

courses of dolichyl monophosphate mannose shown in Fig. 5 indicate that preparations from fructose-fed animals, chow-fed animals and glucose-plus-cholesterol-fed animals have larger endogenous concentrations of dolichyl phosphate than preparations from lard-fed and glucose-fed animals. Because of these apparent differences in dolichyl phosphate concentrations, further kinetic characterization was performed.

The mannosyltransferase activity can be measured by assaying the formation of dolichyl monophosphate mannose under saturating conditions for GDP-mannose and dolichyl phosphate. When such an experiment is performed, the preparations from lard-fed animals are found to have approx. 1.5 times the mannosyl-transfer activity as the other three preparations.

The differences between lard-fed and glucose-fed animals are of interest because of the previous studies demonstrating that the carbohydrates of plasma-membrane glycoproteins of liver and adipose tissue can be modified by these two diets. However, the studies presented here indicating that preparations from lard-fed animals have more enzyme and more endogenous dolichyl phosphate than glucose-fed animals seem to be at variance with the previous studies showing a decreased carbohydrate content of liver plasma-membrane glycoproteins (Henriquez *et al.*, 1979) when animals are fed the lard diet. Although the carbohydrate content of the adipocyte plasma membrane has not been measured directly, its responses to insulin and concanavalin A suggest that its compositional changes in response to diet are similar to those observed in liver. It is possible that the decreases observed in the liver plasma-membrane carbohydrate composition when animals are fed the lard diet can be attributed to decreased transfer of peripheral sugars arising from direct transfer from sugar nucleotides, not involving lipid intermediates. If this is the case, at least part of the changes elicited by diet may be the result of shifts in sugar nucleotide or sugar phosphate pools.

Also of interest are the results obtained when cholesterol is included in the glucose diet. (It should be noted that the lard diet also contains cholesterol.) Dolichol synthesis and cholesterol synthesis share a common pathway up to farnesyl pyrophosphate (Gough & Hemming, 1970a,b) and the inhibition of cholesterol synthesis at hydroxymethylglutaryl-CoA reductase may be expected to have an effect on dolichol synthesis. In fact, Mills & Adamany (1978) have demonstrated that inhibitors of cholesterol synthesis inhibit the formation of dolichyl-linked oligosaccharide and protein glycosylation in smooth-muscle cell cultures. On the other hand, Hemming and his colleagues (Coolbear & Hemming, 1979; Tavares *et al.*, 1979) have observed increases in liver dolichol synthesis and dolichyl monophosphate

mannose and dolichyl monophosphate glucose synthesis on inclusion of cholesterol in the diet. Although the dietary regimens used in our experiments are different from those used by Hemming and his co-workers, we observe similar results. The apparent discrepancy between the results obtained by us and Hemming on the one hand, and Mills and Adamany on the other, may be the result of performing experiments with whole animals as opposed to using cells in tissue culture.

The mechanism by which cholesterol modulates the concentrations of dolichyl phosphate is unknown. Mills & Adamany (1978) were unable to detect any differences in hydroxymethylglutaryl-CoA reductase activity from inhibited and control cells. However, the activities of the enzyme were low and they may not have detected small but significant changes in activity. Coolbear & Hemming (1979) have suggested that cholesterol may be inducing generalized membrane changes that are manifested as apparent differences in the dolichyl phosphate concentrations. These points will need to be studied in more detail before an answer to these questions will be obtained.

In summary, we have presented evidence for the existence of dolichyl monophosphate mannose in adipose tissue and also have shown that the endogenous concentration of dolichyl phosphate and mannosyltransferase activity can be altered in response to dietary manipulation. The modulation of dolichol- and dolichyl phosphate-linked saccharide biosynthesis promises to be an interesting and important area of research.

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