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# *N*-Glycosylation of membrane glycoproteins in retinol-deficient rat liver

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The effect of vitamin A deficiency on *N*-linked oligosaccharides of membrane glycoproteins was studied in rat liver in order to evaluate the suggested role of retinol in protein *N*-glycosylation. First, oligosaccharides of newly synthesized glycoproteins from rough endoplasmic reticulum of vitamin A deficient liver were compared with that of pair-fed controls. Oligosaccharides were metabolically labelled with D-[2-$^3$H]mannose, released from the glycoproteins with endoglycosidase H, purified by reversed phase HPLC and ion exchange chromatography, and were reduced with sodium borohydride. HPLC fractionation of the oligosaccharide alditols showed that the glycoproteins carried mainly four oligosaccharide species, $Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$, in identical relative amounts in the vitamin A deficient and the control tissue. In particular, no increase in the proportion of short chain oligosaccharides was noted in vitamin A deficient liver. Second, the number of *N*-linked oligosaccharides was estimated in dipeptidylpeptidase IV (DPP IV), a major glycoprotein constituent of the hepatic plasma membrane, comparing the newly synthesized glycoprotein from rough endoplasmic reticulum and the mature form of DPP IV from the plasma membrane. No evidence was obtained that retinol deficiency caused incomplete glycosylation of this membrane glycoprotein. From these data, the suggested role of retinol as a cofactor involved in the synthesis of *N*-linked oligosaccharides of glycoproteins must be questioned.

*Keywords*: Vitamin A, retinol, *N*-linked oligosaccharides, membrane glycoproteins, dipeptidylpeptidase IV

*Abbreviations*: DolP, Dolichyl phosphate; DolPP, dolichyl pyrophosphoryl; RetPMan, retinyl phosphate mannose; DPP IV, dipeptidyl peptidase IV (EC 3.4.14.5); endo H, endo-$\beta$-*N*-acetylglucosaminidase H (EC 3.2.1.96); endo F, endo-$\beta$-*N*-acetylglucosaminidase F (EC 3.2.1.96); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The cellular vitamin A status has profound effects on the glycosylation of membrane and secretory glycoproteins [1–3]. The mechanisms by which retinol influences glycosylation, however, are poorly understood. Four observations suggest that retinol is involved in the synthesis of the common precursor oligosaccharide $Glc_3Man_9GlcNAc_2$, which is assembled linked to the polyisoprenoid lipid carrier dolichol [4]: (i) decreased incorporation of [$^{14}$C]mannose into glycoproteins in retinol-deficient animals [5], and (ii) increased incorporation of [$^{14}$C]mannose but not of [$^3$H]galactose into glycoproteins by excess vitamin A [3], (iii) the identification of retinyl phosphate mannose in the endoplasmic reticulum [6–8], and (iv) the accumulation of the shorter-chain oligosaccharide intermediate $DolPPGlcNAc_2Man_5$ in vitamin A deficient rat liver [9].

From the above findings, it was concluded that RetPMan functions as a cofactor in the transfer of the four outer mannose residues to $DolPPGlcNAc_2Man_5$. However, the recent discovery that the endogenous mannolipid so far regarded as RetPMan is not RetPMan but phosphatidylmannose raised doubts as to the physiological relevance of this assumption [10]. Moreover, it has not been proven so far that shorter chain oligosaccharides in vitamin A deficient cells are indeed transferred from DolPP to newly synthesized

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glycoproteins. Studies using isolated microsomal membranes as an *in vitro* system failed to detect shorter chain oligosaccharides in microsomal glycoproteins obtained from vitamin A deficient animals [11]. However, the glycoproteins studied in this *in vitro* system were obtained from incubation of isolated vitamin A deficient membranes with exogenous GDP-[$^{14}$C]mannose. Since vitamin A deficiency has been suggested to impair the synthesis of endogenous GDP-mannose [12], addition of exogenous GDP-[$^{14}$C]mannose could conceal this metabolic effect of vitamin A depletion. Moreover, oligosaccharides transferred to the glycoproteins were not processed in this *in vitro* system, indicating that the system does not completely reflect *in vivo* conditions.

In an attempt to evaluate the suggested role of retinol in protein glycosylation *in vivo*, we therefore comparatively characterized the oligosaccharide chains transferred to newly synthesized glycoproteins in intact liver of vitamin A deficient rats and pair-fed controls after metabolic labelling with D-[2-$^{3}$H]mannose. Furthermore, the effect of vitamin A deficiency on the glycosylation of individual glycoproteins was assessed using the plasma membrane ectoenzyme dipeptidylaminopeptidase IV as a model glycoprotein.

## Materials and methods

### *Chemicals and enzymes*

Radiochemicals were from Amersham Buchler (Braunschweig, Germany), endoglycosidases from Boehringer Mannheim (Mannheim, Germany), protease inhibitors, antipain and chymostatin, from Sigma (Deisenhofen, Germany), pepstatin and leupeptin from Boehringer Mannheim, and Kallikrein inhibitor from Bayer AG (Leverkusen, Germany). Mega-10 (decanoyl-*N*-methylglucamide) was obtained from Calbiochem (Frankfurt/Main, Germany) and Protein A-Sepharose was from Pharmacia (Freiburg, Germany). All other reagents were of analytical grade and were purchased either from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Sigma.

### *Preparation of vitamin A deficient and pair-fed rats*

Male Wistar rats (Fü-Albino SPF, Nafag 854) bred in the laboratories of Hoffmann-La Roche (Basel, Switzerland) were weaned when they had reached a body weight of 30 g. The animals were fed a vitamin A free diet (Klingenthalmühle, Basel, Switzerland) based on a highly purified casein (Hoffmann-La Roche, Basel, Switzerland). The rats were checked daily for retardation of growth and skin disorders. Retinyl acetate dissolved in arachis oil was given by stomach tube in doses of 0–5 IU daily in order to attain a body weight of 120–140 g within 6–10 weeks. Control animals were kept pair-fed on the same vitamin A free diet, but were given 100 IU retinyl acetate daily. Vitamin A content in liver and serum was determined using an HPLC method [13]. Retinol concentration in the livers of vitamin A depleted rats (206 ± 68 ng per g dry weight) was reduced to about 0.5% of that of pair-fed controls. Retinol concentrations in the livers of pair-fed controls were within the normal range (44 420 ± 17 770 ng per g dry weight). Vitamin A deficient animals, but not pair-fed controls showed symptoms of vitamin A deficiency, e.g., keratinization of the mucociliary epithelium of tracheas, xerophthalmia and paralysis of hind legs. The animals were kept in windowless rooms at 24°C with constant humidity, and light from 07.30 to 19.30.

### *Isolation of subcellular fractions*

Endoplasmic reticulum, Golgi apparatus and plasma membranes were isolated from the livers of vitamin A deficient and pair-fed rats as described [14]. Purity of all fractions was checked by assay of marker enzymes with methods given in the references: glucose 6-phosphatase [15], galactosyltransferase [16], and 5′-nucleotidase [17] (Table 1).

**Table 1.** Specific activities of marker enzymes in the homogenate and in subcellular fractions.[a].

| *Fraction* | *5′-Nucleotidase*[b] | *Glucose 6-phosphatase*[b] | *Galactosyl-transferase*[c] |
|---|---|---|---|
| *Homogenate* | | | |
| Vitamin A deficiency | 1.72 | 3.61 | 1.55 |
| Pair-fed | 1.88 | 4.35 | 1.88 |
| *Endoplasmic reticulum* | | | |
| Vitamin A deficiency | 1.92 (1.1) | 15.5 (4.3) | 5.01 (3.2) |
| Pair-fed | 2.58 (1.4) | 17.5 (4.0) | 3.74 (2.0) |
| *Golgi apparatus* | | | |
| Vitamin A deficiency | 7.73 (4.5) | 9.93 (2.7) | 64.9 (41.9) |
| Pair-fed | 6.92 (3.7) | 15.3 (3.5) | 133.6 (71.1) |
| *Plasma membrane* | | | |
| Vitamin A deficiency | 49.1 (28.5) | 1.86 (0.5) | 2.48 (1.6) |
| Pair-fed | 37.2 (19.7) | 1.81 (0.4) | 3.96 (2.1) |

[a] Subcellular fractions were prepared from the livers of vitamin A deficient rats and pair-fed controls, and enzyme activities were assayed as described in the Materials and methods section. Units of specific activity are μmol of inorganic phosphate formed per h per mg protein for 5′-nucleotidase and glucose 6-phosphatase, and nmol of galactose transferred to acceptor per h per mg protein. Means of 5 preparations, SD being in the range below 20% of the mean. Values in parentheses are ratios of specific activities in the isolated fractions to that of the homogenate.
[b] μmol $h^{-1}$ $mg^{-1}$.
[c] nmol galactose transferred $h^{-1}$ $mg^{-1}$.

*In vivo labelling of animals*

Vitamin A deficient rats and pair-fed controls (120–140 g) were intravenously injected with either D-[2-$^3$H]mannose (600 µCi per 100 g body weight, 18.3 Ci mmol$^{-1}$, Amersham) or L-[$^{35}$S]methionine (500 µCi per 100 g body weight, 1020 Ci mmol$^{-1}$, Amersham). In pulse-chase experiments with L-[$^{35}$S]methionine, 100 mg of unlabelled L-methionine were given as a chase 10 min after injection of the isotope. Livers were perfused with saline, containing 2 mM $CaCl_2$, and removed under slight ether anaesthesia.

*Isolation of oligosaccharides from membrane glycoproteins*

*Delipidation.* Samples of isolated endoplasmic reticulum labelled with D-[2-$^3$H]mannose were thawed by ultrasonication for 5 min at 4°C and sonicated for a further 15 min. Secretory glycoproteins were removed by centrifugation at 45 000 × **g** for 30 min at 4°C. The pellet containing membrane bound glycoproteins was resuspended in distilled water (6 mg protein in 3 ml), homogenized by ultrasonication for 15 min at 4°C, frozen and lyophilized. Traces of water were removed by careful drying over phosphorus pentoxide in a desiccator. Glycolipids were separated from the glycoprotein fraction by mixing the dried pellet in chloroform–methanol (2:1 by vol) at 4°C for 10 min followed by ultrasonication for 15 min. After centrifugation at 45 000 × **g** for 30 min, the overlayer was carefully removed from the pellet and the extraction procedure was repeated. Extracts were combined and counted for radioactivity. More polar glycolipids were extracted twice with chloroform–methanol–water (10:10:3 by vol). After a final centrifugation and careful removal of the overlayer, 1 ml methanol was immediately added to the pellet prior to freezing at −20°C to avoid drying of the subsequent glycoprotein fraction leading to incomplete solubilization in the following step.

*Release of oligosaccharides by endo-β-N-acetylglucosaminidase H* (endo *H*). Pellets were solubilized in 1.5 ml 2% (w/v) sodium dodecylsulfate (SDS) plus 200 mM mercaptoethanol, and methanol was removed at 40°C in a stream of nitrogen. Residual traces of methanol were removed by performing twice the addition of 1 ml of distilled water followed by concentration in a stream of nitrogen. Samples were then completely solubilized by three cycles of ultrasonication for 15 min and incubation at 80°C for 5 min, and were diluted to 30 ml (0.1% SDS, 10 mM mercaptoethanol) with distilled water. Low molecular weight contaminants were removed by three cycles of ultrafiltration in a Centriprep-10 concentrator tube (Amicon, Witten, Germany) at 3000 × **g** and 10°C for 2 h and twice washing in 0.1% SDS plus 10 mM mercaptoethanol. Dilution of solubilized protein to 0.1% SDS is necessary to avoid enrichment of the detergent in the protein fraction during ultrafiltration due to the development of micelles that would inhibit endoglycosidase H in the following deglycosylation step. By ultrafiltration the sample was obtained in 3 ml 0.1% SDS plus 10 mM mercaptoethanol, and was further concentrated to a volume of 300 µl in 1% SDS plus 100 mM mercaptoethanol, in a stream of nitrogen. Samples were then mixed with 90 µl 2% (w/v) bovine serum albumin and 450 µl of 0.2 M sodium citrate buffer, pH 5.5, containing 1 M sodium thiocyanate and 1% Mega-10. To prevent proteolysis and growth of bacteria, 90 µl leupeptin, pepstatin, chymostatin, antipain and Kallikrein inhibitor (0.5 mg ml$^{-1}$ each) and 5 µl of toluene were added. N linked oligosaccharides of the high mannose type were released by incubation with 50 mU endo-H at 37°C for 64 h. Released oligosaccharides were separated from protein by centrifugation in a Centricon-10 microconcentrator (Amicon) (4000 × **g**, 2 h). The rate of deglycosylation was estimated by measuring the radioactivities in the filtrate containing the released oligosaccharides and in the protein fraction. Filtrates were evaporated under reduced pressure in an Univapo centrifuge 150 H (Uniequip, München, Germany) at 40°C.

*Reversed phase HPLC and ion exchange chromatography.* Detergents and salts were removed from the oligosaccharide preparation by reversed phase chromatography on an Inertsil ODS II column (Gynkotek, München, Germany) (5 µm, 0.46 cm × 25 cm), using distilled water as eluent at a flow rate of 0.55 ml min$^{-1}$ at 65 °C. 20 Fractions of 2.2 ml were collected and monitored for radioactivity. Fractions 2–6 containing the labelled oligosaccharides were pooled and evaporated to dryness. Samples were redissolved in 1 ml distilled water and passed through a column (0.6 cm × 10 cm) containing 500 µl Dowex 50 AG 50W-X2 ($H^+$-form) (Bio-Rad, München, Germany) and 500 µl AG 3-X4A ($OH^-$-form). The column was washed seven times with 1 ml of distilled water, and the effluent and washings, containing the reducing high-mannose oligosaccharides, were concentrated to dryness.

*Preparation of oligosaccharide alditols.* Oligosaccharides dissolved in 100 µl 0.2 M sodium borate, pH 9.1, were reduced by addition of 100 µl of sodium borohydride (30 mg ml$^{-1}$ in 0.1 N NaOH) and incubation for 4 h at 30 °C. An additional 100 µl of freshly prepared $NaBH_4$ solution (30 mg ml$^{-1}$ in ice cold sodium borate, pH 9.1) was added and the reaction was continued for a further 2 h at 30 °C. The solution was adjusted to pH 6.0 by dropwise addition of 1 N acetic acid. Sodium ions were separated from oligosaccharide by passage through an AG 50-X12 ($H^+$) column (1 ml bed volume). The column was washed with five bed volumes of distilled water and the combined filtrates were evaporated to dryness at 30 °C. Boric acid was removed by five times evaporation with 1 ml of methanol, and traces of acetic acid were removed by drying the oligosaccharides over sodium hydroxide in a desiccator.

*Fractionation of oligosaccharides*

*Oligosaccharide standards.* The oligomannosidic glycans $Man_5GlcNAcOH$, $Man_6GlcNAcOH$, $Man_7GlcNAcOH$,

$Man_8GlcNAcOH$ and $Man_9GlcNAcOH$, metabolically labelled with D-[2-$^3$H]mannose, were a generous gift of Dr. R. Geyer (Biochemisches Institut, Universität Gießen, Germany). They were prepared from $HA_2$ subunits of influenza virus haemagglutinin by endo-H treatment and $NaBH_4$ reduction [18].

Standard glucose oligomers were prepared by hydrolysis of dextran (mol.wt 210000) in 0.1 N HCl for 4 h at 100 °C as described by Yamashita *et al.* [19]. Labelling of standard glucose oligomers by reductive amination with 8-amino-2-naphthol was performed as will be described in detail elsewhere. Briefly, 300 µl (30 mg) of hydrolysed dextran were lyophilized, dried over phosphorus pentoxide and incubated with 47.8 mg aminonaphtol (0.3 mM, Aldrich, Steinheim, Germany) dissolved in 1.2 ml dry dimethylsulfoxide in a screw capped vial with Teflon sealing at 82 °C for 30 min. The mixture was allowed to come to room temperature, and 19 mg (0.3 mmol) sodium cyanoborohydride in 1.5 ml methanol and 150 µl acetic acid were added. After stirring at 82 °C for 1.5 h, additional equal amounts of sodium cyanoborohydride and acetic acid were added and stirring was continued for 2 h. Cyanoborohydride was removed by passing through a column of AG 3-X4 A (acetate), and the filtrate was evaporated to dryness. Labelled oligosaccharides were dissolved in 2 ml distilled water, separated from aminonaphthol by filtration through a Minisart SRP 15 filter (Sartorius, Göttingen, Germany), and were finally purified from residual traces of the reagent by reversed phase HPLC in an increasing gradient of acetonitrile in water.

*HPLC fractionation of oligosaccharides.* Chromatography was performed using a Bio-Rad Model 700 chromatography workstation equipped with two Bischoff (Leonberg, Germany) Model 2200 pumps, a Knauer (Berlin, Germany) dynamic mixing chamber and a Shimadzu fluorescence HPLC monitor RF-535. Samples were dissolved in 20 µl distilled water, mixed with 10 µl of fluorescent labelled glucose oligomers as internal standard and introduced into the column by a Knauer A 0263 injection valve carrying a 500 µl loop containing 100% acetonitrile. The oligosaccharide mixture was separated on an APS Hypersil column (0.46 cm × 25 cm; Bischoff Analysentechnik, Leonberg, Germany), equilibrated with 65% acetonitrile and 35% 0.015 M sodium phosphate buffer, pH 5.2, at a flow rate of 1.5 ml min$^{-1}$ at room temperature. After injection of a sample, the proportion of acetonitrile was decreased to 45% over 100 min and then to 30% over 1 min. Flurometric detection of glucose oligomers was employed with an excitation wavelength of 335 nm and an emission wavelength of 415 nm. Fractions of 750 µl were collected and monitored for radioactivity. Figure 1 summarizes the isolation and fractionation procedures.

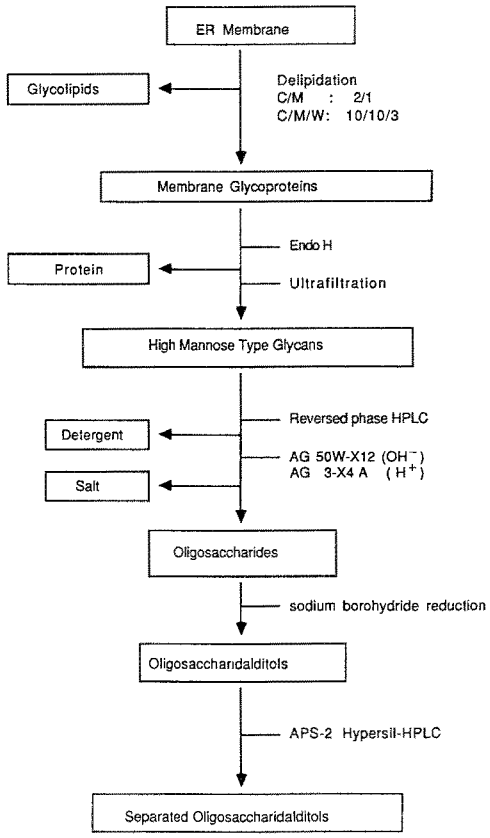


**Figure 1.** Isolation and fractionation of oligomannose type oligosaccharides from ER glycoproteins. For details see the Materials and methods section.

*Determination of radioactivity*

Radioactivity was determined by liquid scintillation counting using a Tri-Carb 1900 CA liquid scintillation analyser (Canberra Packard). Counting efficiency was monitored by external standard and channels ratio method. Protein-bound radioactivity of subcellular fractions was determined by a modification [16] of the method of Mans and Novelli [20]. Protein was determined by the method of Lowry *et al.* [21].

*Immunoprecipitation*

Dipeptidylpeptidase IV was immunoprecipitated from endoplasmic reticulum and plasma membranes metabolically labelled with L-[$^{35}$S]methionine as described [22] using the monoclonal anti-DPP IV antibody MAB De 13.4 [23]. Digestion of immunoprecipitated DPP IV with endo-H (EC 3.2.1.96, of *Streptomyces plicatus* from *Streptomyces lividans*) or with a preparation of endo-$\beta$-*N*-acetylglucosaminidase F (endo-F) (EC 3.2.1.96) containing glycopeptidase F activity (EC 3.2.2.18) (from *Flavobacterium meningosepticum*) was done as described [22].

**Table 2.** Radioactivity in disintegrations $min^{-1}$ per mg protein.

| *Isolated membranes from* | *Chloroform–methanol extracts (2:1 by vol)*[a] | *Chloroform–methanol–water (10:10:3 by vol) extracts*[a] | *Glycoprotein fraction*[a] | *Oligosaccharides released from glycoproteins by endo-H (% of protein bound radioactivity)*[a] | L-[$^{35}$S]*Methionine labelled proteins*[b] |
|---|---|---|---|---|---|
| Vitamin A deficient rats | 1834 (1.5) | 12799 (10.7) | 104642 (87.7) | 96479 (92.2) | 34325 |
| Pair-fed controls | 2762 (1.6) | 11636 (6.8) | 156680 (91.6) | 151509 (96.7) | 28460 |

[a] Retinol deficient rats and pair-fed controls were i.v. injected with D-[2-$^3$H]mannose (600 μCi per 100 g body weight). 30 min after labelling, endoplasmic reticulum was isolated from the pooled livers of four vitamin A deficient or three pair-fed rats. After removal of soluble secretory glycoproteins, membranes were successively extracted with chloroform–methanol (2:1 by vol) and chloroform–methanol–water (10:10:3 by vol). The delipidated glycoprotein fraction was completely solubilized in 0.1% SDS plus 10 mM mercaptoethanol and oligomannosidic *N*-glycans were released by endo-H, as described in the Materials and methods section. Solvent extracts, delipidated glycoprotein fractions and released oligosaccharides were measured for radioactivity.
[b] Endoplasmic reticulum was isolated 120 min after i.v. injection of L-[$^{35}$S]methionine (500 μCi per 100 g body weight), and protein bound radioactivity was determined in the total isolated membrane proteins.

*SDS-PAGE*

SDS-PAGE was carried out according to the procedure of Laemmli [24] with some modifications [25]. $M_r$ standards were carbonic anhydrase ($M_r$ 29000), ovalbumin ($M_r$ 45000), bovine serum albumin ($M_r$ 66000), phosphorylase b from rabbit muscle ($M_r$ 97400), β-galactosidase from *E. coli* ($M_r$ 116000) and myosin from rabbit muscle ($M_r$ 205000). Radiofluorographic analysis of gels was done as described by Bonner and Laskey [26] using Kodak XAR-5 film.

## Results

*Characterization of oligomannosidic* N*-glycans transferred to membrane glycoproteins*

Endoplasmic reticulum could be isolated at comparable levels of fraction purity from the livers of both vitamin A deficient rats and pair-fed controls (Table 1). Enrichment factors were similar to those obtained by other reported isolation procedures [27]. Thirty minutes after metabolic labelling of animals with D-[2-$^3$H]mannose, approximately 1.5% of labelled mannose was incorporated into chloroform–methanol (2:1 by vol) extracts of the isolated ER membranes (Table 2). Approximately 10% became soluble in chloroform–methanol–water (10:10:3 by vol), and approximately 90% was recovered in the protein fraction. Chloroform–methanol (2:1 by vol) has been shown to extract DolPMan and RetPMan [11, 28], while chloroform–methanol–water (10:10:3 by vol) is a solvent known to extract lipid-linked oligosaccharides [11, 29, 30]. In vitamin A deficient rats, incorporation into both chloroform–methanol (2:1 by vol) extracts and the protein fraction was reduced to about 66% of pair-fed controls, whereas incorporation into material solubilized by chloroform–methanol–water (10:10:3 by vol) was not changed. Incorporation of L-[$^{35}$S]methionine into ER membrane proteins determined as a measure of protein biosynthesis showed no difference between vitamin A deficient rats and pair-fed controls (Table 2).

Oligosaccharides labelled with D-[$^3$H]mannose were nearly quantitatively released from the delipidated membrane glycoproteins by endo-H (Table 2). Control incubations performed for 24 h at 37 °C without endo-H resulted in a release of less than 10% of the [$^3$H]mannose label. The released oligosaccharides were separated from glycoproteins by ultrafiltration and were further purified from salts and detergent by reversed phase HPLC and ion exchange chromatography.

Following reduction with $NaBH_4$, oligosaccharide alditols were separated by HPLC on an APS Hypersil column and the size of the oligomannosidic species was determined by comparison with standard structures (Figs 2, 3). Oligosaccharide alditols obtained from both vitamin A deficient rats and pair-fed controls yielded four major peaks, I, II, III, and IV. Peak I co-eluted with oligosaccharide standard $Man_7GlcNAcOH$, peak II with $Man_8GlcNAcOH$ and peak III with $Man_9GlcNAcOH$. Peak IV eluted at the position of 13.2 aminonaphthol-derivatized glucose oligomer units and most likely represents $Glc_1Man_9GlcNAcOH$. As estimated from the distribution of [$^3$H]mannose radioactivity in the four peaks, the oligosaccharide of peak III representing $Man_9GlcNAcOH$ was the principal structure in both retinol deficient rats and controls, followed by that of peak II ($Man_8GlcNAcOH$) > peak IV ($Glc_1Man_9GlcNAcOH$) > peak I ($Man_7GlcNAcOH$). No differences in the estimated ratios of the four oligosaccharide structures were noted when comparing vitamin A deficient rats and pair-fed controls.

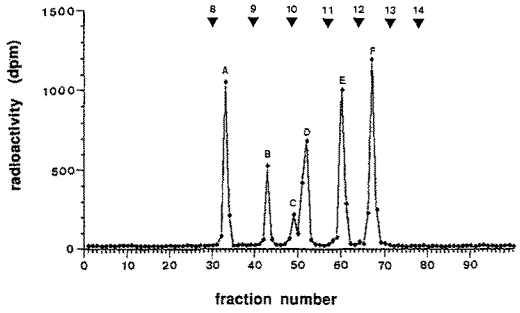


**Figure 2.** Separation of authentic oligomannose type oligosaccharide alditols. Authentic oligomannose type oligosaccharide alditols $Man_5GlcNAcOH$ (A), $Man_6GlcNAcOH$ (B), $Man_7GlcNAcOH$ (C), $Man_7GlcNAcOH$ (D), $Man_8GlcNAcOH$ (E) and $Man_9GlcNAcOH$ (F) were separated on APS-2 Hypersil (3 μm, Shandon) in a decreasing gradient of acetonitrile in 15 mM sodium dihydrogen phosphate buffer, pH 5.2. For further conditions see the Materials and methods section. Black arrows on the top indicate the eluting position of glucose oligomer units fluorescence labelled by reductive amination with 2-amino-8-naphthol.

### *Glycosylation of dipeptidylpeptidase IV from ER and plasma membranes*

The reduced incorporation of [$^3$H]mannose into microsomal acceptor proteins in vitamin A deficient liver reported by several investigators [5, 11, 12] and also found in this study for the total protein fraction of endoplasmic reticulum (Table 2) has been suggested to reflect a defect in the transfer of oligosaccharide units from the lipid carrier to the newly synthesized glycoproteins [11]. We have tested if vitamin A deficiency results in incomplete protein glycosylation using DPP IV, a major constituent of the hepatocytic plasma membrane, as a model glycoprotein [23, 31]. Since individual glycoproteins could not be labelled sufficiently with D-[$^3$H]mannose *in vivo*, the following experimental approach has been applied. DPP IV was labelled biosynthetically with L-[$^{35}$S]methionine *in vivo* and isolated from subcellular fractions by immunoadsorption using the monoclonal anti-DPP IV antibody 13.4 [23]. Numbers of complex type and oligomannose type *N*-glycans in DPP IV were estimated from the decrease in $M_r$ following enzymatic deglycosylation with either endo-H cleaving *N*-glycans of the oligomannose type [32], or with a mixture of glycopeptide-*N*-glycosidase and endo-F [33] cleaving all types of asparagine bound *N*-glycans [34]. DPP IV was denatured by boiling in SDS, incubated with excess of the endoglycosidases and was thereafter separated by SDS-PAGE and visualized by radiofluorography. In order to eliminate the possibility that differences in glycosylation were due to processing reactions, DPP IV was analysed in the endoplasmic reticulum 10 min after labelling with L-[$^{35}$S]methionine. DPP IV from the endoplasmic reticulum of both vitamin A deficient liver and that of pair-fed controls migrated with an idential $M_r$ of 100 000 in SDS polyacrylamide gels (Fig. 4(a)). Digestion with endo H for

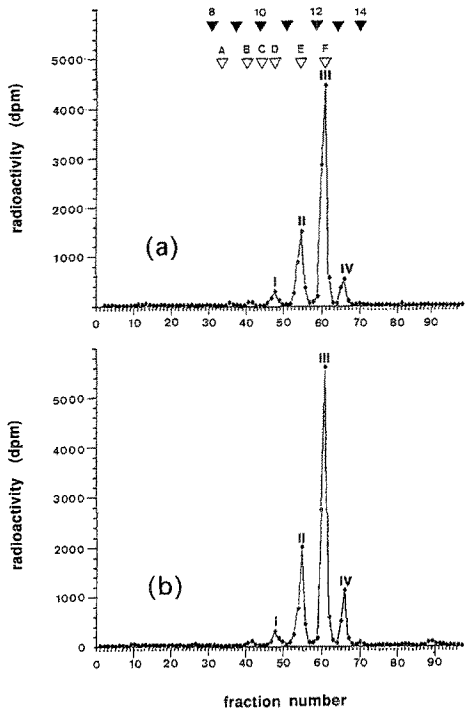


**Figure 3.** Oligomannose type oligosaccharide alditols from rat liver endoplasmic reticulum of retinol deficient (a) and pair-fed control rats (b). Endoplasmic reticulum was isolated from the livers of vitamin A deficient (a) and pair-fed control rats (b) 30 min after intravenous injection of D-[2-$^3$H]mannose (0.6 mCi per 100 g body weight). After delipidation of the isolated membranes oligomannose-type oligosaccharides were released from the glycoproteins by endo-H. Oligosaccharides were converted to their corresponding oligosaccharide alditols by reduction with sodium borohydride and were analysed by HPLC on APS-2 hypersil (for details see the Materials and methods section). White arrows on the top indicate the eluting position of authentic oligosaccharide alditols $Man_{5-9}GlcNAcOH$ (A to F), see also Fig. 2. Black arrows indicate the eluting position of glucose oligomer units fluorescence labelled with 2-amino-8-naphthol.

16 h converted this nonprocessed form of DPP IV to an $M_r$ 88 000 species. A similar size ($M_r$ 87 000) has been reported for the nonglycosylated form of rat liver DPP IV immunoprecipitated from cell-free translation products [35].

DPP IV immunoprecipitated from plasma membranes after a 2 h chase period had an $M_r$ of 105 000 in vitamin A deficient rats and pair-fed controls. Plasma membrane DPP IV from each of the two tissues was resistant to endo-H, and was converted to species of $M_r$ 90 000 and $M_r$ 88 000 by digestion with a mixed preparation of endo-F and peptide-*N*-glycosidase (Fig. 4(a, b)).

## Discussion

The results of this study support two conclusions concerning the role of retinol for the *N*-glycosylation of membrane proteins.

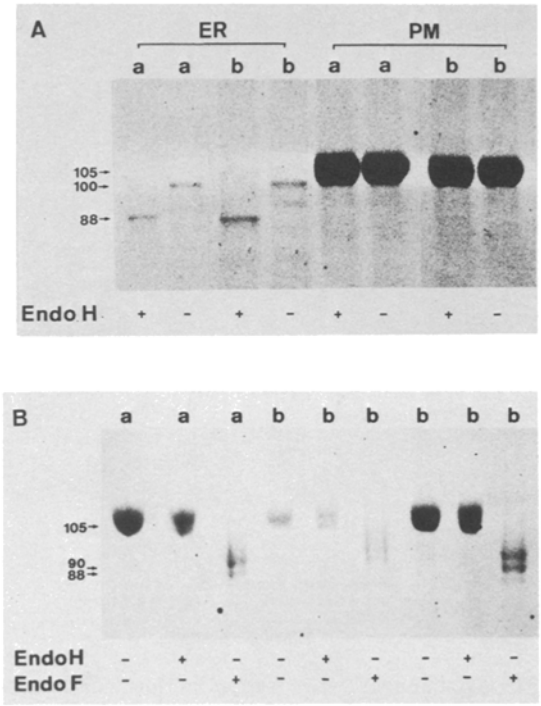


**Figure 4.** Digestion of DPP IV from endoplasmic reticulum and plasma membranes of vitamin A deficient (a) and pair-fed control (b) liver with endo-H and endo-F/glycopeptidase F. Endoplasmic reticulum and plasma membranes were isolated from retinol deficient (a) and pair-fed control (b) liver 10 min (endoplasmic reticulum) and 120 min (plasma membranes) after intravenous pulse-chase labelling of the animals with L-[$^{35}$S]methionine (0.45 mCi per 100 g body weight). DPP IV was immunoprecipitated from the isolated membranes, digested with either endo-H or endo-F/glycopeptidase F, separated on 7.5% SDS/polyacrylamide gels and fluorographed. (A) Endoplasmic reticulum and plasma membranes; (B) plasma membranes.

First, retinol deficiency had no detectable influence on the size of *N*-glycans on newly synthesized acceptor proteins in the endoplasmic reticulum. Glycoproteins isolated from the endoplasmic reticulum 30 min after injection of D-[2-$^{3}$H] mannose carried typical intermediates of *N*-glycan processing ($Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$) in identical relative amounts in both vitamin A deficient liver and that of pair-fed controls. These glycans are generated from $Glc_3Man_9GlcNAc_2$ by glucosidase I and II and mannosidase I [4]. Especially, no increase in the proportion of short chain oligosaccharides was noted in glycoproteins from vitamin A deficient liver. An accumulation of short chain lipid-linked intermediates, especially of $DolPPGlcNAc_2Man_5$, has been reported for retinol deficient liver [9], and had led to the assumption that retinol is involved in the elongation of lipid linked oligosaccharides from $DolPPGlcNAc_2Man_5$ to $DolPPGlcNAc_2Man_9$. Our results give no evidence that an increased proportion of such short chain intermediates is transferred to glycoproteins in the endoplasmic reticulum. Our *in vivo* results are consistent with *in vitro* studies on glycoprotein biosynthesis in isolated microsomal membranes from vitamin A deficient rat liver [11]. In isolated microsomes from both vitamin A deficient liver and controls, only $Glc_3Man_9GlcNAc_2$ was transferred from endogenous lipid linked oligosaccharides to acceptor proteins. Moreover, by contrast to previous *in vivo* studies Chan and Wolf could not find an increased pool of smaller oligosaccharide-lipids in the isolated microsomes [11]. Thus, it is very unlikely that both *in vivo* and *in vitro* retinol has a significant influence on the elongation of lipid linked oligosaccharides. The role of retinol as a mannose carrier has also been questioned by the finding that the endogenous mannolipid so far regarded as RetPMan is at least to a larger part not RetPMan but phosphatidylmannose [10].

Second, estimation of the number of *N*-linked chains of the complex type and the oligomannose type on DPP IV, a major glycoprotein constituent of hepatic plasma membranes, gave no evidence that retinol deficiency causes incomplete glycosylation of plasma membrane glycoproteins. DPP IV from endoplasmic reticulum and plasma membranes of both vitamin A deficient liver and that of controls had the same $M_r$ and showed the same shifts in $M_r$ upon *N*-deglycosylation with endoglycosidases. The $M_r$ of the *N*-deglycosylated form of DPP IV corresponded to that of the nonglycosylated DPP IV polypeptide immunoprecipitated from cell-free translation products [35]. This comparison is valid for DPP IV, since the signal peptide of DPP IV is not cleaved off during biosynthesis and is hence present both in cellular DPP IV and in DPP IV translated in cell free systems [35]. Assuming an $M_r$ contribution of approximately 2000 per oligomannose oligosaccharide, shifts in $M_r$ upon deglycosylation with endo-H of DPP IV from the endoplasmic reticulum exclusively glycosylated with oligomannose structures, corresponded to about 5 or 6 *N*-linked oligosaccharides per polypeptide chain. This finding is in accord with more detailed analyses of the carbohydrate moiety of DPP IV from rat liver plasma membranes (in preparation) and from rat kidney brush-border plasma membranes [36], both suggesting the presence of 5 or 6 oligosaccharide chains per molecule. Hence, at least with respect to DPP IV, vitamin A deficiency does not inhibit the transfer of oligosaccharides from the lipid carrier to newly synthesized glycoproteins to a significant extent. This result is different from the findings of Chan and Wolf [11] that transfer of $Glc_3Man_9GlcNAc_2$ $^{3}$H labelled in the glucose residues from exogenous $DolPPGlcNAc_2Man_9Glc_3$ to microsomal protein acceptors from vitamin A deficient liver is reduced by about 50%. However, this inhibition was found in an *in vitro* microsomal model that differed from intact liver tissue with respect to several reactions in the biosynthesis of lipid linked

oligosaccharides. Whereas synthesis of DolPMan and lipid linked oligosaccharides was increased two- to threefold in vitamin A deficient membranes in the *in vitro* microsomal model, synthesis of DolPMan during vitamin A deficiency was decreased *in vivo*, as shown in livers from hamster [5, 12] and rat (Table 2). The effect of vitamin A deficiency on the *in vivo* synthesis of lipid linked oligosaccharides was less expressed compared to that on the synthesis of DolPMan. Whereas synthesis of lipid linked oligosaccharides was moderately decreased in vitamin A deficient hamster liver [12], no change was observed in rat liver in the present study.

The mechanisms by which vitamin A deficiency causes the reduction in the amount of labelled mannose incorporated into glycoproteins of intact tissues ([5] and Table 2) and of isolated microsomal membranes [11] is not clear. The present study shows that *N*-glycans on newly synthesized glycoproteins in the endoplasmic reticulum have the same size in vitamin A deficient liver and in controls (Fig. 3). Hence, it can be ruled out that the decreased metabolic labelling of glycoproteins with radiolabelled mannose reflects the transfer of small-size oligosaccharides in vitamin A deficient tissues. Moreover, since endoplasmic reticulum DPP IV carried the same number of *N*-glycans in vitamin A deficient liver and in controls (Fig. 4), it is very unlikely that during vitamin A deficiency glycosylation, sites of glycoproteins are only partially glycosylated. As recently shown in hamster liver, the decreased incorporation of D-[$^3$H]mannose into DolPMan, lipid linked oligosaccharides and glycopeptides of hamster liver during retinol deficiency is accompanied by a reduced incorporation of [$^3$H]mannose into GDP-mannose. Inhibition of GDP-mannose synthesis was therefore suggested to be a major consequence of retinol deficiency [12].

In addition to effects of vitamin A deficiency on the synthesis of lipid linked oligosaccharides or on oligosaccharide transfer to proteins, it is likely that retinol deficiency may also affect later steps of glycoprotein biosynthesis. Vitamin A deficiency of liver has been shown to be associated with a decreased activity of galactosyltransferase [37], and with a reduced content of protein-bound carbohydrates, including galactose and sialic acid, in lysosomal membranes [38]. As concluded from the resistance of plasma membrane DPP IV against endo-H (Fig. 4) mature DPP IV contains almost exclusively complex type *N*-glycans and none or only a very small portion of oligomannose chains, consistent with previous reports [31]. The proportion of complex type and oligomannose type glycans was not significantly changed in vitamin A deficient livers, indicating that vitamin A deficiency does not inhibit processing of oligomannose intermediates to complex type structures. However, more refined alterations in the structure of complex-type glycans cannot be excluded. Therefore, possible effects of retinol deficiency on single processing reactions are under current investigation.

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