

In Vivo Synthesis of Lipid-linked Oligosaccharides in the Livers of Normal and Vitamin A-deficient Rats*

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[1-¹⁴C]Glucosamine, [2-³H]mannose, or [1-¹⁴C]galactose were injected into vitamin A-deficient and pair-fed control rats at an early stage of deficiency. The livers were homogenized, centrifuged, and the oligosaccharide-lipids extracted into chloroform:methanol:water (10:10:3). This fraction, when labeled with glucosamine, reached a maximum at 35 min after injection, and remained constant to 110 min. The fraction was chromatographed on a DEAE-cellulose-acetate column and was eluted at an ammonium acetate concentration of 20 mM, corresponding to oligosaccharide bound to dolichylpyrophosphate. Fractionation on Bio-Gel P-4 of the oligosaccharide produced by mild acid hydrolysis of the oligosaccharide-lipid, gave a major peak (I) followed by a broad minor peak (II) of smaller molecular weight. Peak II from deficient liver was always greater than from normal liver (25.91 ± 8.89 versus $6.28 \pm 5.46\%$ of total oligosaccharide). In a recovery experiment, vitamin A was given intragastrically to deficient rats. It caused virtual disappearance of peak II between 4 and 8 h after administration. Rechromatography of peak II on a longer column yielded several distinct peaks of smaller molecular weight than peak I, with the principal peak (peak F) having a K_d of 0.486, corresponding to that of a standard oligosaccharide (mannose)₅(N-acetylglucosamine)₂. Peak I had a K_d of 0.285, corresponding to that of a standard oligosaccharide (glucose)₃(mannose)₅(N-acetylglucosamine)₂. Both peaks were α -mannosidase-digestible: 48% of the radioactivity of [³H]mannose-labeled peak I was liberated as [³H]mannose; whereas 79% of the label of mannose-labeled peak F was liberated as labeled mannose. Peak F, the (mannose)₅(N-acetylglucosamine)₂-like component of peak II, was resistant to endo-N-acetylglucosaminidase-H hydrolysis, whereas peak I was digested by this enzyme under the same conditions. [2-³H]Mannose and [1-¹⁴C]galactose were injected simultaneously into vitamin A-deficient and normal rats, yielding double-labeled peaks I and II, the latter only in the deficient. Double-labeled peak II, upon refractionation, showed the same pattern of oligosaccharides as [1-¹⁴C]glucosamine-labeled peak II, including a major peak corresponding to (mannose)₅(N-acetylglucosamine)₂. We conclude that normal rat liver accumulates

an oligosaccharide linked to dolichylpyrophosphate consistent with the structure (glucose)₃(mannose)₅(N-acetylglucosamine)₂, whereas vitamin A deficiency causes an increased pool of smaller molecular weight oligosaccharide-lipids, the principal one being consistent with (mannose)₅(N-acetylglucosamine)₂-dolichylpyrophosphate.

Results from several laboratory studies (1-3) have shown that synthesis of complex oligosaccharide chains of glycoproteins involves transfer of glucose-containing mannose-rich oligosaccharide chains as a unit from a lipid carrier to the protein. The peptide-bound oligosaccharide is then processed (i.e. some sugars are removed and others added) to form the complex glycoprotein.

The major lipid-linked oligosaccharide intermediate in *in vivo* glycoprotein synthesis contains 2 N-acetylglucosamine, 9 mannose, and 3 glucose residues linked through pyrophosphate to dolichol (4, 5). Although these studies indicated that the oligosaccharide moiety is assembled on the dolichol pyrophosphate, which participates as the carrier lipid, stepwise addition of mannose to the lipid carrier has not yet been demonstrated.

It has been observed repeatedly (6) that vitamin A deficiency caused decreased incorporation of glucosamine and mannose into some glycoproteins of various tissues. Recently, Haars and Pitot (7) demonstrated a severe reduction in the synthesis of the mannose-containing glycoprotein α_{2u} -globulin in hepatocytes from vitamin A-deficient rats; and Adhikari and Vakil (8) found a decrease in the carbohydrate content of the glycoproteins in lysosomal membranes of vitamin A-deficient rats.

Since lipid-linked oligosaccharides are intermediates in glycoprotein synthesis, it seemed important to investigate their synthesis in the livers of vitamin A-deficient and normal rats *in vivo*.

MATERIALS AND METHODS

Preparation of Animals—Vitamin A deficiency was induced in 21-day-old male Holtzman rats (40 to 60 g) by the method of Wolf *et al.* (9). Rats were fed a vitamin A-deficient diet (9) *ad libitum* for 4 weeks. Half the rats were randomly designated as controls and given a weekly supplement of 2000 I.U. of vitamin A in the form of retinyl acetate dissolved in cottonseed oil at a concentration of 10 mg/ml. When rats were 7 weeks old, they were ranked according to weight and paired, deficient to control. Deficient rats were fed the vitamin A-deficient diet *ad libitum*; each control rat was fed the same amount of diet eaten by the deficient rat of its pair on the previous day. Body weight and amount of food eaten were determined daily. Only healthy animals were sampled; deficient rats showing signs of infection were discarded. The animals were used for experimentation when they had lost 10 to 20% of body weight without any signs of infection. Rats

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were injected intraperitoneally with an amount of a radioactively labeled sugar, specified under "Results," in 0.9% saline (total volume, 0.3 ml).

For the recovery experiment, a solution of 100 μ g of retinyl acetate in 0.3 ml of cottonseed oil was intubated into the stomach of vitamin A-deficient rats, and the animals killed at specified times thereafter, 45 min after the intraperitoneal injection of labeled glucosamine.

Extraction of Oligosaccharide-Lipid—Rats were decapitated at times after injection, specified under "Results." The liver was removed, weighed, minced, and suspended in 0.15 M Tris buffer at pH 7.4 (5 ml of buffer/g of liver), containing 4 mM magnesium chloride. The suspension was homogenized by six strokes of a Teflon pestle with a motor-driven homogenizer. The homogenate was centrifuged at 38,000 rpm for 35 min in the Beckman model L ultracentrifuge (Beckman Instruments, Fullerton, CA). The supernatant was decanted. The pellets were suspended in the ice-cold buffer to a final volume of 1.5 ml/g of original tissue. Chloroform and methanol were then added to achieve a mixture of chloroform:methanol:water (3:2:1). The mixture was shaken at room temperature for 10 min and centrifuged at $500 \times g$ for 10 min at 10°C ; the upper (F1) and lower phases were separated from the interphase. To obtain the oligosaccharide-lipid, the interphase pellet was then extracted three times with chloroform:methanol:water (10:10:3) (F2); 5 volumes of solvent/g of original tissue were used each time.

Mild Acid Hydrolysis of Oligosaccharide-Lipid—A total of 10 ml of chloroform:methanol:water (10:10:3) extract was dried in a flash evaporator under nitrogen. The residual lipid was redissolved in 0.2 ml of *n*-propyl alcohol and 1.8 ml of 0.02 M HCl was added. Hydrolysis was done for 20 min at 100°C . The reaction was stopped by adding 4 ml of cold water and evaporating to dryness in a flash evaporator. The hydrolysate was redissolved in 1 to 2 ml of water. The insoluble material was removed by centrifugation and the solution was ready for column fractionation.

α -Mannosidase Hydrolysis—Enzyme solution contained 18 units/mg/0.1 ml of jackbean α -mannosidase in 0.02 M acetate buffer (pH 7.5). The sample was lyophilized to dryness; rehydrated, if necessary, to remove the bicarbonate or acetic acid residue; and dissolved in 0.2 ml of 0.1 M acetate buffer (pH 4.5) with 3×10^{-4} M ZnCl_2 , 10 μ l of 5000 units/ml of penicillin, and 5 mg/ml of streptomycin; and 25 μ l of α -mannosidase (18 units/mg/0.1 ml) were added. Additional enzyme (25 μ l) was added after 6 and 24 h. Total incubation time was 2 days.

Endo-N-acetyl- β -D-glucosaminidase-H—Samples were lyophilized and redissolved in 0.1 M sodium citrate buffer (pH 5.5). The endo-H enzyme (30 mg/ml) was added in a ratio of (20:1) sample to enzyme. Incubation was for 6 h at 37°C .

Column Fractionation—The hydrolyzed oligosaccharide sample was first fractionated on a Bio-Gel P-4 column (200 to 400 mesh), measuring 1.4×113 cm. Fractions were eluted with 0.1 M acetic acid and 2 to 4 ml/tube/15 min were collected. Fractions containing the major oligosaccharide (peak I) and the minor broad peak (peak II) of oligosaccharide were pooled separately. The pooled samples were lyophilized for further fractionation or enzymatic treatment.

The rechromatography was done on a Bio-Gel P-4 column (1.2×200 cm). Fractions were eluted with 0.1 M bicarbonate solution containing 0.5 mM NaN_3 . Exclusion and inclusion volume markers were bovine serum albumin (0.5 mg) and [^{14}C]mannose, respectively. Twenty drops (0.9 to 0.98 ml) per tube were collected.

The individual peaks were pooled separately. The particular peak selected for enzymatic treatment was lyophilized after treatment with 0.1 M acetic acid until excess bicarbonate was released as CO_2 .

DEAE-cellulose-Acetate Fractionation—The column was prepared by the method of Dankert *et al.* (10). The resin was suspended in 1 N NaOH, kept at room temperature overnight, and washed successively with water, 95% ethanol, and absolute methanol. The cellulose was then dried by pressing between sheets of filter paper, suspended, and stored in glacial acetic acid. Before use, it was packed into a column (1.7×10 cm) and washed with 800 ml methanol, 400 ml of chloroform, 500 ml of methanol, and 500 ml of chloroform:methanol:water (10:10:3). Then the oligosaccharide-lipid sample was applied and washed with 2 column volumes of chloroform:methanol:water (10:10:3). The oligosaccharide-lipid was then eluted with a linear gradient of 0.0 to 0.1 N ammonium acetate (total volume, 800 ml).

The oligosaccharide fractions obtained from oligosaccharide-lipid before and after DEAE-cellulose-acetate chromatography behaved identically when chromatographed on Bio-Gel P-4. Therefore, in most experiments, the oligosaccharide-lipid (F2) was not subjected to

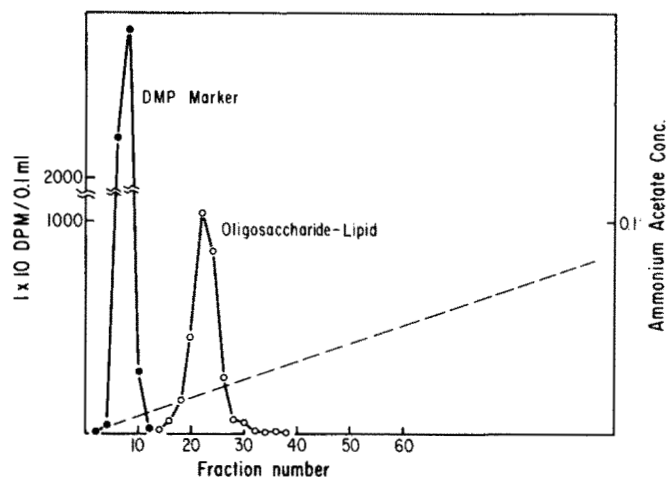


FIG. 1. Fractionation of [^3H]glucosamine-labeled 10:10:0.3 (chloroform:methanol:water) fraction by DEAE-cellulose-acetate. The label was eluted as dolichylpyrophosphate oligosaccharide at 20 mM ammonium acetate; dolichyl[^{14}C]mannosyl phosphate was used as a marker (eluted at 7 mM ammonium acetate). Recovery was 67% of label applied to the column. The labeled DMP was prepared from GDP-[^{14}C]mannose and rat liver microsomes (11). \bullet — \bullet , ^{14}C ; \circ — \circ , ^3H .

DEAE-cellulose chromatography, but hydrolyzed directly to oligosaccharide.

Sources of Materials—D-[6- ^3H]Glucosamine hydrochloride (18.8 Ci/mmol), D-[1- ^{14}C]glucosamine hydrochloride (56.04 mCi/mmol), D-[1- ^{14}C]galactose (56.5 mCi/mmol), and D-[2- ^3H]mannose (14.1 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA. α -Mannosidase was obtained from Sigma Chemical Co., St. Louis, MO. Endo-H-glucosaminidase and the standard M_5N_2^1 were gifts from Drs. S. C. Hubbard and P. W. Robbins, Center for Cancer Research, Massachusetts Institute of Technology. Bio-Gel P-4 columns (200 to 400 mesh) were obtained from Bio-Rad Laboratories, Richmond, CA. The DEAE-cellulose-acetate was purchased from Eastman-Kodak Co., Rochester, NY.

RESULTS

Oligosaccharide-Lipid from Rat Liver *in Vivo*—Since labeling of oligosaccharide-lipid in normal rat liver *in vivo* has not yet been reported, the time for optimal uptake of labeled precursor had to be determined. Upon injection of 75 μCi of [^3H]glucosamine into a normal rat, uptake into the oligosaccharide-lipid fraction (F2, see "Materials and Methods") reached a maximum at 35 min (0.21 μCi /total liver of 9.4 g), and remained constant for at least 110 min. The fraction containing the monosaccharide-lipid (F1) declined from 6.04 μCi (15 min) to 2.9 μCi (110 min) throughout the time of the experiment.

Chromatography of the oligosaccharide-lipid fraction (F2) on a DEAE-cellulose-acetate column with an ammonium acetate gradient, showed elution of the glucosamine-labeled lipid at 20 mM salt concentration, consistent with the presence of a pyrophosphate group, with authentic dolichylmannosyl phosphate used as a marker eluting at 7 mM (Fig. 1). Mild acid hydrolysis of the oligosaccharide-lipid produced an oligosaccharide fraction that was chromatographed on a Bio-Gel P-4 column.

Effect of Vitamin A Deficiency on Oligosaccharide-Lipid Synthesis—[^{14}C]Glucosamine (75 μCi) was injected into a vitamin A-deficient rat 1 week after the weight-plateau stage,

¹ The abbreviations used are: M_5N_2 , (mannose)₅(N-acetylglucosamine)₂; similarly, for M_1N_2 , M_6N_2 etc., M = mannose, N = N-acetylglucosamine; $\text{G}_3\text{M}_5\text{N}_2$, (glucose)₃(mannose)₅(N-acetylglucosamine)₂; DMP, dolichylmannosyl phosphate; MRP, mannosylretinyl phosphate.

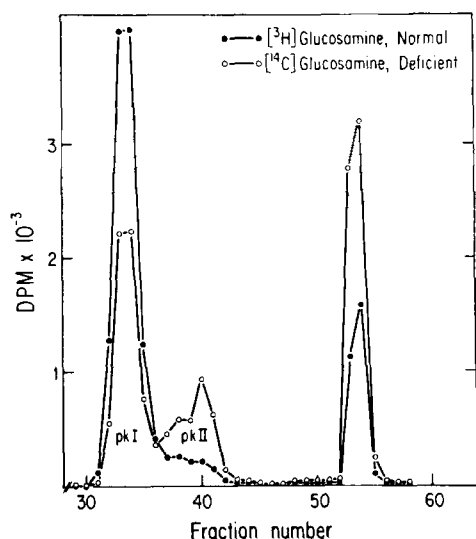


FIG. 2. Fractionation of combined [^{14}C]glucosamine- and [^3H]glucosamine-labeled oligosaccharides by Bio-Gel P-4 filtration column (1.4×113 cm). [^{14}C]Glucosamine ($75 \mu\text{Ci}$) was injected into vitamin A-deficient rats and $75 \mu\text{Ci}$ of [^3H]glucosamine into normal control rats. The oligosaccharides were released from the lipid by mild acid hydrolysis. Tubes 31 to 36 were pooled as peak I. Tubes 37 to 44 were pooled as peak II.

when the animal had lost about 10% of its body weight, and [^3H]glucosamine ($75 \mu\text{Ci}$) was injected into a control rat, paired to the deficient rat. The animals were killed 45 min later and the oligosaccharides from the oligosaccharide-lipids were obtained from their livers as described under "Materials and Methods." Total radioactivity in oligosaccharides was always higher in normal compared to deficient livers, but because of the smaller weights of the deficient livers, radioactivity per g of liver was similar in normal and deficient ($0.02 \mu\text{Ci/g}$ wet weight). Approximately equal portions of radioactive oligosaccharides from normal (79×10^3 dpm of ^3H label) and deficient (104×10^3 dpm of ^{14}C label) livers were combined and fractionated on a Bio-Gel P-4 column (Fig. 2). Two principal oligosaccharide peaks were observed: peak I, which is larger in the fraction from normal liver; and peak II, a pronounced broad peak in the fraction from deficient liver, but only a shoulder on the peak I from normal liver. The peak that eluted in the later fractions was shown by paper chromatography to be largely glucosamine.

Peak II (Fig. 2) represented about 34%² of the total oligosaccharide label in the deficient fraction and about 10% in the normal fraction. The inverse experiment ([^3H]glucosamine injected into the deficient rat and [^{14}C]glucosamine into the normal rat) gave the same result, a pronounced peak II (about 15% of the total oligosaccharide label) in the fraction from deficient liver and almost no peak II in the oligosaccharides of normal liver. This result, peak II of 15 to 34% in deficient and of only 2 to 10% in normal liver, was obtained in five separate experiments (deficient, 25.91 ± 8.89 S.E. %; normal, 6.28 ± 5.46 S.E. %; $p < 0.005$). It should be noted that similar amounts of total labeled oligosaccharides from normal and deficient liver were applied to the gel filtration columns. Therefore, the increased label in peak II of the deficient livers corresponds to a decreased label in peak I, as clearly discernible in Fig. 2.

The increase in peak II is not due to a difference in food intake, since the control rats were pair-fed to the deficient rats. Moreover, the same result was obtained whether the animals received a glucose supplement (0.1 g of glucose/ml 48

h before killing) in their drinking water or not. Peak II, characteristic of deficient liver, was almost absent when [^3H]glucosamine of high specific activity ($18.8 \text{ Ci}/\mu\text{mol}$) was injected. To generate peak II in deficient liver with [^3H]glucosamine, the latter had to be diluted with unlabeled glucosamine to the same specific radioactivity as the [^{14}C]glucosamine used ($56.0 \text{ mCi}/\text{mmol}$). Evidently, a certain minimum concentration of glucosamine has to be present in the liver to lead to accumulation of peak II.

A recovery experiment was done to determine the earliest time at which peak II of the deficient rats could be observed to decline to normal levels: [^{14}C]glucosamine ($75 \mu\text{Ci}$) was injected into vitamin A-deficient rats 45 min before killing. Retinyl acetate ($100 \mu\text{g}$) was given intragastrically in oil solution and the liver oligosaccharides were isolated and fractionated as described under "Materials and Methods." Peak II was 14.5%² in the vitamin A-deficient liver, and remained at this level 4 h after vitamin A administration; 8 h after administration, peak II had declined to 7.0% and was still close to this level 24 h after administration. In the control liver, peak II was 6.0% of total oligosaccharide. Thus, recovery was complete between 4 and 8 h after feeding the vitamin.

We had to guard against the eventuality of an artifactual breakdown of a higher molecular weight oligosaccharide-lipid during isolation. Hubbard and Robbins (5) reported breakdown of oligosaccharide-lipid when cells were harvested and homogenized in buffer instead of in chloroform:methanol:water. To ensure absence of artifactual breakdown, the vitamin A-deficient liver in one of our experiments was homogenized directly in methanol. This homogenate was brought to 3:2:1 (chloroform:methanol:water) and extracted as usual, as described under "Materials and Methods"; peak II was still present as 31% of total oligosaccharide label.

Refractionation of Peak II—Oligosaccharides that differ from each other by only one sugar moiety can be separated on a Bio-Gel P-4 column (1×200 cm) (4). Accordingly, accumulated [^{14}C]glucosamine-labeled peak II oligosaccharides, obtained from the livers of two vitamin A-deficient rats, were pooled and rechromatographed. Fig. 3 shows that this peak contained oligosaccharides of various sizes, with the K_d values given in Table I. The principal component of peak II from deficient liver is peak F. This corresponds in size to the oligosaccharide containing 5 mannose and 2 *N*-acetylglucosamine residues (M_5N_2); other peaks present corresponded to M_8N_2 , M_7N_2 , and M_6N_2 (Table I). Part of peak I was also present in peak II from deficient liver, which reveals, upon refractionation, the oligosaccharide corresponding to $\text{G}_3\text{M}_9\text{N}_2$. Fig. 3 shows that peak F is completely lacking in the oligosac-

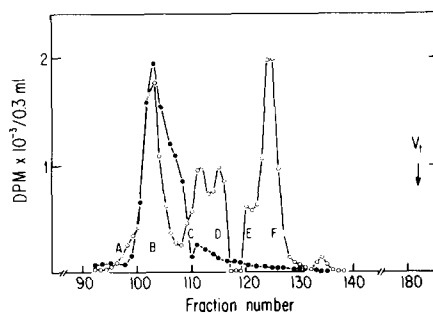


FIG. 3. Fractionation of peak II of [^{14}C]glucosamine-labeled oligosaccharides synthesized by vitamin A-deficient and normal rat liver on Bio-Gel P-4 columns (1×200 cm). Equal amounts (48,000 dpm) of peak II from deficient and normal livers were placed on the columns. The predominant peak in deficient (peak F) had a K_d value of 0.486 corresponding to M_5N_2 . ○—○, oligosaccharides of deficient livers; ●—●, oligosaccharides of normal livers.

² Oligosaccharide label is expressed as peak II/peak I + II $\times 100$, throughout.

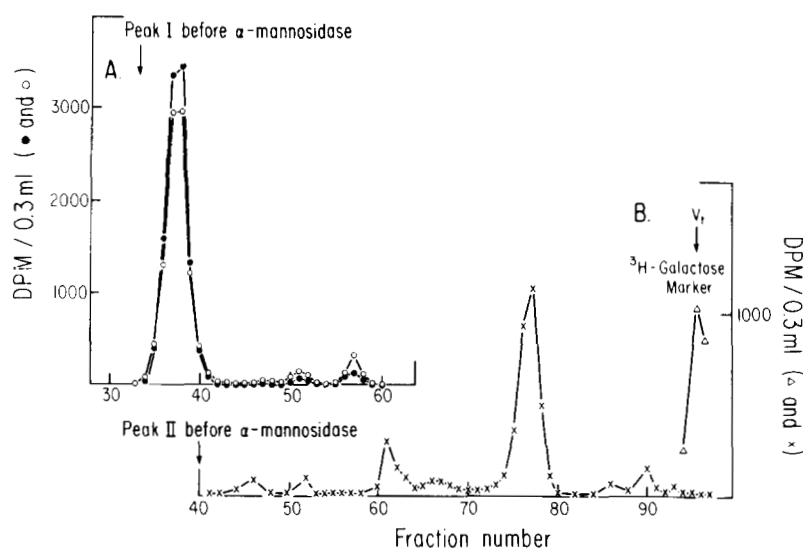


FIG. 4. Bio-Gel P-4 chromatography on columns (1.4 × 113 cm) of products of α -mannosidase treatment. A, peak I (Fig. 2) from normal (●—●, [3 H]glucosamine) and deficient (○—○, [14 C]glucosamine) rats after α -mannosidase treatment; B, peak II (Fig. 2) labeled with [14 C]glucosamine from deficient rats (×—×), after α -mannosidase treatment. The principal peak has K_d 0.60, corresponding to M_1N_2 . [3 H]Galactose is a marker (Δ—Δ). Arrows show positions of peaks before α -mannosidase treatment.

TABLE I

Relative elution coefficients of oligosaccharides derived from oligosaccharide-lipids

Peaks	K_d found	K_d reported ^a	Oligosaccharide
A	0.249	0.240	Pre-oligosaccharide ^b
B	0.285	0.279	$G_3M_9N_2$ ^a
C	0.362	0.383	M_8N_2
D	0.390	0.408	M_7N_2
E	0.448	0.445	M_6N_2
F	0.486	0.474	M_5N_2

^a S. C. Hubbard and P. W. Robbins,³ reported these K_d values for the oligosaccharides indicated (G = glucose, M = mannose, N = N-acetylglucosamine).

^b Pre-oligosaccharide represents a shoulder also found by Hubbard and Robbins as always eluting before the largest oligosaccharide ($G_3M_9N_2$), probably an artifact of isolation.

charide fraction of normal liver, upon rechromatography of peak II taken from a normal rat.

A standard oligosaccharide of 5 mannose and 2 N-acetylglucosamine units (M_5N_2) was chromatographed on the same column; it showed a K_d of 0.490 and corresponded well to peak F (K_d 0.486, Fig. 3), the largest component of deficient peak II.

α -Mannosidase Sensitivity of Peaks I and II—In order to establish some of the structural properties of the oligosaccharides from deficient and control livers, the following experiments were carried out. Combined peak I material labeled with [3 H]glucosamine (normal) and [14 C]glucosamine (deficient) was treated with α -mannosidase and refractonated (Fig. 4A) on Bio-Gel P-4, resulting in a slight shift to a smaller molecular weight (see arrow, Fig. 4A). On the other hand, when the [14 C]glucosamine-labeled peak II from deficient liver was subjected to α -mannosidase, this shift was pronounced (see arrow, Fig. 4B). The K_d value (0.60) of the main peak of the α -mannosidase product from peak II corresponded to that observed by Hubbard and Robbins³ for M_1N_2 (0.64), suggesting that peak II contained no glucose end groups.

Endo-N-acetyl- β -D-glucosaminidase-H Sensitivity of Peaks B and F—Peaks B and F (Fig. 3) were treated separately with endo-N-acetyl- β -D-glucosaminidase-H, as described under "Materials and Methods." Peak B was sensitive to this enzyme, since its K_d value changed from 0.285 to 0.363 (A, Fig. 5); it therefore had substituted α -1,6-linked mannose.

A small peak (C) of glucosamine was detectable, liberated by the enzyme from peak B. By contrast, peak F was insensitive to the same enzyme under the same conditions (B, Fig. 5), suggesting that its α -1,6-linked mannose was unsubstituted.

[3 H]Mannose and [14 C]Glucose-labeled Oligosaccharides—In order to analyze glucose and mannose labeling directly, the oligosaccharide-lipid was double labeled *in vivo*. 2-[3 H]Mannose (300 μ Ci), 1-[14 C]galactose (200 μ Ci), and unlabeled glucosamine (0.29 mg) were injected into each of two deficient and two pair-fed control rats. [14 C]Galactose was used as a glucose label, since galactose is converted to UDP-galactose and hence to UDP-glucose, and is therefore not diluted by the body's large glucose pools as [14 C]glucose is (5). Labeling ratios, $^3H:^{14}C$, of the oligosaccharide-lipid fraction from normal and deficient livers were similar (1.45 and 1.54, respectively). Fig. 6, A and B, shows the chromatogram on Bio-Gel P-4 of the 3H - and ^{14}C -labeled oligosaccharides resulting from mild acid hydrolysis of the corresponding oligosaccharide-lipids. As with glucosamine labeling, a broad peak II and a large peak I, corresponding to $G_3M_9N_2$, appeared in the oligosaccharide fraction from deficient liver. Double labeled peak II was absent in the fraction from normal liver. Rechromatography of the combined double labeled peak II on Bio-Gel P-4 column (1 × 200 cm) showed resolution into peaks A through F (Fig. 7), closely resembling the pattern of the glucosamine-labeled peak II (Fig. 3). Notice that radioactivity from both [3 H]mannose and [14 C]galactose appeared in all fractions, which shows conversion of galactose not only

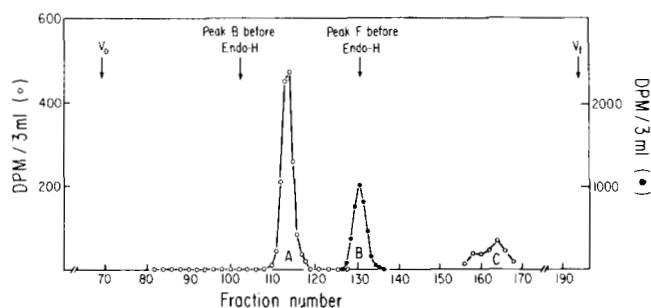


FIG. 5. Fractionation on Bio-Gel P-4 columns (1 × 200 cm) of the products of endo- β -glucosaminidase-H treatment. A, peak B (Fig. 3) labeled with [14 C]glucosamine after endo-H treatment. K_d changed from 0.285 to 0.363; B, peak F (Fig. 3) after endo-H treatment. K_d unchanged at 0.483; peak F is therefore not sensitive to endo-glucosaminidase-H. Arrows indicate positions of peaks before endo-H treatment; C, glucosamine liberated from peak B.

³ Hubbard, S. C., and Robbins, P. W. (1980) *J. Biol. Chem.* **255**, 11782–11793.

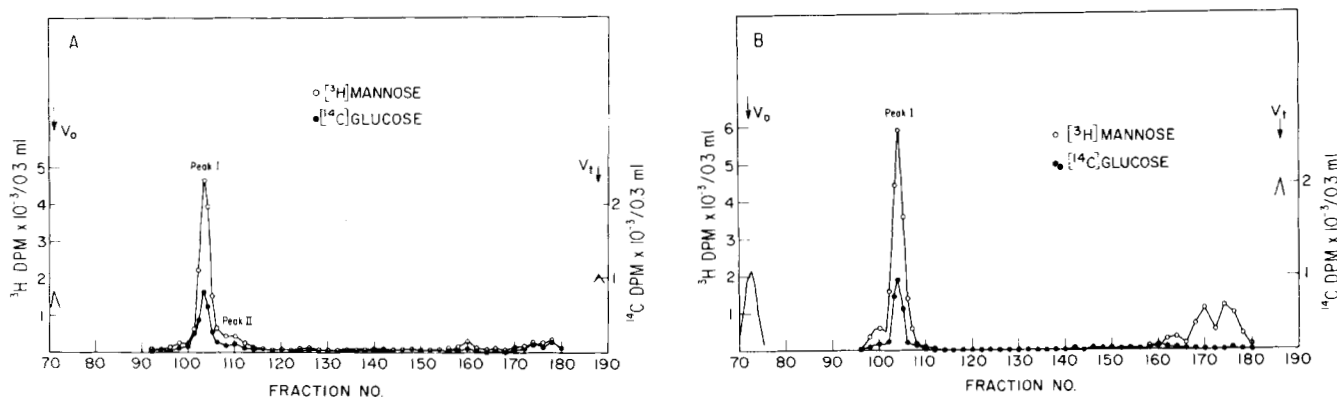


FIG. 6. Fractionation on Bio-Gel P-4 column (1 × 200 cm) of double labeled oligosaccharides from vitamin A-deficient rat liver (A) and normal liver (B) after injection of 2-[³H]mannose (300 μCi), 1-[¹⁴C]galactose (200 μCi), and 0.29 mg unlabeled glucosamine. Total oligosaccharide applied to column: 20,000 dpm of ³H label and 7,000 dpm of ¹⁴C label. Fraction tubes 100 to 108 pooled as peak I. Tubes 109 to 120 pooled as peak II.

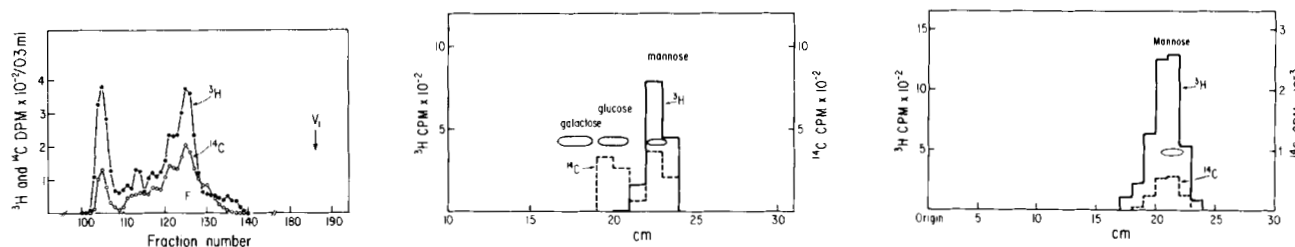


FIG. 7 (left). Rechromatography on Bio-Gel P-4 column (1 × 200 cm) of the collected peak II from Fig. 6A. ³H-labeled oligosaccharide ●—●, ¹⁴C-labeled oligosaccharide, ○—○.

FIG. 8 (center). Paper chromatography of the acid hydrolysate of double labeled peak I (Fig. 6A). Peak I, labeled by injection with [³H]mannose and [¹⁴C]galactose as described in the legend to Fig. 6, was treated with 2 N trifluoroacetic acid at 110 °C for 2 h in the presence of 1 μmol each of unlabeled mannose, galactose, and glucose. The solution was then neutralized and aliquots were applied

to filter paper and chromatographed in butanol:pyridine:0.1 N HCl (5:3:2). After drying, the paper was cut into 1-cm strips and counted in toluene-Triton X-100 after standing in 1 ml of water overnight. Unlabeled sugars were visualized by an AgNO₃ spray. —, ³H label; ---, ¹⁴C label.

FIG. 9 (right). Paper chromatography in butanol:pyridine:0.1 N HCl (5:3:2) of the product of α-mannosidase-treated peak I (Fig. 8A). 48% of the [³H]mannose was released after this treatment. Chromatogram shows only released mannose. —, ³H; ---, ¹⁴C.

to glucose, but also to mannose and probably glucosamine, by rat liver *in vivo*, in contrast to Chinese hamster ovary and fibroblast cells in culture which convert [¹⁴C]galactose mainly to glucose, with only 6% going to mannose (5). When fractions corresponding to peak II were taken from the chromatogram of oligosaccharides from normal liver and refractionated, very little radioactivity (less than 50 dpm) was detected in fractions corresponding to peaks C through F.

Acid hydrolysis of double labeled peak I (Fig. 8) showed that, *in vivo*, labeled galactose injection did label both glucose and mannose to an approximately equal degree, whereas mannose injection labeled only oligosaccharide mannose.

α-Mannosidase Sensitivity of ³H- and ¹⁴C-labeled Peaks I and F—Fig. 9 shows a chromatogram of the monosaccharide released from double labeled peak I by α-mannosidase. It is clearly mannose, with 48% of the ³H activity released as monosaccharide (52% remained at the origin as presumably unchanged oligosaccharide). When double labeled peak F (Fig. 7) was treated with α-mannosidase, 79% of the radioactivity was released as mannose.

DISCUSSION

These results show that in normal rat liver, labeling of oligosaccharide-lipid reaches a maximum 35 min after injection of labeled glucosamine, compared to 2.5 min in cell culture (5). The oligosaccharide-lipid was identified by (a) labeling with glucosamine and mannose; (b) extraction into the 10:10:3 chloroform:methanol:water fraction; (c) chromatography on DEAE-cellulose-acetate and its elution position with respect to that of authentic DMP; and (d) hydrolysis to oligosaccharides by mild acid.

The oligosaccharides obtained from the oligosaccharide-lipid, when fractionated from normal liver, revealed the labeling pattern already observed by Hubbard and Robbins (5) for cells grown in culture. They found that the major lipid-linked oligosaccharide had the composition G₃M₉N₂ and was probably a result of the rapid addition of sugar moieties to dolichylpyrophosphate. The properties of peak I, the major oligosaccharide linked to lipid which we found in normal rat liver, correspond very closely to those of G₃M₉N₂, reported by Hubbard and Robbins (5). The characteristics of peak I which suggest this structure, are the following: (a) its size, derived from *K_d* values, compared to authentic standards; (b) its hydrolysis to a smaller size by α-mannosidase, with only partial release of mannose (48%). G₃M₉N₂ is known to be sensitive to α-mannosidase digestion which removes the 6'-pentamannosyl branch, liberating 50% of the label as mannose. Hubbard and Robbins (5) reported 45% mannose liberated from G₃M₉N₂. An oligosaccharide without glucose would yield M₁N₂ after α-mannosidase treatment (see below); (c) its labeling with glucosamine. After injection of labeled mannose and galactose, mannose and glucose were found labeled in this oligosaccharide; (d) its sensitivity to endo-glucosaminidase-H, producing a smaller size oligosaccharide and glucosamine. Therefore, it had a substituted α-1,6-linked mannose. Although these properties do not by themselves prove the structure of this oligosaccharide, they are consistent with that also found as the major lipid-linked oligosaccharide in cell culture, namely G₃M₉N₂.

The vitamin A-deficient liver accumulated a series of smaller lipid-linked oligosaccharides (peak II, Fig. 2), when compared to normal liver. Upon fractionation, the principal

oligosaccharide was found to be peak F (Fig. 3). We suggested the structure for this peak to be M_5N_2 for the following reasons: (a) its size, which was directly compared by gel filtration with an authentic sample; (b) its hydrolysis by α -mannosidase to an oligosaccharide corresponding in size to M_1N_2 , with liberation of 79% of the labeled mannose. It therefore has no glucose end group; (c) its labeling with glucosamine and mannose; (d) its resistance to endo-glucosaminidase-H, showing absence of α -1,3- and α -1,6-linked mannose, or both, substituted on the α -1,6-mannose (12). These properties, although they do not prove the oligosaccharide to have the structure of M_5N_2 , are consistent with it.

The conclusion from the above results is that vitamin A deficiency leads to the accumulation of smaller oligosaccharide-lipids in liver than the normal $G_3M_9N_2$ derivative, principally an oligosaccharide-lipid with properties of lipid-linked M_5N_2 .

Four other conditions have been reported under which oligosaccharide-lipids smaller than the $G_3M_9N_2$ derivative accumulate: (a) Mohinder and Elbein (13) showed that when dolichylmannosyl phosphate synthesis was inhibited by the antibiotic amphotycin, M_5N_2 lipid accumulated; (b) glucose deprivation in virus-infected baby hamster kidney cells in culture results in shortened oligosaccharide-lipid chains (14); (c) Datema and Schwarz (15) found that when DMP synthesis was inhibited by 2-deoxyglucose, smaller oligosaccharides were formed; and (d) Chapman *et al.* (16) showed that a mutant strain of thymoma cells (E Thy-1 negative) had lost the ability to synthesize DMP, but made the M_5N_2 -lipid nonetheless; the product was then glucosylated, transferred to the polypeptide as $G_3M_5N_2$, and processed. Recently, Shidoji *et al.* (17) found that the above thymoma mutant could make neither DMP nor mannosylretinyl phosphate. Under all the above conditions, the first 5 mannose residues are therefore not derived from either DMP or MRP. Similarly, since we show here that an M_5N_2 -like oligosaccharide-lipid accumulates in vitamin A-deficient liver, one can conclude that it can be synthesized by a mechanism independent of either DMP or MRP. Spencer and Elbein (18) recently showed that at least some of the α -linked mannose residues in M_5N_2 are derived from GDP-mannose. The 4 additional mannose units of $G_3M_9N_2$ come from lipid-bound mannose. Whether MRP participates in the elongation of oligosaccharide-lipids remains to be investigated. Certainly, the liver pool of GDP-mannose appears to be unaffected by vitamin A deficiency, at least in the hamster (6). It is possible that vitamin A affects DMP synthesis and the synthesis of dolichol itself. Endogenous dolichyl phosphate appears to be rate-limiting in the glycosylation of protein via oligosaccharide-lipids (19), and its level depends on the rate of dolichol synthesis, which seems to be influenced by dietary cholesterol (20), fat (21), or glucose (22). In our experiments, control and deficient animals were fed a strictly equal diet (except in the amount of vitamin A), quantitatively and qualitatively, through pair-feeding. Nonetheless, to circumvent possible adverse effects of the low food intake during the last few days of pair-feeding, we tested the inclusion of glucose in the rats' drinking water during the last 48 h of their lives. It had no effect on the appearance of peak II in the deficient rats. Furthermore, the recovery experiment, which showed that the accumulation of peak II in deficient rats declined to normal levels between 4 and 8 h after vitamin A administration, disposes of the argument that fasting causes this effect. Clearly, the action is rapid enough not to be caused by secondary phenomena attendant upon changes in food intake.

The functional significance of the accumulation of an abnormally small oligosaccharide-lipid with respect to glycopro-

tein synthesis cannot be ascertained at present. Although De Luca *et al.* (23) report a 70% decrease in labeled mannose incorporation into glycoproteins of vitamin A-deficient hamster liver, work from our laboratory⁴ showed a smaller decline in glucosamine uptake into total liver glycoprotein of the vitamin A-deficient rat. On the other hand, when isolating a specific glycoprotein from serum, such as α_1 -macroglobulin (24), which was found to be synthesized by the liver,⁵ we showed that a 30% decrease in uptake of labeled glucosamine or mannose occurred reproducibly in this, but not in all other serum glycoproteins. Similar effects on specific glycoproteins were observed for vitamin A-deficient trachea and cornea (reviewed, 6).

The relatively small effect observed in the increased accumulation of label in peak II (with the corresponding decrease in peak I) may cause a block in the synthesis of only one or several, but not of all, glycoproteins of vitamin A-deficient liver. A functional consequence would then be a decline in the level of only one or several particular liver glycoproteins.

This study, the first of oligosaccharide-lipid synthesis in the living rat, may offer a clue to the systemic function of vitamin A in the mammalian organism. We can now point to an effect of vitamin A deficiency on a definite intermediate, rapidly reversed by administration of the vitamin, providing a likely explanation for the role of the vitamin in glycoprotein synthesis.

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REFERENCES

1. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1976) *J. Biol. Chem.* **251**, 6420
2. Turco, S. J., Stetson, B., and Robbins, P. W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 4411-4414
3. Li, E., Tabas, I., and Kornfeld, S. (1978) *J. Biol. Chem.* **253**, 7762-7770
4. Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C., and Robbins, P. W. (1979) *J. Biol. Chem.* **254**, 4554
5. Hubbard, S. C., and Robbins, P. W. (1979) *J. Biol. Chem.* **254**, 4568-4576
6. De Luca, L. M. (1977) *Vitam. Horm.* **35**, 1-56
7. Haars, L. J., and Pitot, H. C. (1979) *J. Biol. Chem.* **254**, 9401-9407
8. Adhikari, H. R., and Vakil, U. K. (1980) *Biochim. Biophys. Acta* **633**, 465-478
9. Wolf, G., Lane, M. D., and Johnson, B. C. (1957) *J. Biol. Chem.* **225**, 995-1008
10. Dankert, M., Wright, A., Kelley, W. S., and Robbins, P. W. (1966) *Arch. Biochem. Biophys.* **116**, 425-435
11. Quill, H. R., and Wolf, G. *Ann. N. Y. Acad. Sci.* **359**, 331-344
12. Kobata, A. (1979) *Anal. Biochem.* **100**, 1-14
13. Kang, M. S., Spencer, J. P., and Elbein, A. D. (1978) *J. Biol. Chem.* **253**, 8860-8866
14. Turco, S. J. (1980) *Arch. Biochem. Biophys.* **205**, 330-339
15. Datema, R., and Schwarz, R. T. (1979) *Biochem. J.* **184**, 113-123
16. Chapman, A., Fujimoto, K., and Kornfeld, S. (1980) *J. Biol. Chem.* **255**, 4441-4446
17. Shidoji, Y., Sasak, W., Silverman-Jones, C. S., and DeLuca, L. (1981) *Ann. N. Y. Acad. Sci.* **359**, 345-357
18. Spencer, J. P., and Elbein, A. D. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 2524-2527
19. Harford, J. B., Waechter, C. J., and Earl, F. L. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1036-1043
20. Coolbear, T., and Hemming, F. W. (1979) *Biochem. Soc. Trans.* **7**, 370-372

⁴ N. A. Butler (1974) Ph.D. thesis, Massachusetts Institute of Technology.

⁵ T. C. Kiorpes (1978) Ph.D. thesis, Massachusetts Institute of Technology.

21. Henriquez, D. S., Tepperman, H. M., and Tepperman, J. (1979) *J. Lipid Res.* **20**, 624-630
22. Lucas, J. J., Tepperman, H., and Tepperman, J. (1980) *Biochem. J.* **186**, 791-798
23. De Luca, L. M., Silverman-Jones, C. S., and Barr, R. M. (1975) *Biochim. Biophys. Acta* **409**, 342-348
24. Kiorpes, T. C., Molica, S. J., and Wolf, G. (1976) *J. Nutr.* **106**, 1659-1667