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The Biosynthesis of Mannolipids and Mannose-Containing Complex Glycans by the Retina

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Large-scale incubations were carried out with homogenates of the retinas of the 15–16-day-old chick embryo in the presence of GDP[U-^{14}C]mannose, from which there were isolated a mannolipid (Lipid I), oligosaccharide-lipids (Lipid II), and glycoprotein (residue). These incubations were performed in the presence of endogenous acceptors as well as dolichyl phosphate. [^{14}C]Mannolipid I was subjected to chromatography on DEAE cellulose and silicic acid. The response to these, as well as TLC, enzymatic, and chemical treatments, were consistent with the product being dolichyl phosphomannose. [^{14}C]Lipid II was purified by DEAE cellulose chromatography and gel filtration on LH-20. Responses to these treatments, as well as TLC and paper chromatography, were consistent with this product being of the class of the oligosaccharide-pyrophosphate-lipids. The residue remaining after removal of the lipids was shown to contain glycoproteins by conversion of high-molecular-weight radioactive material to low-molecular-weight [^{14}C]mannose-containing glycopeptides by the action of pronase. These reactions and their products are consistent with there being in the retina, the pathway for glycoprotein synthesis involving the participation of the lipid-activated carbohydrates.

When the incubations were performed in the presence of ATP or ADP there was a decrease in the labeling of Lipid I, accompanied by an increase in the labeling of Lipid II and glycoprotein. When incubated in the presence of dolichyl phosphate and detergent, however, the stimulatory effect of ATP did not occur. The effect on these activities of a variety of other nucleotide phosphates was also examined.

Key words: dolichyl phosphomannose, glycoproteins, mannosyltransferases, polyprenyl phosphosugars, retina

It has been suggested (1) that the pathway involving the lipid activation of carbohydrates via their polyprenyl monosaccharides and oligosaccharides is involved in the biosynthesis of the core region of membrane-bound as well as secretory glycoproteins in animal tissues. The major protein in the membranes of the discs of the rod outer segments

Abbreviations: TES – N-tris[hydroxylmethyl]methyl-2-aminoethanesulfonic acid; TX-100 – Triton X-100; C/M – chloroform/methanol (volume to volume); TCA/PTA – trichloroacetic acid/phosphotungstic acid; SDS – sodium dodecyl sulfate; TLC – thin layer chromatography.

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of the retina is the unique glycoprotein, rhodopsin. This molecule has been shown to contain 9 moles of mannose and 5 moles of glucosamine per mole (2). Studies from this laboratory have shown the presence of, optimal conditions for, and many of the properties of lipid and glycan mannosyltransferases in preparations from the retinas of several species (3–7). Three major classes of products are formed: a mannolipid, oligosaccharide(-pyrophospho-)lipids, and mannose-containing glycoproteins. The present report is concerned with aspects of the characterization of these materials synthesized by large-scale preparations from the retina of the embryonic chick. These reactions and products are consistent with the presence in the retina of the pathway involving the participation of the lipid-activated carbohydrates in the biosynthesis of its glycoproteins. Further studies may reveal whether these types of reactions are involved in the biosynthesis of the carbohydrate chains of rhodopsin. This report also describes the differential responses by these mannosyltransferases to the presence of nucleotides, principally ATP, in the formation of these 3 products.

MATERIALS AND METHODS

Enzyme Preparation

Retinas were obtained from the eyes of 15–16-day-old chick embryos (White Leghorn chickens), after the eggs had been incubated at 37°C. From 120 eggs, about 4 g wet weight of retinas can be recovered. The tissue was homogenized in 0.25 M sucrose (2 volumes of sucrose to 1 g of tissue) in the cold using a hand operated Ten Broeck all-glass homogenizer. The homogenate was dialyzed in the cold for 4 h against at least a 100-fold volume excess of 0.01 M TES buffer, pH 7.0, with one change of dialysis medium. After dialysis, the retentate was rehomogenized and either used at once or stored at −20°C.

Assay for Mannosyl Transferase Activities

Incubations were carried out as described previously (3–7) in 12-ml glass centrifuge tubes for 5 min at 37°C. The mixture contained: GDP[$U^{14}C$]mannose, 3.3 μM (specific activity 160–221 μCi/μmole); $MnCl_2$, 3.3 mM; TES buffer, 0.2 M, pH 7.0; and enzyme (0.6–0.9 mg protein) in a total volume of 0.15 ml. When incubations were performed in the presence of exogenously supplied dolichyl phosphate, the lipid was dispersed in TX-100 [final concentration, 0.24% (wt/vol)] by vigorous agitation with the Vortex. The reaction was stopped by the addition of 3 ml of a mixture of cold 6% trichloracetic acid/0.5% phosphotungstic acid (TCA/PTA) (8). All of the assay steps were carried out in the cold, except where indicated. The mixture, after standing 15–30 min was centrifuged at 1,000 × g for 5 min, and the supernatant solution discarded. The pellet was rewashed twice by resuspending in 2 ml of TCA/PTA, followed by centrifugation. After the excess fluid had been drained for a few minutes, the assay could be interrupted at this point and the pellet stored at −20°C. Lipids were extracted from the washed pellet by vigorous mixing with 2 ml of C/M (2:1) on the Vortex. After centrifugation for 5 min at 1,000 × g the C/M extract was transferred to a ground-glass centrifuge tube. The C/M extraction of the pellet was carried out 3 times. The pooled C/M extract was then equilibrated with either water or 0.1 M KCl, and the lower phase washed twice with theoretical upper phase prepared with water or KCl, according to the procedures described by Folch, Lees, and Sloane-Stanley (9). The washed lower phase, containing Lipid I, was evaporated to dryness in counting vials and the amount of radioactivity present determined by liquid scintillation spectrometry.

The acid-washed, C/M (2:1) extracted pellet was then mixed vigorously with 2 ml of C/M/H_2O (10:10:3) and the supernatant solution recovered after centrifugation. This extraction was performed twice, and this material (Lipid II) was evaporated to dryness in counting vials on a steam bath, and the amount of radioactivity measured.

The residual pellet (referred to as the "residue") was then dissolved in 0.5 ml of Unisol overnight at room temperature. After the addition of 0.25 ml of methanol, the solution was transferred to a counting vial and the radioactivity determined in the presence of 5 ml of "Complement" (the Unisol-Complement scintillation counting system was obtained from Isolab, Inc., Akron, Ohio).

Every incubation was performed in duplicate, accompanied by a zero time control, i.e., a complete incubation to which enzyme was added after the addition of TCA/PTA. Values obtained with the latter type of incubation were similar to that obtained using boiled enzymed controls.

CHEMICALS

All solvents used in the chemical synthesis and in the preparative procedures for the mannolipids were redistilled before use. GDP[$U^{14}C$]Mannose was purchased from New England Nuclear Corporation, Boston, Massachusetts. Nucleotides were obtained from Cal Biochemicals, LaJolla, California and Sigma Chemical Company, St. Louis, Missouri. DEAE-cellulose Selectacel, No. 70, Standard type, was purchased from Schleicher and Schuell Company, Keen, New Hampshire. Sephadexes G-200, G-25, and LH-20, were purchased from Pharmacia, Piscataway, New Jersey. Silicic acid (Unisil) was obtained from Clarkson Chemical Company, Inc., Williamsport, Pennsylvania.

Dolichyl phosphate was prepared according to the procedure described by Wedgewood, Strominger, and Warren (3, 8) using O-phenylene phosphochloridate as phosphorylating reagent (purchased from Aldrich Chemical Company, Milwaukee, Wisconsin).

PREPARATION AND PURIFICATION OF MANNOLIPIDS

Large scale incubations were carried out using either the endogenous acceptor alone or in the presence of exogenously supplied dolichyl phosphate. When the latter compound was used, it was evaporated to dryness with nitrogen, then dispersed by vigorous vortex mixing in the presence of TX-100. The large scale incubations were either multiple incubations performed as indicated in Materials and Methods, or incubations increased proportionately in scale.

Purifications of the products of the enzymatic reactions were carried out by the chromatographic procedures described in general below, and in more detail in the legends to the pertinent figures.

Lipid I

Mild deacylation: DEAE-cellulose and silicic acid chromatography. The C/M (2:1) extract of the acid-washed pellet was partitioned according to the procedure of Folch et al (9), and the lower phase was then subjected to a mild deacylation according to the method of Lester and Steiner (11). After deacylation, the lipid phase was again partitioned according to the procedure of Folch et al (9), and then placed over a column of DEAE-cellulose acetate prepared as described by Rouser, Kritchevsky, and Yamamoto (12) or by Dankert, Wright, Kelley, and Robbins (13) (see Figs. 1 and 2).

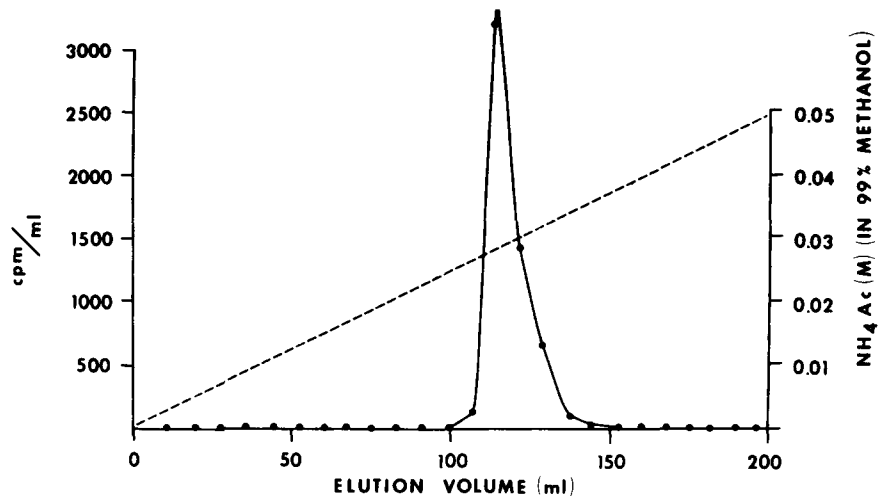

Fig. 1. Elution profile of Lipid I from DEAE-cellulose acetate. The "Folch washed," C/M (2:1) extract of the pellet obtained after treating large-scale incubations with TCA/PTA as described in Materials and Methods, was placed onto a DEAE-cellulose column prepared as described by Rouser et al (12). After washes with C/M (2:1), methanol, and 99% methanol, a linear gradient between 99% methanol and 99% methanol containing 0.1 M NH_4Ac was performed. The total volume of the system was 400 ml. Gradient (- - -).

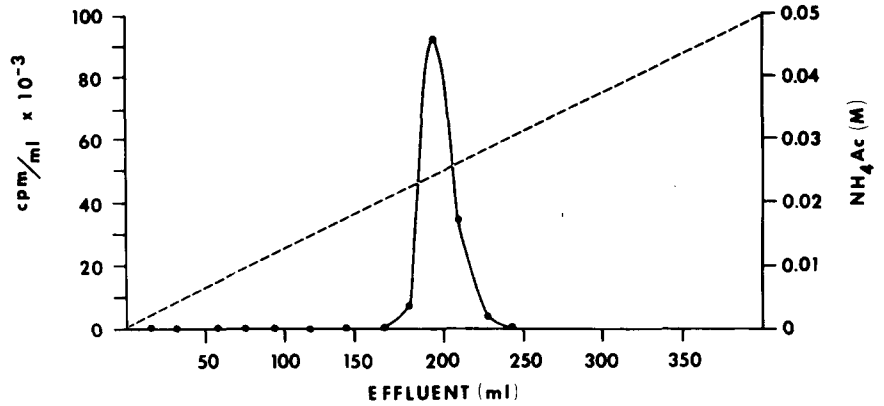

Fig. 2. Elution profile of dolichyl phosphomannose from DEAE-cellulose. The "Folch washed" C/M (2:1) extract of the TCA/PTA pellet obtained after large-scale incubations were performed in the presence of dolichyl phosphate, as described in Materials and Methods, was placed onto a column of DEAE-cellulose, After washes with C/M (2:1), methanol, and 99% methanol, a linear gradient was performed between 99% methanol (400 ml) and this solvent containing 0.1 M NH_4Ac (400 ml). Gradient (- - -).

The product obtained from DEAE-cellulose was evaporated to dryness, redissolved in C/M (2:1), and partitioned according to the procedure of Folch et al (9). The washed lower phase was evaporated to dryness, redissolved in C/M (95:5), and placed over a column of silicic acid (Fig. 3).

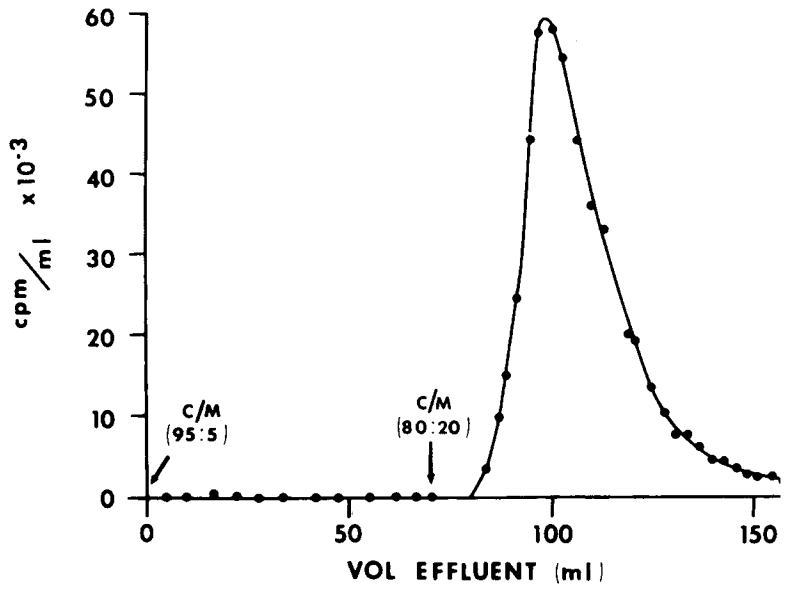

Fig. 3. Silicic acid chromatography of dolichyl phosphomannose synthesized by the retina. The radioactive material obtained after DEAE-cellulose chromatography, described in Fig. 2, was placed onto a column of silicic acid (Unisil) in C/M (95:5). Where indicated by the arrow, the solvent was changed to C/M (80:20). The flow rate was maintained at 0.6 ml/min, and 3-ml fractions were collected.

Lipid II

DEAE-cellulose chromatography and gel filtration on Sephadex LH-20. As described in the assay procedure (Materials and Methods), Lipid II is the material extracted with C/M/H_2O (10:10:3) from the pellet remaining after the extensive extraction with TCA/PTA and C/M (2:1). The C/M/H_2O (10:10:3) extract was then fractionated on DEAE-cellulose acetate (see Fig. 5). The product recovered from DEAE-cellulose was evaporated to dryness on a rotary evaporator, redissolved in 5ml of C/M/H_2O (10:10:3), and placed over a column (2.5 X 41 cm) of Sephadex LH-20, which had been packed and equilibrated with C/M/H_2O (10:10:3). This same solvent was used to elute the product from the gel, the flow rate being regulated at about 0.5 ml/min. Lipid II isolated by these procedures was stored at −20°C.

The elution patterns from these columns were monitored by measuring the amount of radioactivity in aliquots of the fractions, after evaporating to dryness in counting vials.

Residue: treatment with SDS and pronase. The residue remaining after TCA/PTA treatment followed by extraction with C/M (2:1), and C/M/H_2O (10:10:3) was solubilized by incubating with 1% SDS at 50°C for 4 h. The solubilized material was examined by gel filtration on a column of Sephadex G-200 (Fig. 8).

Chromatography and electrophoresis. Thin layer chromatography (TLC) was carried out on precoated plates of silica gel 60 (without fluorescent indicator) 0.25 mm thick, (EM Reagents, Merck, Darmstadt, Germany), of dimensions 20 X 20 cm or 5 X 20 cm. TLC was also performed with 0.25 mm thick plates of microcrystalline cellulose (Avicel, Analtech, Inc., Newark, Delaware). The following solvents were used: A) C/M/acetic acid/H_2O (25:15:4:2), B) isobutyric acid/concentrated ammonium hydroxide/H_2O (57:4:39), C) 1-butanol/95%

ethanol/H_2O (10:1:2), D) 1-butanol/pyridine/0.1 N HCl (5:3:1). Paper electrophoresis was carried out with a Gilson high voltage electroforator using borate buffer as described previously (14). Lipids were visualized after TLC by exposure to iodine vapor, spraying with anisaldehyde reagent [anisaldehyde/sulfuric acid/95% ethanol (1:1:18)] followed by heating the plate to about 150°C, or spraying with the phosphate reagent described by Dittmer and Lester (15). Carbohydrates were visualized on paper by the benzidine-periodate (16) and the alkaline silver nitrate procedures (17). Radioactivity was detected after TLC by scraping zones into counting vials and adding 10 ml of a TX-100 toluene solution described previously (18), or 10 ml of a similar solution obtained commercially, Formula 950A (New England Nuclear Corporation, Boston, Massachusetts), and measuring the amount of radioactivity by scintillation spectrometry. Radioactivity was detected after paper chromatography and electrophoresis by scanning the strips.

OTHER PROCEDURES

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (19). Phosphodiesterase was measured as described previously (18). Pyrophosphatase activity was determined by following the cleavage of β-DPN in the presence of L+ lactate (Hohorst, Ref. 20). α-Mannosidase was purified from jack bean meal and assayed as described by Li and Li (21).

RESULTS

Isolation of Lipid I and Dolichyl Phosphomannose Synthesized by the Retina

DEAE-cellulose and silicic acid chromatography. The product formed from GDP-[^{14}C] mannose when incubated in the presence of exogenously added dolichyl phosphate and in its absence (Lipid I) were both retained by DEAE-cellulose. No radioactivity was eluted from the columns with the washes of C/M (2:1), methanol, or 99% methanol. Quantitative recovery of radioactivity was obtained by the linear gradient elution, typical patterns of which are seen in Figs. 1 and 2. In different preparations both mannolipids were eluted between 0.025 and 0.04 M ammonium acetate in 99% methanol using DEAE-cellulose prepared by the procedure of Dankert et al (13) or by that of Rouser et al (12).

After extraction by the procedure of Folch et al (9) Lipid I and dolichyl phosphomannose from DEAE-cellulose were subjected to silicic acid (Unisil) chromatography, a typical elution pattern of which is seen in Fig. 3. A single peak of radioactive material was obtained in the C/M (80:20) fraction with dolichyl phosphomannose (Fig. 3) and with Lipid I (data not shown). A similar elution pattern was obtained when carried out by gradient elution. Of the radioactivity placed onto the column in C/M (95:5), quantitative recovery was obtained in the C/M (80:20) fraction. The components in the peak tubes were pooled, evaporated to dryness, redissolved in C/M (2:1) and partitioned according to the procedure of Folch et al (9).

TLC. The product synthesized by the retina using dolichyl phosphate as acceptor, and the product from the endogenous acceptors, Lipid I, extracted with C/M (2:1) from the TCA/PTA pellet, migrated on TLC in several solvent systems in a manner similar to one another and to standard dolichyl phosphomannose as seen in Fig. 4, using the acidic solvent system, A. While the migrations in neutral and basic solvents were greatly reduced compared to the acidic system, the 2 products showed identical R_f values (3, 4, 7).

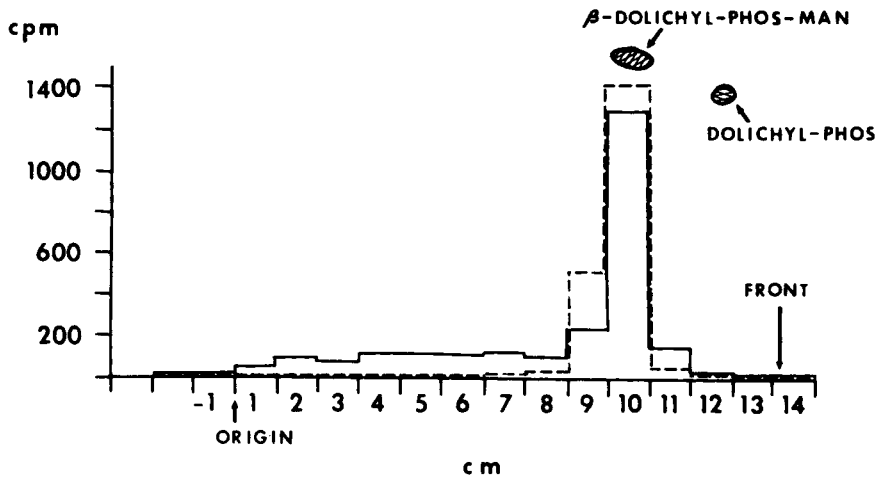

Fig. 4. TLC of mannolipid I and dolichyl phosphomannose. After purification by the column chromatographic procedures described in Materials and Methods, [^{14}C] mannolipid I and dolichyl phosphate-[^{14}C] mannose synthesized by the retina were chromatographed on silica gel plates using as solvent system, C/M/acetic acid/H_2O (25:15:4:2). Their migrations were detected by scintillation spectrometry of 1-cm zones of the gel scraped into counting vials. The standards were visualized by the anisaldehyde spray reagent. Lipid I (- - -); dolichyl phosphomannose (——).

Acid hydrolysis. After strong acid hydrolysis (2 N HCl, 100°C, 4 h, sealed tube), of Lipid I or dolichyl phosphomannose, the hydrolysate was placed over a mixed bed resin column (Ag-50- X 8(200–400 mesh)H^+ and Ag-1- X 8(200–400 mesh) formate). The radioactivity was recovered quantitatively in the water eluate of the column. When examined by borate electrophoresis and by paper chromatography in solvent systems B, C, and D, only one radioactive spot was observed which migrated with standard mannose. Lipid I and dolichyl phosphomannose synthesized by the retina were rapidly hydrolyzed by dilute acid (0.1 N HCl) at room temperature ($t_{1/2}$, about 12 min) (3).

Resistance to phosphodiesterases; α-mannosidase. The mannolipids synthesized by the retina were not cleaved by phosphodiesterase preparations from snake venom either in the presence or absence of detergents. In addition to phosphodiesterase these preparations contained alkaline phosphatase and inorganic and nucleotide pyrophosphatases. The action of these enzymes on the mannolipids synthesized by the retina was also examined by the procedure described by Ghalambor, Warren, and Jeanloz (22) in which a chaotropic agent, KCNS, was present to aid in the availability of the substrate to the enzyme. The retina mannolipid was also resistant to cleavage when treated in this manner. Neither Lipid I nor dolichyl phosphomannose synthesized by the retina were cleaved by treatment with α-mannosidase purified from jack bean meal incubated at either pH 6.0 or 4.5 in the presence or absence of detergents. Control studies with all of these enzymes showed that the mannolipids did not interfere with their activities.

In addition to the chromatographic properties described above, Lipid I and the product synthesized using dolichyl phosphate as exogenous acceptor showed identical responses to treatment with mild and strong acids and bases to those described for dolichyl-β-mannosyl phosphate (3, 7).

Lipid II – Chromatography

The C/M/H_2O (10:10:3) extract of the acid-washed, C/M (2:1) extracted pellet was chromatographed on DEAE-cellulose (Fig. 5). No radioactivity was obtained in the preliminary wash of the column with C/M/H_2O (10:10:3), but the produce was recovered by the linear gradient using 0.2 M ammonium acetate in this solvent. The radioactive material eluted as a single peak from ~ 0.08 to 0.11 M ammonium acetate, with recoveries of 80–95% in different preparations. Using a linear gradient of NH_4CHO_2 in C/M/H_2O (10:10:3) the radioactivity was eluted at 0.11 M salt, with a recovery of about 75% of the radioactivity applied to the column (data not shown).

Lipid II, recovered from DEAE-cellulose, was desalted by gel filtration on a column of LH-20 (2.5 X 20 cm) packed in C/M/H_2O (10:10:3) and eluted with this solvent. Over 90% recovery of radioactivity was obtained in a single sharp peak at about 0.36 of the calculated V_t.

The migration of purified Lipid II after paper chromatography in solvent system B was also similar to that of the oligosaccharide-lipid described by Lucas, Waechter, and Lennarz (23).

TLC of Lipid II (recovered from DEAE-cellulose) on microcrystalline cellulose (Avicel) as described by Chambers and Elbein (24) showed the possible presence of 2 components which migrated slower than dolichyl phosphomannose in this system (Fig. 6). This pattern was similar to, but simpler than that seen with, the oligosaccharide-lipid derived from aorta.

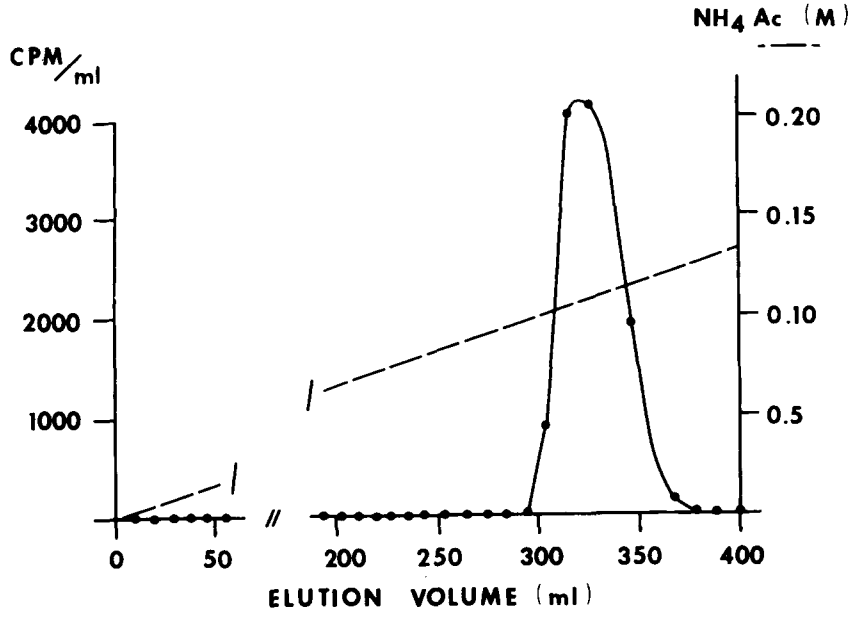

Fig. 5. DEAE-cellulose chromatography of Lipid II. The C/M/H_2O (10:10:3) extract of the TCA/PTA pellet obtained after a large-scale incubation using the endogenous acceptors, as described in Materials and Methods was placed over a column of DEAE-cellulose acetate prepared by the method of Rouser et al, (12), and packed in 10:10:3. After an initial wash with 200 ml of this solvent, a linear gradient was carried out using 200 ml of 10:10:3 in the mixing chamber and 300 ml of this solvent containing 0.2 M NH_4Ac in the reservoir. Fractions of 5 ml each were collected at a flow rate of 1 ml/min. Gradient (- - -).

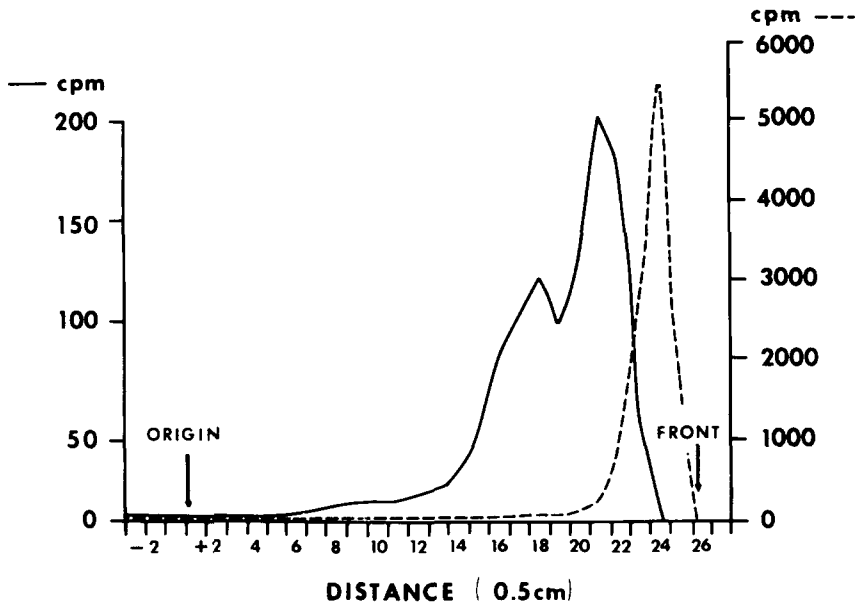

Fig. 6. TLC of Lipid II. Lipid II, purified by chromatography of DEAE-celluose and gel filtration of LH-20, was applied to a 250 micron thick layer of microcrystalline cellulose (Avicel) and the plate irrigated with solvent system B. Dolichyl phosphate-[^{14}C] mannose was also applied to the same plate. After air drying, 0.5-cm zones were scraped into counting vials and the radioactivity determined by liquid scintillation spectrometry. The ordinate on the left refers to Lipid II (——); that on the right, to dolichyl phosphomannose (- - -).

Strong acid hydrolysis of purified Lipid II followed by borate electrophoresis and paper chromatography (solvent system D), showed that the radioactivity was present in a single component which migrated with standard mannose.

These chromatographic characteristics of Lipid II are consistent with the suggestion that this product is an oligosaccharide(-pyrophospho-)lipid similar to those synthesized by other tissues (23, 24). Studies are in process to characterize this product further.

Residue

The residue remaining after the isolation of Lipid I and Lipid II, was solubilized in 1% SDS as described in Materials and Methods. Within 4 h at 50°C, ~ 70% of the radioactivity was solubilized. The elution pattern of this material from Sephadex G-200 is seen in Fig. 7. Two large radioactive components can be seen: one eluting at the void volume and a second for which an apparent molecular weight of 110,000 was calculated. After treatment with pronase, the high-molecular-weight components were converted to those of lower molecular weight. When examined further by gel filtration on Sephadex G-75, a major radioactive component was obtained whose apparent molecular weight was calculated to be 7,000 (data not shown). Quantitative recovery of radioactive material was obtained from both columns.

These findings, of the conversion of the high-molecular-weight components to a low-molecular-weight component by the action of pronase, together with the observation that

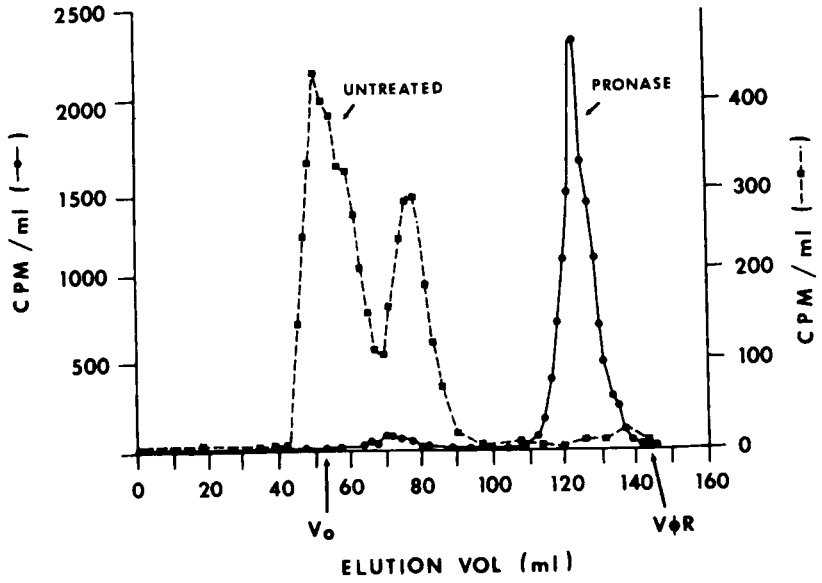

Fig. 7. Elution patterns from Sephadex G-200 of the "residue" in SDS before and after treatment with pronase. The residue (containing 15,400 cpm) remaining after the removal of Lipid I and Lipid II, was dissolved in 4 ml of 1% SDS by heating at 50°C for 4 h. The solution was fractionated on a Sephadex G-200 column packed in and eluted with 0.005 M Tris-HCl buffer, pH 7.0, containing 0.2% SDS. A separate sample of the residue (containing 65,000 cpm) was incubated at 37°C with 0.3 mg pronase in 0.2 M Tris-HCl, pH 7.9, containing 0.0015 M $CaCl_2$. After 4 h, 78% of the radioactivity was solubilized. The mixture was centrifuged and the supernatant solution adjusted to 1% SDS and then kept at 50°C for 4 h. The solution was then fractionated on Sephadex G-200 as above. The column dimensions were: 1.5 × 84 cm; V_0 = 52 ml; V(phenyl red) = 143 ml. The flow rate was maintained at 8 ml/h. Treated with pronase (•); not treated with pronase (■).

the only radioactive component was mannose (3), are consistent with the conclusion that the sugar was a component of glycoproteins in the fraction described in this report as the "residue."

Effect of Nucleotides

The effect of nucleotides on these enzymatic activities was examined. ATP inhibited the incorporation of radioactivity from GDP[^{14}C]mannose into Lipid I, while stimulating the incorporation into Lipid II and the residue (Fig. 8). ATP at greater than 1 mM stimulated the incorporation of radioactivity into Lipid II by twofold and into the residue by 30%, while the incorporation into Lipid I was inhibited by 50%. A similar inhibition of mannolipid formation by ATP has been observed previously by Richards and Hemming (25) although the effect on the other products was not noted. However, stimulation, instead of the inhibition observed here, of the formation of mannolipids by ATP (26) has been reported.

When the incubations were performed in the presence of 20 μM dolichyl phosphate, which stimulated the incorporation of radioactivity into the fraction extracted by C/M (2:1) (dolichyl phosphomannose) about 27-fold over the endogenous level, the same extent

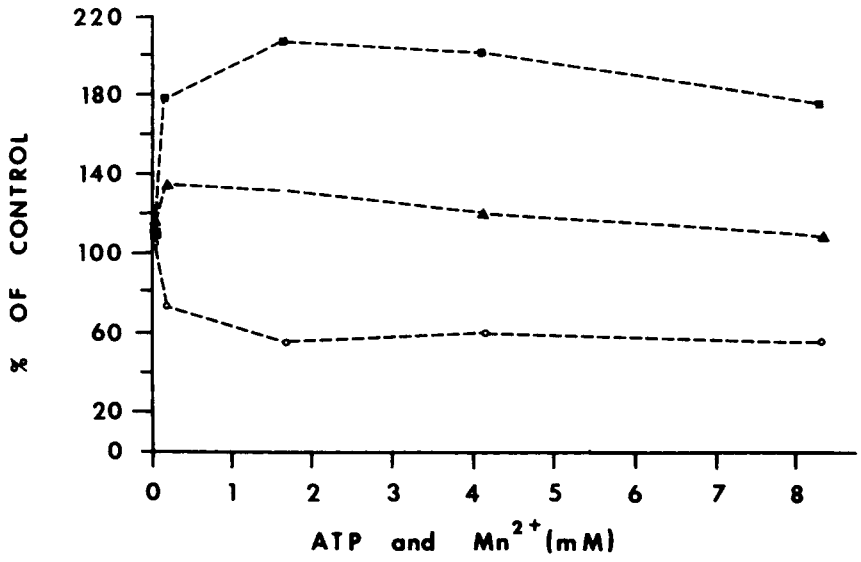

Fig. 8. Effect of the concentration of ATP on the endogenous incorporation of radioactivity into the 3 products. Incubations were performed for 5 min using GDP[U-^{14}C]mannose, TES buffer, pH 7.0, and enzyme (0.80 mg protein) and assays were carried out as described in Materials and Methods. In addition to the usual concentration of Mn^{2+} (3.3 mM), additional $MnCl_2$ was added equal to that indicated for ATP. The control values in the absence of added ATP were: Lipid I, 3,120 cpm; Lipid II, 1,380 cpm; Residue, 3,400 cpm. Lipid I, (○); Lipid II, (■); residue, (▲).

of inhibition in the presence of ATP was seen (Fig. 9). Unlike the response with the endogenous acceptors, however, the stimulation by ATP of the incorporation of radioactivity into the oligosaccharide-lipid fraction (Lipid II) and the residue did not occur.

The loss of the stimulatory effect of ATP was probably the result of the inhibition by TX-100 on the incorporation of radioactivity into Lipid II and the residue. Thus, using the endogenous acceptor system in the presence of 0.24% TX-100, there was a 32% decrease in the labeling of Lipid II and a 62% reduction in the radioactivity incorporated into the residue. These decreases in incorporation were not overcome by the presence of optimal concentrations of ATP (1.7 mM). These observations suggest that it may be necessary for the polyprenyl-monosaccharide as well as the oligosaccharide-lipid and glycoprotein acceptors to be present in a membrane-bound form in order for the stimulation by nucleotides to be exerted.

In the present studies, additional amounts of $MnCl_2$ equal to that of ATP were also present during the incubations. Similar results were observed, however, over this same range in ATP concentration without the addition of additional Mn^{2+} over that usually present (3.3 mM). The time course of these reactions in the presence of ATP (1.67 mM) was the same as in its absence (3, 7). These effects were not observed for $MnCl_2$ alone at these concentrations.

Studies were also performed in the presence of a constant concentration of ATP (1.67 mM) with varying amounts of Mn^{2+} (0–6.67 mM) (data not shown). In the absence of metal, all 3 activities were inhibited. While inhibition of the incorporation of radioactivity into Lipid I persisted as the ratio of Mn^{2+} to ATP was increased to 4:1, the stimulation

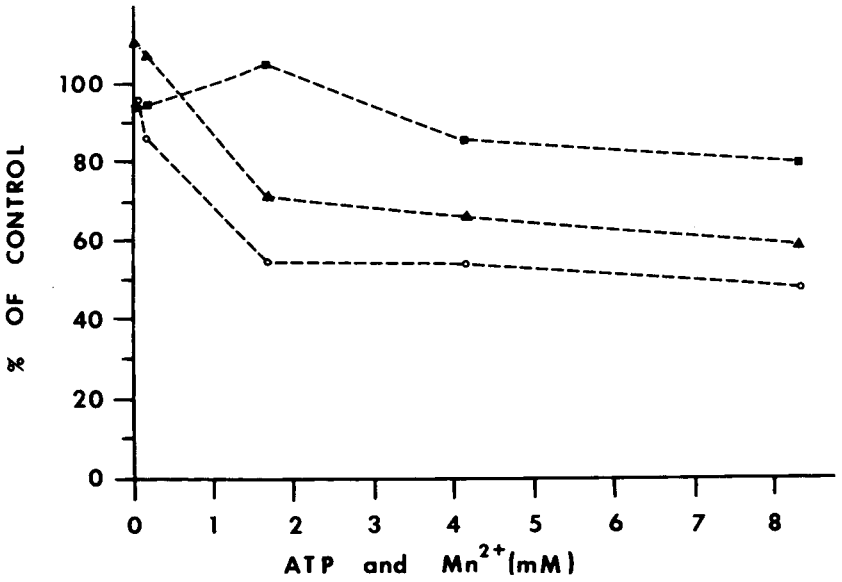

Fig. 9. Effect of ATP on the mannosyltransferases incubated in the presence of dolichyl phosphate. Incubations were carried out for 5 min as described in Fig. 8, (using a different enzyme preparation), but contained in addition, dolichyl phosphate (19 μM) and TX-100 (0.24%). The control values, in the absence of added ATP were: Lipid I, 80,600 cpm; Lipid II, 1,720 cpm; Residue, 3,292 cpm. Lipid I (○); Lipid II (■); residue (▲).

into Lipid II and into the residue occurred as previously. Thus, the inhibition of Lipid I activity was not due solely to chelation effects of ATP on the metal, but was due to an effect on the activity of the enzyme itself.

When ADP was added to 1.67 mM, the stimulation of incorporation of label into Lipid II and the residue, and the inhibition of incorporation into Lipid I were similar to those observed with ATP (Table I). AMP, cyclic-AMP, and inorganic phosphate had little effect on these activities.

The inhibition by ATP and ADP of the formation of Lipid I, accompanied by the stimulation of incorporation into Lipid II and the residue, suggested that ADP[^{14}C]-mannose might have been formed by the reversal of the reaction and that this compound might be more directly involved in the formation of Lipid II and the glycoproteins present in the residue. That this was probably not the case may be concluded from experiments in which up to 150-fold molar excess of nonradioactive ADP-mannose over GDP[^{14}C]-mannose was present during the incubations. There was no effect on the incorporation of radioactivity into Lipid I, II, or the residue from the presence of ADP-mannose.

In Table I also is shown the effects of a variety of other nucleotides examined at this same concentration. Although these experiments were carried out under conditions approximating the initial rates, their interpretation in terms of specificity is difficult due to the possible influence of phosphokinases and phosphatases in the enzyme preparation. Nonetheless, some differential response to these reagents on the formation of the 3 products was evident. Of the compounds tested GDP and GTP inhibited all of these activities to the greatest extent. Some of this inhibition may be due to reversal of the reaction. This has been observed by incubating dolichyl phosphate [^{14}C]mannose in the presence of GDP,

TABLE I. Influence of Various Nucleotides on the Enzyme Activities*

Addition (1.67 mM)	Percentage of radioactivity in products		
	Lipid I	Lipid II	Residue
ATP	57	207	126
ADP	71	229	125
AMP	99	98	98
Cyclic-AMP	85	97	91
CTP	96	125	89
dCTP	96	168	103
CDP	82	164	118
GTP	4	7	7
GDP	3	4	5
GMP	29	27	22
Cyclic-GMP	103	104	94
ITP	22	51	50
TTP	100	108	97
UTP	85	47	61
UDP	81	49	52
PEP	98	–	117[a]
P_i	89	78	98

*Incubations were carried out for 5 min using 0.84 mg of dialyzed homogenates of embryonic chick retina, and assayed for the incorporation of radioactivity into products as indicated in Materials and Methods. The added compounds were all present at 1.67 mM. The data is presented in terms of the percentage of the radioactivity incorporated compared to the controls incubated in absence of these compounds which in different enzyme preparations and different experiments varied from: Lipid I, 2,800–3,500 cpm; Lipid II, 1,300–1,800 cpm; residue, 1,960–3,300 cpm.

[a]Refers to Lipid II plus residue.

and following the appearance of a new uv quenching, radioactive area which migrated with GDP-mannose (3) (data not shown). However, in addition to reversal of the reaction which produced dolichyl phosphomannose, an inhibitory effect on the mannosyltransferases by the guanosine nucleotides may also be involved. The decrease in the labeling of Lipid II and the residue observed in the presence of UDP and UTP with relatively little effect on Lipid I also indicates influences by these compounds other than reversal of reaction. While cyclic-GMP was without effect, GMP also resulted in considerable inhibition.

DISCUSSION

The endogenous acceptor-lipid in the retina was shown to have the properties of dolichyl phosphate as indicated by the characteristics of the product of the mannosyltransferase reaction. This material and that synthesized using exogenously added dolichyl phosphate were identical to one another by a variety of criteria, and to authentic dolichyl phosphomannose. Studies from this laboratory with the retina from the embryonic chick have revealed that dolichyl phosphate was the only polyprenyl phosphate among a variety tested, including retinyl phosphate, which was capable of stimulating the incorporation of mannose from GDP[^{14}C]mannose into material extractable by C/M (2:1) (3, 7). These findings, in addition to the formation of material which had the properties of the oligosaccharide-pyrophosphate-lipids described in other tissues, are consistent with the presence

in the retina of the pathway for the biosynthesis of its glycoproteins involving the participation of the lipid-activated carbohydrates as intermediates.

In a previous report from this laboratory [Kean and Plantner, (27)] the biosynthesis by preparations from bovine retina of a high-molecular-weight mannose, glucosamine-containing oligosaccharide-lipid was observed. It was suggested that this component may function as an intermediate in the biosynthesis of the carbohydrate groups of bovine rhodopsin.

Several nucleotide di- and triphosphates have been shown in this study to influence these mannosyltransferase reactions. Most extensively studied was the effect of ATP. The mechanism whereby its presence inhibited the incorporation of mannose into Lipid I, while at the same time stimulating over twofold the labeling of the oligosaccharide-lipid fraction, Lipid II, and also stimulating the labeling of the glycoprotein, is not known. The stoichiometry of these relationships was not simple. Much more (up to twofold) radioactivity was found incorporated into the complex glycans than could be accounted for by the decrease in labeling of Lipid I after a 5 min incubation carried out in the presence of ATP. The similarity in response to ADP as with ATP may reflect the presence of adenylate kinase in the preparation from the retina. The nucleotides may function as participants in the formation of as yet unknown intermediates for the biosynthesis of the oligosaccharide-(-pyrophosphate-)lipids (Lipid II) and glycoproteins, or act as modifiers of these enzymatic reactions.

Previous studies have shown (3, 7) that detergent is necessary in order to obtain stimulation from exogenously added dolichyl phosphate. The concentration of TX-100 which was optimal for this stimulation (0.24%) was, however, inhibitory for the endogenous labeling of Lipid II and the residue. This probably accounts for the failure of ATP to stimulate the labeling of Lipid II and the residue when the reactions were performed in the presence of dolichyl phosphate and TX-100. Nonetheless, the inhibition of the labeling of dolichyl phosphomannose when incubated in the presence of ATP was still observed, and to the same proportionate extent as with the endogenous product, Lipid I. These observations suggest that in order for the nucleotide stimulation of the formation of the complex glycan products to be effected, the activated lipids may be required to be present in the form of membrane-bound components rather than as detergent suspensions, a relationship which may reflect more accurately the situation within the cell. Further studies are in process to examine more fully the influence of the nucleotides on these reactions.

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