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Synthesis of P^1-Dolichyl P^2-α-D-Mannopyranosyl Pyrophosphate. The Acid and Alkaline Hydrolysis of Polyisoprenyl α-D-Mannopyranosyl Mono- and Pyrophosphate Diesters†

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ABSTRACT: P^1-Dolichyl P^2-α-D-mannopyranosyl pyrophosphate (**9**) has been chemically synthesized by a method developed for the corresponding citronellyl derivative, which also contains a saturated α isoprene residue. In each case, the P^1-polyisoprenyl P^2-diphenyl pyrophosphate was treated with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate to give a fully acetylated pyrophosphate diester, which was purified chromatographically and subsequently deacetylated. The citronellyl and dolichyl pyrophosphate diesters were compared with the previously synthesized citronellyl and dolichyl α-D-mannopyranosyl phosphate, respectively, by chromatography and by hydrolysis experiments. Good separations of the monophosphate from the corresponding pyrophosphate were achieved by silica gel tlc in a variety of solvent systems. Brief dilute acid hydrolysis of both the mono- and pyrophosphate diesters gave D-mannose and no α-D-mannosyl phosphate, the other products being polyprenyl phosphate and pyrophosphate, respectively. When the polyprenyl α-D-mannopyranosyl mono- and pyrophosphate diesters were treated with hot dilute alkali, the major products were polyprenyl phosphate and substances arising from the breakdown of D-mannose, indicating that the α-D-mannosyl phosphate bond was the most labile linkage in both compounds. However, the formation of a small proportion of free dolichol indicated that α-D-mannosyl phosphate was also formed to a minor extent. The interpretation of the results of the alkaline hydrolysis was complicated by the instability of D-mannose under basic conditions, it being almost completely degraded by even a brief treatment.

The recent demonstration that polyisoprenyl mannolipids are formed *in vitro* by enzymic systems obtained from mammalian and avian sources has prompted the study of the probable role of these compounds as intermediates in glycoprotein biosynthesis (Richards and Hemming, 1972; Baynes *et al.*, 1973; Waechter *et al.*, 1973). Partly on the basis of chromatographic and hydrolytic data, the compounds have been identified as phosphate diesters of D-mannose and of either dolichol or a very similar, long-chain isoprenol. In several instances, strong evidence for a monophosphate diester bridge was obtained from chromatographic comparison of the endogenous mannolipid phosphates with synthetic dolichyl α-D-mannopyranosyl phosphate (Warren and Jeanloz, 1973a,c). The compounds characterized in this way were formed by preparations from pig-liver endoplasmic reticulum (Evans and Hemming, 1973), calf pancreas (Tkacz *et al.*, 1974), human lymphocytes (Wedgwood *et al.*, 1974), and hen oviduct and bovine

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thyroid (Waechter *et al.*, 1973). However, the possibility remained that the compounds might be pyrophosphates, as it was not possible to predict whether or not the chromatographic differences associated with the mono- and pyrophosphate groups would be masked by the presence of such a long aliphatic chain (approximately 100 carbon atoms).

The identification of the products of dilute acid treatment of polyisoprenyl D-mannosyl phosphates has been used as a method to distinguish between mono- and pyrophosphate diesters, on the assumption that pyrophosphate diesters would undergo preferential hydrolytic scission between the two phosphate groups (Jung and Tanner, 1973; De Luca *et al.*, 1973). Since the dolichyl phosphate bond is known to be stable to mild acid treatment, it was suggested that formation of D-mannosyl phosphate would indicate the presence of a pyrophosphate, whereas the absence of D-mannosyl phosphate would indicate a monophosphate. However, no standard compounds have been available to test this hypothesis.

Although it is generally accepted that enzymically formed polyisoprenyl mannolipids are rapidly hydrolyzed by sodium hydroxide above 60°, the nature of the products formed is cause for controversy, possibly because of variations in the conditions of hydrolysis from one group of workers to another. In some cases, D-mannosyl phosphate was reported as the product of hydrolysis of mannolipid phosphates obtained from mammalian and avian systems (Waechter *et al.*, 1973; Baynes *et al.*, 1973), or from the compound formed by a particulate enzyme from cotton fibers (Forsee and Elbein, 1973); the latter workers also reported that only 25% of the released ^{14}C label was bound to an anion-exchange resin, and could therefore have been D-mannosyl phosphate. On the other hand, Evans and Hemming (1973) hydrolyzed the mannolipid phosphate formed by pig-liver endoplasmic reticulum, as well as synthetic dolichyl α-D-mannopyranosyl phosphate (**11**), and reported D-mannose as the only carbohydrate product obtained from both compounds, and dolichyl phosphate as the lipid product. Cawley *et al.* (1972) hydrolyzed a yeast phosphomannan containing α-D-mannosyl phosphate residues and did not obtain any D-mannosyl phosphate. Furthermore, Behrens *et al.* (1971) reported that the nature of the mannose-derived products from the alkaline hydrolysis of a mannolipid phosphate formed by rat-liver microsomes had yet to be investigated, indicating that no clear, single compound was obtained. Recent work (Herscovics *et al.*, 1974) has shown that the polyprenyl mannosyl phosphates formed by calf pancreas and human lymphocytes contain a β-D-mannosyl phosphate residue, and that α- and β-linked compounds yield dolichyl phosphate and dolichol, respectively, on treatment with alkali. Furthermore, this work has shown that alkali treatment of the β-linked compound yields β-D-mannosyl phosphate as well as mannose 2-phosphate. It is therefore also possible that the contrasting results detailed here reflect the presence of different anomeric configurations in the mannolipid phosphates formed in different systems.

In the work presently reported, we have undertaken the chemical synthesis of P^1-dolichyl P^2-α-D-mannopyranosyl pyrophosphate (**9**) and have investigated the chromatographic separation of this compound from the previously synthesized monophosphate **11** (Warren and Jeanloz, 1973a,c). The behavior of compounds **9** and **11** on treatment with dilute acid and dilute base was studied with a view to establishing the mechanisms of hydrolysis of these two phosphate diesters, and to determine if mono- and pyrophosphate diesters can be distinguished on the basis of their hydrolysis products.

Results and Discussion

Conditions for the chemical synthesis of polyisoprenyl pyrophosphate diesters were established by the use of citronellol, a readily available compound, as a short-chain model isoprenoid alcohol containing an α-saturated isoprene unit. The sodium salt of the synthetic product **8** was a water-soluble solid, thus, hydrolysis experiments could be performed in aqueous solutions, avoiding the use of organic solvents, which are necessary to solubilize glycolipid phosphates that contain long aliphatic chains.

Citronellyl phosphate (**4**) was prepared (a) as one of the starting materials for the synthesis of P^1-citronellyl P^2-α-D-mannopyranosyl pyrophosphate (**8**), and (b) as a chromatographic standard for the subsequent hydrolysis experiments. Citronellyl pyrophosphate was also required for (b). Application of the method of phosphorylation (Popjak *et al.*, 1962; Warren and Jeanloz, 1972) that consists of treating the alcohol with bis(triethylammonium) phosphate and trichloroacetonitrile gave both citronellyl pyro- and monophosphate. Compound **4** has been prepared previously (Warren and Jeanloz, 1973c) by the *o*-phenylene phosphorochloridate method (Khwaja *et al.*, 1970) and the compounds made by the two methods gave identical main spots on tlc; the by-products were also the same, except for citronellyl pyrophosphate, which was present only in the preparation by the method of Popjak *et al.* (1962). This observation eliminates the possibility that the *o*-phenylene phosphorochloridate method of phosphorylation (Khwaja *et al.*, 1970), which involves lead tetraacetate oxidation (Warren and Jeanloz, 1972; Warren *et al.*, 1973; Warren and Jeanloz, 1973c; Wedgwood *et al.*, 1974), could degrade the highly unsaturated alcohol. As citronellol has only one double bond, such an effect, if present, would be particularly marked.

Citronellyl phosphate (**4**) was converted into the diphenyl pyrophosphate **2** which was unexpectedly no more stable in the presence of anhydrous pyridine than the corresponding allylic compounds (Warren and Jeanloz, 1972), and immediately decomposed. Therefore, compound **2** was treated with 2,3,4,6-tetra-*O*-acetyl α-D-mannopyranosyl phosphate (**1**) in a solution of 1,2-dichloroethane containing a small proportion of pyridine. These reaction conditions were a modification of those originally employed by Michelson (1964) for the preparation of nucleoside pyrophosphate diesters. The resulting acetylated pyrophosphate diester **6** was purified by chromatography and converted into the solid sodium salt for characterization (tlc, ir spectrum, and elemental analysis). Deacetylation by the standard procedure gave P^1-citronellyl P^2-α-D-mannopyranosyl sodium pyrophosphate (**8**) as a water-soluble solid, pure according to tlc in a variety of solvent systems, and showing the ir spectrum and elemental analysis expected for a pyrophosphate diester of D-mannose and citronellol. It was much more stable than the allylic pyrophosphate diesters prepared previously (Warren and Jeanloz, 1972), so that it could, if necessary, be purified by preparative tlc.

The conditions for the reaction of P^1-diphenyl P^2-dolichyl pyrophosphate (**3**) with compound **1** were based on the preparation of the citronellyl derivative. The resulting acetylated pyrophosphate diester **7** was purified by chromatography and characterized by its ir spectrum and tlc in sev-

Table I: Thin-Layer Chromatography on Silica Gel of P^1-Citronellyl P^2-α-D-Mannopyranosyl Pyrophosphate (8), P^1-Dolichyl P^2-α-D-Mannopyranosyl Pyrophosphate (9), Citronellyl α-D-Mannopyranosyl Phosphate (10), and Dolichyl α-D-Mannopyranosyl Phosphate (11).

Solvent System[a]	R_F[b]			
	8	9	10	11
Chloroform–methanol–water (60:25:4)(A)		0.18	0.18	0.54
Chloroform–methanol–water (60:35:6)(B)	0.23	0.47	0.40	0.83
Chloroform–methanol–water (10:10:3)(C)	0.70	0.89	0.87	
2,6-Dimethyl-4-heptanone–acetic acid–water (20:15:2)(D)		0.43	0.20	0.67
2-Propanol–15 M ammonium hydroxide–water (6:3:1)(E)	0.67	0.62[c]	0.70	0.83[c]
Chloroform–methanol–15 M ammonium hydroxide–water (65:35:4:4)(F)		0.20	0.20	0.45

[a] All proportions are volume to volume. [b] R_F value was calculated from measurement of the distance from the origin of the chromatogram to the point of maximum intensity of the spot (anisaldehyde spray). [c] With tailing.

eral different solvent systems. Deacetylation gave P^1-dolichyl P^2-α-D-mannopyranosyl pyrophosphate (**9**) and, as tlc showed the presence of only traces of contaminants, further purification was not performed. The allylic pyrophosphate derivatives of D-galactose and 2-acetamido-2-deoxy-D-glucose, synthesized previously (Warren and Jeanloz, 1972; Warren *et al.*, 1973), were too unstable to tolerate preparative tlc without some decomposition. However, as compound **9** is much more stable, preparative tlc could be used, if it is necessary to obtain an absolutely pure sample. Compound **9** was further characterized by its ir spectrum and elemental analysis.

Compound **9** was compared with dolichyl α-D-mannopyranosyl phosphate (**11**) (Warren and Jeanloz, 1973a,c). Both compounds were well separated on silica gel tlc in four different solvent systems as well as on DEAE-paper (acetate form), with a range of concentrations of ammonium acetate in methanol as the eluent (Table I).

The behavior of the synthetic compounds in hydrolysis experiments was examined, and the results are summarized in Table II. When the monophosphate diesters **10** and **11**, and pyrophosphate diesters **8** and **9** were treated with dilute acid, the main reaction was splitting of the glycosidic bond, and the hydrolysis rates of both types of compound were similar. In order to confirm the formation of dolichyl pyrophosphate from **9**, a standard sample was prepared. Prolonged reaction times resulted in hydrolysis of the polyprenyl pyrophosphate derived from **8** or **9**, yielding the corresponding polyprenyl phosphate. Therefore it is apparent that, in the pyrophosphate compounds, the α-D-mannopyranosyl phosphate bond is more labile under acidic conditions than the pyrophosphate bond, and in both types of compound the α-D-mannopyranosyl phosphate bond is more labile than the lipid–phosphate bond. This is in contrast to the properties of allylic derivatives, such as ficaprenyl α-D-mannopyranosyl phosphate (Warren and Jeanloz, 1973b), where α-D-mannopyranosyl phosphate is usually obtained. From these results, it is concluded that the nature of the terminal (α) isoprene residue of the lipid moiety is the crucial factor in determining whether or not α-D-mannopyranosyl phosphate will be a product of mild acid hydrolysis. When this isoprene residue is unsaturated, as in bacterial undecaprenol and retinol, α-D-mannopyranosyl phosphate or pyrophosphate is obtained, and when it is saturated, as in dolichol, no α-D-mannosyl phosphate or pyrophosphate is formed from either a mono- or a pyrophosphate type of compound.

Recent work (Warren, C. D., Liu, I. Y., Herscovics, A., Wedgwood, J. F., and Jeanloz, R. W., unpublished results) has shown that α- and β-D-mannopyranosyl phosphate have similar stability under acidic conditions, hydrolysis of the β-linked derivative being only slightly faster than the α, and that the rates of acid hydrolysis of the mannosyl phosphate bond in dolichyl α- and β-D-mannopyranosyl phosphate are similar. Therefore, it can be reasonably expected that the arguments presented here for the acid hydrolysis of α-D-linked compounds will be equally valid for polyprenyl mannosyl phosphates having a β-D linkage.

When the monophosphate diesters **10** and **11** were treated with hot, dilute alkali, the formation of a polyprenyl phosphate showed that the α-D-mannopyranosyl phosphate bond was the site of primary hydrolytic cleavage. Only traces of α-D-mannopyranosyl phosphate were formed, and D-mannose was not a product. In order to determine whether the D-mannose moiety had been degraded by the alkaline conditions, a sample of the free sugar was subjected to a comparable brief treatment with alkali. This confirmed that D-mannose is almost totally decomposed under these conditions, to yield a mixture of unidentified compounds (some of which being most probably saccharinic acids). These results suggest that α-D-mannosyl phosphate could be the only readily identifiable ^{14}C-labeled compound obtained in the hydrolysis of a biosynthetically formed mannolipid phosphate of this type, although it might represent only a small proportion of the total D-mannose present in the original α-D-mannopyranosyl phosphate moiety.

When the pyrophosphate diesters **8** and **9** were hydrolyzed by dilute alkali, the α-D-mannopyranosyl phosphate bond was again the most labile bond in the molecule, as shown by the fact that very little α-D-mannopyranosyl phosphate was obtained. In order to demonstrate that this compound had not been formed to any extent during the hydrolysis and subsequently degraded, a sample was subjected to alkaline conditions more vigorous than those used for the hydrolysis, and shown to be unaffected. The rates of hydrolysis of the monophosphate **11** and pyrophosphate **9** were similar, so these results did not support the hypothesis that pyrophosphates are always more labile under basic conditions than monophosphates, or that scission invariably occurs mainly between the two phosphate groups (Clark and Villemez, 1973).

Obviously, a very different situation holds when the glycosyl moiety contains a hydroxyl group that can participate in cyclic 1,2-phosphate formation (Khorana *et al.*, 1957; Paladini and Leloir, 1952). In this case, as for example with derivatives of D-galactose (Dankert *et al.*, 1966; Troy *et al.*, 1971; Warren and Jeanloz, 1972), very mild alkaline treatment can yield a cyclic 1,2-phosphate and a lipid phosphate,

Table II: Acid Hydrolysis of Citronellyl α-D-Mannopyranosyl Pyrophosphate (8) and Phosphate (10) and P^1-Dolichyl P^2-α-D-Mannopyranosyl Pyrophosphate (9).

Compd	Phosphate	Solvent	Temp (°C)	Time (min)	Hydrolysis (%)	Products (R_F, tlc solvent)[a]
8	Pyro	0.01 M HCl–H_2O	80	1	0	None
8	Pyro	0.01 M HCl–H_2O	80	5	>50	Man[b](0.27, B; 0.45, C; 0.32, E), Cit-PP(0.14, B; 0.53, E) derivative of Cit-PP(0.10, B)
8	Pyro	0.1 M HCl–H_2O	85	5	100	Man, Cit-PP, Cit-P[c](0.46, B)
8	Pyro	0.1 M HCl–H_2O	85	60	100	Cit-P, P_i[d](0.0, all solvents)
10	Mono	0.01 M HCl–H_2O	80	1	0	None
10	Mono	0.01 M HCl–H_2O	80	5	>50	Man,[b] Cit-P, derivative of Cit-P (0.37, B)[e]
9	Pyro	$CHCl_3$–CH_3OH–0.08 M HCl (10:10:3)	80	5	>95	Man, [b]Me-Man,[f] Dol-PP (0.14, A; 0.58, E) Dol-P[c] (0.63, A; 0.76, E)
9	Pyro	$CHCl_3$–CH_3OH–0.08 M HCl (10:10:3)	80	60	100	Man, Me-Man,[f] Dol-P

[a] Abbreviations: Man, D-mannose; Man-P, α-D-mannopyranosyl phosphate; Me-Man, methyl D-mannopyranoside; Cit-P, citronellyl phosphate; Cit-PP, citronellyl pyrophosphate; Dol-P, dolichyl phosphate; Dol-PP, dolichyl pyrophosphate; P_i, inorganic phosphate. [b] No detectable Man-P (0.23, C; 0.19, E; anisaldehyde and phosphate sprays). [c] Only a trace obtained. [d] Detected with phosphate spray. [e] See Warren and Jeanloz (1973c). [f] Migrates ahead of Man in C and E.

the latter moiety acting as an efficient leaving group. β-Linked derivatives of D-mannose, such as dolichyl β-D-mannopyranosyl phosphate (Herscovics *et al.*, 1974; Warren, C. D., Liu, I. Y., Herscovics, A., Wedgwood, J. F., and Jeanloz, R. W., unpublished results), can form a 1,2-phosphate on treatment with alkali. However, the leaving group is in this case an alcohol (dolichol) and the conditions necessary for hydrolysis are much more vigorous.

The glycosyl phosphate bond in P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (Warren and Jeanloz, 1974) was relatively stable under alkaline conditions, presumably owing to the fact that the presence of an acetamido group at C-2 of the sugar residue results in a loss of electropositive character at C-1. This would hinder nucleophilic attack by a hydroxyl ion at P-1, and cleavage of the O-1–P bond. Also, the possible alternative mechanism for the hydrolysis of the α-linked D-mannose derivative, in which trans attack by the C-2 hydroxyl group leads to the formation of a 1,2-anhydro derivative (which may undergo further reaction) with expulsion of the dolichyl phosphate moiety, is obviously ruled out in the case of the *N*-acetylglucosamine compound.

Experimental Section

Materials. Citronellal was a gift of Dr. F. W. Hemming, dolichol, of Dr. J. L. Strominger. Citronellol was prepared from citronellal (Warren and Jeanloz, 1973c), and P^1-dolichyl P^1-diphenyl pyrophosphate (**3**), from **5** (Warren and Jeanloz, 1974). Dolichyl phosphate (**5**) was synthesized by the method of Wedgwood *et al.* (1974), 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (**1**) by the method of Warren and Jeanloz (1973b), and dolichyl α-D-mannopyranosyl phosphate by the method of Warren and Jeanloz (1973a,c). All other compounds were obtained from the commercial sources indicated.

General Methods. Optical rotations were determined in 1-dm semi-micro tubes with a Perkin-Elmer Model 141 polarimeter. Infrared (ir) spectra were recorded with a Perkin-Elmer spectrophotometer, Model 237. The cation exchange resin used was AG50W-X8 (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.). In all cases, the amount of resin used was in at least a twofold excess over the necessary quantity to obtain complete ion exchange. Evaporations were carried out under reduced pressure, with an outside bath temperature kept below 30°. The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Toluene and pyridine were dried over calcium hydride before use.

Chromatographic Methods. Tlc was performed on precoated plates of Silica Gel G (E. Merck A.G., Darmstadt, Germany) or Cellulose F (Merck) used without pretreatment; the plates supplied were 20 × 20 cm and were cut to a length of 6 cm before use. In experiments where one or more of the samples were applied to the plate in aqueous solution, all the other spots were treated with water (1–2 μl) before the plate was dried in a current of air. R_F refers to tlc on silica gel, unless otherwise stated. Preparative tlc was carried out on precoated plc plates, Silica Gel F 254 (Merck). The spray reagent used, unless otherwise stated, was anisaldehyde–sulfuric acid–ethanol (1:1:18) (Dunphy *et al.*, 1967), and the plates were heated to 125°. Unsaturated bonds were detected with 1% aqueous potassium permanganate in 2% aqueous sodium carbonate, and phosphate groups with the reagent described by Dittmer and Lester (1964). Solvents A, B, and C for tlc were: chloroform–methanol–water (60:25:4), (60:35:6), and (10:10:3), respectively; other solvents were (D) 2,6-dimethyl-4-heptanone–acetic acid–water (20:15:2), (E) 2-propanol–15 M ammonium hydroxide–water (6:3:1), (F) chloroform–methanol–15 M ammonium hydroxide–water (65:35:4:4), and (G) 1-propanol–15 M ammonium hydroxide (1:1); all proportions are v/v. Reversed phase tlc was carried out on Kieselguhr F (Merck), the plates being impregnated with a solution of 5% liquid paraffin in hexane.

Citronellyl Phosphate (**4**). Citronellol (0.3 g) was dissolved in acetonitrile (25 ml) and treated with trichloroacetonitrile (2.5 ml) and bis(triethylammonium) phosphate (1.3 g; Popjak *et al.*, 1962). The mixture was stirred until

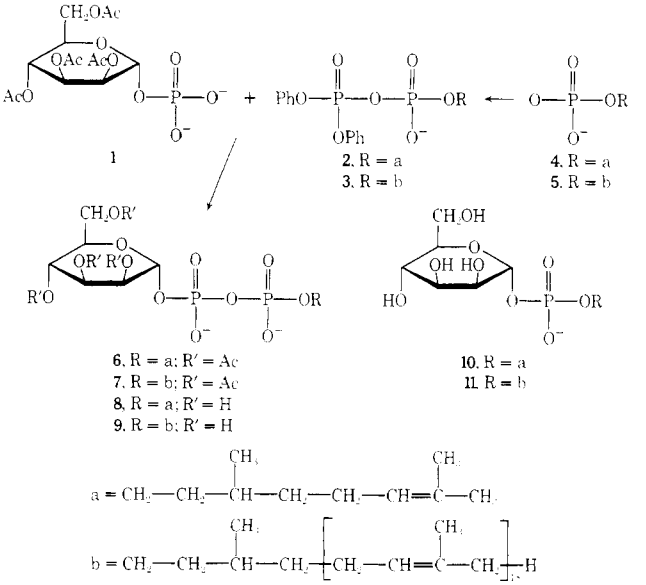
all solid had dissolved, and the solution was kept at room temperature for 2 hr, when tlc (solvent B) showed the presence of citronellyl phosphate (R_F 0.46) and pyrophosphate (R_F 0.14) as well as unchanged citronellol (near solvent front). The mixture was poured into ether (100 ml) and extracted three times with a solution of 10% pyridine in water (20 ml). The combined aqueous extracts were evaporated, the residue was dissolved in methanol (20 ml), and the solution was treated with ether (200 ml). After being kept overnight, the clear supernatant was decanted from a syrupy precipitate and evaporated, to give a mixture (0.4 g) of citronellyl phosphate (**4**) and citronellyl pyrophosphate (pyridinium forms). Tlc (solvent B) showed that the ratio of mono- to pyrophosphate was approximately 5:1 and that the product contained residual inorganic phosphate (at origin).

In order to recover the unchanged citronellol, the ether solution (100 ml, after extraction with aqueous pyridine) was washed with saturated aqueous potassium chloride solution, dried ($MgSO_4$), and evaporated. The solid residue was triturated with hexane, filtered off, and washed with more hexane. The combined solutions were kept overnight at room temperature, decanted from precipitated solid, and evaporated to give citronellol (0.2 g), identical by tlc (toluene–methanol 49:1) with the starting material (R_F 0.3).

Compound **4** (mixed with citronellyl pyrophosphate) was compared chromatographically with a noncrystalline sample of citronellyl phosphate prepared by the *o*-phenylene phosphorochloridate method (Warren and Jeanloz, 1973c). In each of the three solvents used, the major component of each product cochromatographed, R_F 0.46 (solvent B), R_F 0.6 (solvent D), and R_F 0.73 (solvent E). Both products contained minor components, which also appeared to be identical, while only compound **4** contained an appreciable proportion of citronellyl pyrophosphate, R_F 0.14 (solvent A), 0.08 (solvent D), and 0.53 (solvent E).

P^1-Citronellyl P^2-Diphenyl Pyrophosphate (**2**). Citronellyl phosphate (**4,** crude, mixed with citronellyl pyrophosphate, pyridinium form, 0.4 g, obtained from 0.1 g of citronellol) was dissolved in methanol and treated with tributylamine (0.4 g). Evaporation, followed by three additions and evaporations of toluene (2 ml), gave citronellyl phosphate (crude, tributylammonium form), which was dissolved in 1,2-dichloroethane (5 ml) and treated with a further quantity of tributylamine (0.4 g). The solution was cooled to −10° and a solution of diphenyl phosphorochloridate (0.4 g, Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added dropwise with stirring, under anhydrous reaction conditions. After the solution had been kept for 2 hr at room temperature, tlc (chloroform–methanol, 5:1) showed the formation of a product (R_F 0.8), together with traces of slow-running materials. Methanol (4 ml) was added, and after a further 30 min at room temperature, the mixture was evaporated. Three additions and evaporations of toluene gave syrupy compound **2.** Examination by tlc of a solution of compound **2** (a nonallylic diphenyl pyrophosphate, R_F 0.8, chloroform–methanol, 5:1) in anhydrous pyridine indicated immediate conversion into at least two products (R_F 0.1–0.2), no starting material remaining after 2 min at room temperature.

P^1-Citronellyl P^2-2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl Disodium Pyrophosphate (**6**). 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (**1,** pyridinium form, 25 mg) was converted into the tributylammonium form by the method described for 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (Warren and Jeanloz, 1974). A mixture of compound **1** and compound **2** (from 12.5 mg of citronellol) was dried by three additions and evaporations of toluene, then dissolved in a mixture of 1,2-dichloroethane (0.5 ml) and pyridine (5 mg), and kept at room temperature for 48 hr. A small sample treated with a cation-exchange resin (pyridinium form), and examined by tlc (solvent B), showed that compound **2** (R_F 0.95) had been almost completely converted into a new product (R_F 0.64), together with a small proportion of compounds having lower mobility (possibly derived from citronellyl pyrophosphate). The main product did not cochromatograph with citronellyl phosphate, R_F 0.46; therefore, the reaction solution was diluted with chloroform (20 ml), and extracted three times with water (5 ml) in order to remove the excess of compound **1,** dried ($MgSO_4$), and evaporated to yield a



crude product (130 mg) containing compound **6.** Preparative tlc in solvent B, on two plates (20 × 20 cm and 6 × 20 cm) gave a band containing **6** located with the phosphate specific spray (this was the *lower* of the two bands giving a positive phosphate reaction—the upper one containing diphenyl phosphate). The silica gel was removed from the plate, extracted by stirring overnight with solvent C, filtered off, and washed, and the combined filtrates were evaporated to dryness. The residue was extracted with chloroform–methanol (5:1), and filtration and evaporation gave compound **6** (14 mg, tributylammonium form) as a syrup, showing a single spot on tlc in solvent B (R_F 0.64), solvent A (R_F 0.39), and solvent D (R_F 0.43), together with a trace of material having a lower R_F (in solvents A and B), and a higher R_F (in solvent D), after spraying with the anisaldehyde, potassium permanganate, and phosphate specific reagents. For further characterization, compound **6** was converted into the sodium form by passage of a methanolic solution through a small column of a cation-exchange resin (pyridinium form), and by stirring the solution (and methanol washings from the resin) with a cation-exchange resin (sodium form) for 48 hr at room temperature. The resin was filtered off on a sintered glass, and washed with methanol. Evaporation of the combined filtrates gave a solid residue, which was dissolved in chloroform–methanol (5:1). The solution was filtered and evaporated to yield **6** (disodium salt, 12 mg), a slightly hygroscopic solid: mp 174–175°; $[\alpha]^{24}D$ +26° (*c* 0.5, methanol); ir spectrum ν_{max}^{KBr} 2960 (CH_3, stretching), 2930 and 2860 (CH_2, stretching),

1750 (C=O, acetyl), 1375 (CCH_3), 1225–1260 [P=O, O—C—(C=O)], 1160 and 1130 (CH_3CCH_3), 1070 and 1035 (CH_2—C—O—), and 940 cm^{-1} (P—O—P). *Anal.* Calcd for $C_{24}H_{38}Na_2O_{16}P_2 \cdot 1.5H_2O$: C, 40.17; H, 5.90; P, 8.63. Found: C, 39.90; H, 5.45; P, 8.88.

P^1-Citronellyl P^2-α-D-Mannopyranosyl Disodium Pyrophosphate (**8**). Compound **6** (24 mg, disodium or pyridinium salt) was dissolved in methanol and treated with 1% sodium methoxide in methanol until a strongly basic solution was obtained. After the solution had been kept at room temperature for 30 min, tlc showed that all the starting material (R_F 0.64, solvent B) had been converted into a new product (R_F 0.23). Tlc also indicated the presence of traces of contaminants; therefore, the methanolic solution, without treatment with a cation-exchange resin, was applied to a preparative tlc plate (20 × 6 cm) that was developed in solvent C. The band containing the deacetylated compound **8** was located by the phosphate specific spray, and the extraction of the product from the silica gel was performed as described for the preparation of **6.** Evaporation of the extract gave a solid residue that was triturated with chloroform–methanol (1:1). The resulting solution was filtered (sintered glass) and evaporated to give a slightly hygroscopic solid (12 mg, 48 mg from 100 mg of citronellol) with no definite mp, but decomposing slowly above 168°: $[\alpha]^{24}D$ +38° (*c* 0.6, methanol); ir spectrum ν_{max}^{KBr} 3400 (OH), 2935 and 2860 (CH_2, stretching), 1600 (C=C, stretching), 1375 (CCH_3), 1235 (P=O), 1145 (CH_3CCH_3), 1075 (C—O, adjacent to a saturated residue), and 930 cm^{-1} (P—O—P); for tlc, see Table I. *Anal.* Calcd for $C_{16}H_{30}Na_2O_{12}P_2 \cdot 1.5H_2O$: C, 34.99; H, 6.01. Found: C, 34.70; H, 5.82.

P^1-Dolichyl P^2-2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl Pyrophosphate (**7**). A mixture of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (**1**) (tributylammonium form, prepared from 20 mg of **1,** pyridinium form) and P^1-dolichyl P^2-diphenyl pyrophosphate (**3**) (tributylammonium form, prepared from dolichol, 28 mg) was dried by three additions and evaporations of toluene (2 ml), dissolved in a mixture of 1,2-dichloroethane (0.4 ml) and pyridine (4 mg), and kept for 48 hr at room temperature. Tlc (solvent A), performed as described in the preparation of **6,** showed the formation of a product (R_F 0.59) but also indicated the presence of several other compounds having higher mobility. The major spot had the same R_F as the starting material **3,** but was not transformed by a prolonged treatment with an excess of **1.** The tlc did not separate the product (R_F 0.59) from dolichyl phosphate (R_F 0.63), therefore a second tlc was performed in solvent D which showed that the product (R_F 0.62) and dolichyl phosphate (R_F 0.71) were both components of the reaction mixture, the proportion of the latter being approximately 10% of that of the main product. The reaction solution was diluted with chloroform (20 ml) and extracted three times with water (5 ml) in order to remove the excess of **1.** The chloroform solution was dried ($MgSO_4$) and evaporated to yield a crude product (40 mg) containing compound **7.** Purification was achieved by preparative tlc on a 20 × 10 cm plate in solvent A. The band containing **7** was located by spraying a narrow area near the center of the plate with (a) the potassium permanganate spray and (b) the phosphate specific spray. It was essential to avoid confusion of this band with another one moving ahead of it which contained diphenyl phosphate (which gives an especially intense blue color with the phosphate specific spray). The silica gel was removed from the plate and extracted with solvent C as described for the preparation of compound **6.** Evaporation gave a residue which was triturated with chloroform–methanol (5:1). The resulting solution was filtered (sintered glass) and evaporated to give compound **7** (9 mg, tributylammonium form) as a syrup, showing a single spot on tlc (except for a trace of dolichyl phosphate) in solvent A (R_F 0.59) and solvent D (R_F 0.62) with the anisaldehyde, potassium permanganate, and phosphate specific sprays: $[\alpha]^{20}D$ +5° (*c* 0.45, chloroform–methanol 5:1); ir spectrum ν_{max}^{film} 2965 (CH_3, stretching), 2930 and 2860 (CH_2, stretching), 1745 (C=O, acetyl), 1450 (—CH_2, —CH_3), 1375 (CCH_3), 1240 [broad, P=O and O—C—(C=O)], and 930 cm^{-1} (P—O—P).

P^1-Dolichyl P^2-α-D-Mannopyranosyl Pyrophosphate (**9**). To a solution of **7** (6 mg) in chloroform–methanol (2:1) was added 1% sodium methoxide in methanol, until a strongly basic solution was obtained. After 30 min at room temperature, tlc (solvent A) showed that all the starting material (R_F 0.59) had been converted into a new product (R_F 0.18), and a small excess of a cation-exchange resin (which had been prewashed with chloroform–methanol 2:1) was added and the mixture stirred overnight at room temperature. The resin was filtered off and washed with chloroform–methanol (2:1), and evaporation, followed by three additions and evaporations of toluene, gave a syrup (**9,** 5 mg, 7.5 mg from 28 mg of dolichol), showing a single major spot on tlc with the three spray reagents (Table I), as described in the preparation of **7,** a faint spot corresponding to dolichyl phosphate, and one other very faint spot having a lower mobility than the main product: ir spectrum ν_{max}^{film} 3350 (OH), 2965 (CH_3, stretching), 2930 and 2860 (CH_2, stretching), 1725 (unassigned—this peak is also present in dolichyl phosphate and in dolichyl α-D-mannopyranosyl phosphate), 1450 (—CH_2, —CH_3), 1375 (CCH_3), 1240 (P=O), 1070 (C—O, adjacent to a saturated residue), and 930 cm^{-1} (P—O—P). *Anal.* Calcd for $C_{101}H_{167}O_{12}P_2$: C, 74.13; H, 10.31. Calcd for $C_{101}H_{167}O_{12}P_2 \cdot CHCl_3 \cdot 2(CH_3OH)$: C, 68.66; H, 9.77. Found: C, 68.60; H, 10.19.

Chromatographic Comparison of Compound **9** *with Dolichyl α-D-Mannopyranosyl Phosphate* (**11**). Compounds **9** and **11** were compared by tlc in solvents A, B, D, E, and F (see Table I), and also by chromatography on DEAE-paper (Whatman DE81, acetate form) in solutions of ammonium acetate (0.05–1.0 M) in methanol. The paper was immersed in glacial acetic acid for 24 hr, washed six times with methanol, and dried before use. The spots were revealed with the phosphate specific spray. The phosphate diester **11** and pyrophosphate diester **9** were well separated at all salt concentrations. Although separations were best in the range of 0–0.2 M NH_4OAc in methanol, streaking of the spots was most serious at low concentrations, therefore a range of 0.3–0.5 M is preferred. When R_F was plotted against logarithm of salt concentration, a straight line was obtained for both compounds over the range of 0.2–1 M NH_4OAc (Figure 1).

Dolichyl Pyrophosphate. A mixture of dolichol (4 mg), acetonitrile (1.5 ml), toluene (2 ml), trichloroacetonitrile (200 μl), and bis(triethylammonium) phosphate (100 mg, Popjak *et al.*, 1962) was stirred overnight at room temperature. The reaction mixture was treated with pyridine in excess (1 ml) and evaporated. Toluene (2 ml) was added and evaporated three times, to give a residue which was dissolved in methanol (0.2 ml). Ether (2 ml) was added, and after 1 hr the clear supernatant was decanted from the residue and evaporated to yield a crude product (5 mg). For tlc,

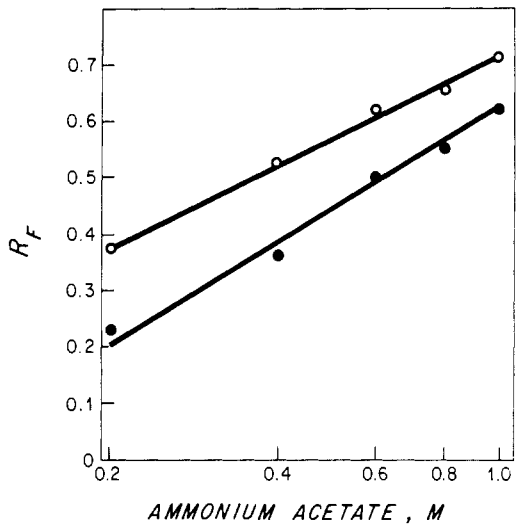


FIGURE 1: Relationship of the chromatographic mobility of dolichyl α-D-mannopyranosyl pyrophosphate (**9**) (●) and phosphate (**11**) (○) on DEAE-paper with log of molar concentration of ammonium acetate in methanol.

the product was triturated with hexane, and the resulting suspension was filtered and examined in solvent F to show two spots (R_F 0.18 and 0.29), the latter migrating with dolichyl phosphate that had been prepared by the *o*-phenylene phosphorochloridate method (Wedgwood *et al.*, 1974), together with unchanged dolichol (at solvent front). The mixture of compounds was chromatographed on a tlc plate (20 × 5 cm) (*not* a thick-layer plate) in solvent F. Three bands corresponding to the three components of the mixture could be located with the potassium permanganate spray. Extraction of the upper band with chloroform–methanol (5:1) gave dolichol (1.5 mg), and extraction of the two lower bands with solvent C gave a low yield of (a) dolichyl phosphate and (b) dolichyl pyrophosphate (pyridinium form). Dolichyl phosphate and pyrophosphate were separated by tlc in solvents A, F, D, and E, but dolichyl pyrophosphate gave some streaking in A (R_F 0.14) and serious streaking in D (R_F 0.66) and E (R_F 0.58).

Acid and Alkaline Hydrolysis of Compounds **8, 9, 10,** *and* **11.** For details of the experiments, see Tables II and III. For each hydrolysis (unless otherwise indicated) 1 mg of synthetic mono- or pyrophosphate diester was dissolved in 0.2 ml of solvent and the mixture heated in a sealed tube. After a brief acid hydrolysis (1–5 min), solutions were neutralized with 1% sodium methoxide in methanol before examination by tlc.

Acid Treatment of Citronellyl Phosphate and Citronellyl Pyrophosphate. A mixture of the two compounds (1 mg) in 0.1 M HCl (0.2 ml) was kept at 85° for 1 hr when tlc (solvent B) showed that all the citronellyl pyrophosphate (R_F 0.14) had been converted into citronellyl phosphate (R_F 0.46) and inorganic phosphate (R_F 0.0, phosphate spray). Tlc in toluene–methanol (49:1) showed that no citronellol had been formed.

Acid Treatment of Dolichyl Pyrophosphate. A solution of the compound (0.1 mg) in a mixture of chloroform–methanol–0.8 M HCl (10:10:3; 20 μl) was kept at 80° for 1 hr in a sealed tube, when tlc (solvent A) showed that all the starting material (R_F 0.14) had been converted into dolichyl phosphate (R_F 0.63) and inorganic phosphate (R_F 0.0, phosphate spray).

Comparison of the Products of Alkaline Hydrolysis with 1,6-Anhydro-β-D-mannopyranose. 2,3,4-Tri-*O*-acetyl-1,6-anhydro-β-D-mannopyranose (0.5 mg) was treated with an excess of a solution of 1% sodium methoxide in methanol, for 30 min at room temperature. After the addition of cation-exchange resin (pyridinium form) to give a neutral solution, tlc (solvent B) showed that 1,6-anhydro-β-D-mannopyranose (R_F 0.64) was not a product of the alkaline hydrolysis of compounds **10** and **8** under any of the conditions employed.

Identification of Dolichol as a Product of Hydrolysis. The products of acid and alkaline hydrolysis of compound **9** and alkaline hydrolysis of compound **11** showed minor components with the approximate mobility of dolichol (R_F 0.4) on tlc (toluene–methanol, 49:1). Therefore, a further comparison was carried out by reversed phase tlc in acetone (Dunphy *et al.*, 1967), which showed that the alkaline hydrolysate of **11** contained at least three of the isoprenologs that comprise pig-liver dolichol, *i.e.*, C_{90}, C_{95}, and C_{100}, the most intense spot (C_{95}) having R_F *ca.* 0.3. This suggests that a small proportion of α-D-mannopyranosyl phosphate had been formed in the alkaline hydrolysis of **11.** The other hydrolysates were shown by reversed phase tlc to contain no dolichol.

Table III: Alkaline Hydrolysis of Citronellyl α-D-Mannopyranosyl Pyrophosphate (**8**) and Phosphate (**10**) and Dolichyl α-D-Mannopyranosyl Pyrophosphate (**9**) and Phosphate (**11**).[a]

Compd	Phosphate	Solvent	Time (min)	Hydrolysis (%)	Products[b]
8	Pyro	0.01 M NaOH–H_2O	60	40	Cit-P, Cit-PP (1:3)
8	Pyro	0.1 M NaOH–H_2O	60	80	Cit-P, Cit-PP (1:1), Man-P[c] (R_F 0.33, solvent G)
8	Pyro	1 M NaOH–H_2O	60	100	Cit-P, Cit-PP (3:2)
8	Pyro	1 M NaOH–H_2O	120	100	Cit-P, Cit-PP[c]
10	Mono	0.01 M NaOH–H_2O	60	50	Cit-P, compound with R_F similar to Man[c]
10	Mono	0.01 M NaOH–H_2O	60	95	Cit-P
9	Pyro	0.1 M NaOH–propanol[d]	5	>95	Dol-P, Dol-PP, P_i[e]
11	Mono	0.1 M NaOH–propanol[d,f]	5	100	Dol-P, compound with R_F similar to Man,[c] dolichol[c]

[a] All hydrolyses were performed at 100°. [b] Abbreviations and R_F values: see Table II. [c] Only a trace of this obtained. [d] Mixture (0.5 ml) of 1-propanol and 1 M NaOH (10:1). [e] Detected with phosphate spray. [f] Neutralization with acetic acid did not alter this result.

Alkaline Treatment of D-*Mannose.* (a) In order to explain the absence of D-mannose in the products of the alkaline hydrolysis of compounds **8** and **10,** D-mannose (1 mg) was treated with 0.1 M NaOH (0.2 ml) at 100° for 30 min. The solution turned brown, indicating decomposition, and tlc showed that D-mannose had been completely degraded to give a faint pink spot, R_F 0.38 (solvent B), R_F 0.81 (solvent C), and R_F 0.72 (solvent E).

(b) This experiment was carried out in an attempt to simulate the conditions of the alkaline degradation of the D-mannosyl moiety liberated in the alkaline hydrolysis of compounds **11** and **9.** D-Mannose (1 mg) was dissolved, by stirring, in a mixture of 1.0 M NaOH and 1-propanol (1:10, 1.4 ml). A portion of the solution was withdrawn and neutralized with glacial acetic acid. The remainder was stirred at 80° for 5 min, then cooled to 0°, and neutralized with acetic acid. Tlc indicated that more than 95% of the original sugar had been converted into substances which did not stain with the anisaldehyde spray, whereas the remainder was revealed as a group of faint spots moving near, or just ahead of, the position of D-mannose as detected with anisaldehyde; these compounds reacted much more strongly than D-mannose with the potassium permanganate spray. The solution was evaporated and the residue dissolved in water and stirred with a cation-exchange resin (pyridinium form) for 30 min. The resin was filtered off, and the filtrate evaporated, the residual acetic acid being removed by three additions and evaporations of toluene. The residue was examined by cellulose tlc in 1-propanol–ethyl acetate–water (7:1:2), with standards of D-mannose and D-galacturonic acid (R_F 0.2). Spots were detected with a spray consisting of a solution of Bromothymol Blue (0.1 g) in a mixture of ethanol (80 ml) and water (20 ml), adjusted to give pH 10 by addition of 1 M NaOH. This showed that three acidic compounds were produced in the alkaline treatment of D-mannose, two minor ones with very similar mobility (R_F 0.55) and a major product (R_F 0.75); D-mannose itself was not detectable with this spray.

Alkaline Treatment of α-D-*Mannopyranosyl Phosphate.* The compound (1 mg) was incubated at 100° with 0.1 M sodium hydroxide solution for 3 hr, when tlc (solvent C) showed that it was unaffected. This result suggests that only trace quantities of α-D-mannopyranosyl phosphate could have been formed in the alkaline hydrolysis of compounds **10** and **8.**

Acknowledgments

The authors thank Dr. J. L. Strominger for the gift of dolichol, and Dr. C. P. J. Glaudemans for the gift of 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-mannopyranose.

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