Chemical Synthesis of Dolichyl α-D-Mannopyranosyl Phosphate and Citronellyl α-D-Mannopyranosyl Phosphate†

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ABSTRACT: 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (pyridinium form, **1**) was coupled with the short-chain, nonallylic isoprenoid alcohol citronellol by the dicyclohexylcarbodiimide and triisopropylbenzenesulfonyl chloride methods. The resulting phosphate diester was deacetylated to give a short-chain analog of an isoprenoid "*lipid intermediate*," citronellyl α-D-mannopyranosyl phosphate (**5**). The same method of condensation was used for the synthesis of dolichyl α-D-mannopyranosyl phosphate (**7**) which appears to be identical with a glycolipid formed by a variety of mammalian biosynthetic systems. Compounds **5** and **7** were characterized by their ir and nmr spectra, chromatographic properties, dilute acid hydrolysis, and catalytic hydrogenation.

Phosphate esters of a novel type of glycolipid have recently been isolated from several mammalian biosynthetic systems. These substances were detected upon the incubation of ^{14}C-labeled sugar nucleotides with microsomal enzyme preparations (for a review, see Lennarz and Scher (1972)) and were obtained in only very small quantities. Consequently, determination of their chemical structure has been very difficult to perform. These glycolipids apparently consist of a moiety having the properties of a long-chain polyprenol which is linked through a phosphate group to a carbohydrate residue. The nature of the lipid has been deduced by indirect methods, which indicate that it is either dolichol or a very similar compound. Dolichol is a mixture of very long-chain polyprenols (C_{80}-C_{105}) which are distributed widely in animal cells (Butterworth and Hemming, 1968), the main isomer in pig liver having a C_{95} composition. It has been shown that exogenous dolichyl phosphate can stimulate the formation of labeled glucolipid (Behrens and Leloir, 1970; Behrens *et al.*, 1971a) and mannolipid (Richards and Hemming, 1972). Evidence has been presented that the glucolipids are either mono- or pyrophosphate diesters (Behrens *et al.*, 1971b; Parodi *et al.*, 1972), and that the mannolipids are monophosphate diesters (Richards and Hemming, 1972).

By analogy with bacterial systems of glycan biosynthesis in which polyprenyl-sugar intermediates are known to participate, it might be expected that these mammalian glycolipids are acting as lipid intermediates also, possibly in glycoprotein biosynthesis. This hypothesis is supported by the report that a dolichol derivative is probably an intermediate in the biosynthesis of yeast mannan (Tanner *et al.*, 1971; Sentandreu and Lampen, 1971); the dolichols of yeast have a chain length only slightly shorter than that of mammalian dolichols (Dunphy *et al.*, 1967).

In an attempt to ascertain the chemical structure of the mannolipids that have recently been isolated from a variety of sources (Evans and Hemming, 1973; Herscovics *et al.*, 1973; Wedgwood and Warren, 1973), we have performed an unambiguous chemical synthesis of dolichyl α-D-mannopyranosyl phosphate. Details of this synthesis and a comparison of the synthetic and naturally formed mannolipids are reported here.

Results and Discussion

The synthesis of dolichyl α-D-mannopyranosyl phosphate was based on the synthesis of a short-chain analog, citronellyl α-D-mannopyranosyl phosphate. Fully acetylated α-D-mannopyranosyl phosphate was coupled with the isoprenoid alcohol in the presence of either dicyclohexylcarbodiimide[1] (DCC) or triisopropylbenzenesulfonyl chloride[1] (iPr_3PhsCl) and the intermediate acetylated phosphate diester was deacetylated to yield the required compound.

Citronellol (**2**) was chosen as a short-chain (C_{10}) model isoprenoid because it contains a terminal saturated residue next to the primary alcoholic group, as does dolichol. It was prepared from citronellal by borohydride reduction, followed by ether extraction (Burgos *et al.*, 1963). The best conditions for condensation with mannosyl phosphate in the presence of DCC were essentially the same as those observed by Cawley and Letters (1971). 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (pyridinium form, **1**) was prepared as previously described (Warren and Jeanloz, 1973b), and condensed in anhydrous conditions with a small excess of citronellol (**2**) in the presence of a large excess of DCC maintained in suspension by a vigorous stirring. A mixture of products was obtained as indicated by tlc and the desired material **4** was identified by a small-scale trial deacetylation. Conditions for the condensation were varied in an attempt to enhance the formation of the desired product, but the by-products could not be completely eliminated. Tlc showed that one of these was a derivative of D-mannosyl phosphate, probably di-P^1,P^2-D-mannopyranosyl pyrophosphate, which was re-

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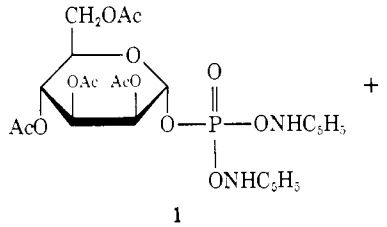
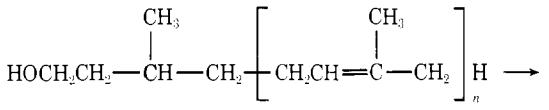
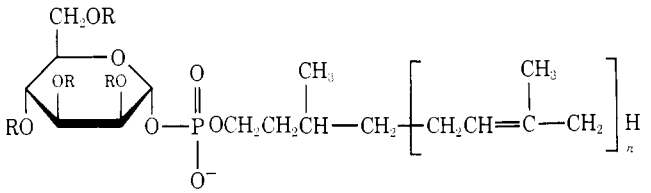
[1] Abbreviations used are: dicyclohexylcarbodiimide, DCC; triisopropylbenzenesulfonyl chloride, iPr_3PhsCl.



1



2, $n = 1$
3, $n = 18$



4, R = Ac; $n = 1$
5, R = H; $n = 1$
6, R = Ac; $n = 18$
7, R = H; $n = 18$

ported by Cawley and Letters (1971) to be a major product when a large excess of DCC was not used (see also the preparation of the dolichol derivative **6**). Other by-products appeared to be derived from **2**, but did not include citronellyl phosphate; they included also a compound which was presumably 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose, as it gave D-mannose after deacetylation. Therefore, it appears that some transphosphorylation occurred during the condensation. For comparison purposes, control experiments containing only one of the reactants and DCC did not produce appreciable quantities of any of the by-products.

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl citronellyl phosphate (**4**) was partially purified by silica gel column chromatography, during the course of which any unreacted alcohol was recovered (which is important when valuable alcohols such as dolichol are used). However, a complete separation of **4** from the by-products could not be achieved. Therefore, the crude material was deacetylated and the phosphate diester **5** obtained pure by preparative tlc. A modification of the procedure where deacetylation was performed without prior column chromatography, and unreacted alcohol recovered by hexane extraction, gave the same product but slightly contaminated with D-mannose; although this method was simpler, the yield was lower.

Triisopropylbenzenesulfonyl chloride (iPr_3PhsCl) has been extensively used as a condensing agent for phosphate diester synthesis in nucleotide chemistry (Lohrmann and Khorana, 1966). When the fully acetylated mannopyranosyl phosphate **1** was treated with citronellol (**2**) in the presence of 1 equiv of iPr_3PhsCl at room temperature in anhydrous pyridine, a very slow reaction took place to give the acetylated phosphate diester **4**; when a large excess of iPr_3PhsCl was used, by-products derived from the alcohol were the main feature of the reaction, and very little of the desired phosphate diester was obtained. Variation of the proportion of alcohol to sugar phosphate showed that even when only a small excess of alcohol was used, 50% of the citronellol remained unchanged after 3 days, and no further formation of the desired product took place. Moreover, nearly all the remaining mannosyl phosphate was in the form of a substance having the chromatographic properties of di-P^1,P^2-D-mannopyranosyl pyrophosphate, the expected by-product of this reaction. However, the side reactions involving the lipid moiety were considerably less than in the DCC experiment. After removal of the derivatives of mannopyranosyl phosphate, and conversion of the crude reaction product into the sodium form, unchanged **2** was recovered by hexane extraction and citronellyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl sodium phosphate was obtained after purification by tlc as a solid, which was characterized by its ir spectrum, optical rotation, and elementary analysis.

Deacetylation of **4** with methanolic sodium methoxide gave citronellyl α-D-mannopyranosyl sodium phosphate (**5**). The compounds obtained by both methods were shown to be pure by tlc in a variety of solvent systems using spray reagents specific for double bonds, isoprenoid alcohol content, and phosphate ester content. The nmr spectrum was recorded on a [$^2H_5$]pyridine solution after conversion of **5** into the pyridinium form. Clear, strong signals were obtained from methyl protons in positions adjacent to (a) saturated and (b) unsaturated carbon–carbo[illegible], and these two doublets were well separated from ea[illegible]. The signals from methylene protons in situations c[illegible]ding to (a) and (b) were also separated, but in this case the individual peaks were not well resolved. The peaks arising from the α-D-mannopyranosyl residue were not readily assignable to individual protons. The compound was further characterized by the ir spectrum which showed peaks due to both of the isomers which comprise citronellol, *i.e.*, those from a $C{=}CH_2$ group and a trisubstituted olefin. This short-chain analog **5** of a "lipid intermediate" of the dolichol type did not exhibit any of the extreme instability which was found to be typical of the allylic pyrophosphate diesters previously synthesized (Warren and Jeanloz, 1972; Warren *et al.*, 1973). Also, the citronellyl phosphate bond was, as expected, quite resistant to catalytic hydrogenolysis in the presence of various platinum catalysts, a reduced phosphate diester being the main product. Dilute acid hydrolysis of **5** in aqueous methanol gave D-mannose, methyl mannosides, and a compound derived from citronellyl phosphate; there was no indication of scission of the citronellol–phosphate bond, in contrast to the high lability to acid of allylic phosphates such as phosphate diesters derived from farnesol and ficaprenol (Warren and Jeanloz, 1972, 1973b). The reference substance, citronellyl phosphate, was synthesized with the *o*-phenylene phosphorochloridate method developed for the preparation of polyprenyl phosphates (Tkacz *et al.*, 1973). The compound, crystallized in the cyclohexylammonium form as optically active needles, and its dilute acid hydrolysis were studied under conditions similar to those employed for **5**.

A pig liver dolichol (**3**), obtained as a mixture of long-chain isoprene analogs ranging from C_{80} to C_{105} with the C_{95} compound predominating (Butterworth and Hemming, 1968), was condensed with **1** to give **6**, as in the citronellol experiment. The totality of the phosphate **1** entered into reaction, but most of **3** did not react. Therefore, additional amounts of DCC and **1** were added at 24-hr intervals, and after 3 days only a small proportion of unreacted **3** remained. The proportion of **3** present in the reaction mixture was always kept greater than that of **1**; it had already been shown in the citronellol experiment that almost none of the desired product was obtained if an excess of **1** was present at the start of the reaction. It was also necessary to maintain the original proportions of DCC to **1**, in order to avoid the formation of di-P^1,P^2-D-mannopyranosyl pyrophosphate, as shown by the

treatment of **1** with an appropriate equivalent amount of DCC in the absence of an alcohol. The major new compound formed was a sugar phosphate that migrated faster than **1**, while only a small amount of the latter compound remained. After deacetylation, this new compound showed on cellulose the properties of a pyrophosphate, presumably di-P^1,P^2-D-mannopyranosyl pyrophosphate (Cawley and Letters, 1971).

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl dolichyl phosphate (**6**) was separated from unreacted **3** by column chromatography, the crude **6** was deacetylated, and the resulting phosphate diester **7** obtained as the syrupy sodium salt by preparative tlc. Two by-products, both apparently being derivatives of dolichol and having chromatographic mobility almost identical with those of **6**, were also obtained. The ir spectrum of **7** showed a contamination due to a derivative of DCC, which required a second preparative tlc in a different solvent mixture. This gave a product pure by tlc in several solvent systems with three spraying reagents. The identity of **7** as dolichyl α-D-mannopyranosyl phosphate was confirmed by elementary analysis and by dilute acid hydrolysis to give derivatives of D-mannose (as in the citronellol experiment) and dolichyl phosphate; dolichol itself was not a product. This is in contrast to the result obtained with allylic phosphate diesters, such as ficaprenyl α-D-mannopyranosyl phosphate (Warren and Jeanloz, 1973b), where scission of the lipid–phosphate bond occurs. Dolichyl phosphate was synthesized separately, as a chromatographic standard, in connection with other studies (Wedgwood and Warren, 1973). The nmr spectrum of synthetic **7**, performed by Evans and Hemming (1973), showed most of the signals expected for a phosphate diester of dolichol and D-mannose.

α-D-Mannopyranosyl dolichyl phosphate (**7**) was also synthesized by the iPr_3PhsCl method. When dolichol (**3**) was treated with equivalent amounts of iPr_3PhsCl and **1** in dry pyridine under anhydrous conditions for 72 hr at room temperature, at least half of **3** was converted into **6**, while nearly all the remaining **1** gave the di-P^1,P^2-D-mannopyranosyl pyrophosphate; in contrast to the DCC experiment, only traces of by-products derived from **3** were present. After removal of the derivatives of **1** by extraction with water, unreacted **3** (40% of the starting quantity) was separated from **6** by chromatography on a column of silica gel. Deacetylation of **6** was performed with sodium methoxide in chloroform–methanol, and the almost pure product **7**, obtained in the syrupy sodium form, was identical in every way with the compound prepared by the DCC method, except for traces of contaminants apparent in the tlc. The yield of **7** based on dolichol was higher with the iPr_3PhsCl method, but the yield based on **1** was higher with the DCC method.

Compound **7** was compared to the ^{14}C-labeled mannolipids produced from GDP-D-[^{14}C]mannose by several biosynthetic systems. (a) The product of a microsomal preparation from calf pancreas was found to cochromatograph with **7** in seven different solvent systems for tlc (Herscovics *et al.*, 1973; Tkacz *et al.*, 1973). (b) The mannolipid produced by a preparation obtained from human lymphocytes also cochromatographed with **7** in two solvent systems for tlc (Wedgwood and Warren, 1973). (c) Synthetic **7** was compared in detail with the mannolipid produced by a microsomal system obtained from pig liver (Evans and Hemming, 1973). The ir and nmr spectra, rate of migration on tlc in two solvent systems, and behavior of the two compounds under conditions of acid and alkaline hydrolysis, and catalytic hydrogenation were found to be identical. Further, the synthetic compound **7** caused a stimulation of the incorporation of D-[^{14}C]mannose from GDP-D-[^{14}C]mannose into endogenous mannolipid in the presence of the microsomal preparation.

Experimental Section

For general and chromatographic methods, see Warren and Jeanloz (1973b). The P analyses and the C and H analysis of compound **7** were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. 37921.

Citronellol (**2**). This preparation is based on the procedure of Burgos *et al.* (1963). A solution of citronellal (1 g), obtained as a mixture of the isomers 3,6-dimethyl-6-octen-1-al and 7-octen-1-al, the 6-octene compound being the major component (Hemming, 1973), in methanol (50 ml) was treated with potassium borohydride (0.4 g), and the mixture was stirred until a clear solution was obtained. After a further 30 min at room temperature, tlc (benzene–methanol, 49:1) showed that reduction of citronellal (R_F 0.58) to citronellol (R_F 0.17) was complete (both substances contained several minor contaminants). The solution was diluted with a large excess of water and extracted three times with ether. The extracts were combined and washed with saturated aqueous potassium chloride solution and dried over magnesium sulfate. Evaporation gave citronellol as a colorless oil (1 g).

Citronellyl α-D-Mannopyranosyl Sodium Phosphate (**5**). (a) 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (**1**, pyridinium form, 62.5 mg; Warren and Jeanloz, 1973b) and citronellol (**2**, 50 mg) were placed with DCC (250 mg, Eastman Organic Chemical, Rochester, N. Y. 14650) in a small round-bottomed flask, equipped with a magnetic stirring bar. Toluene was added and evaporated four times. This method of drying was found more effective than the addition and evaporation of pyridine. Anhydrous pyridine (1 ml) was added and the mixture was stirred for 20 hr at room temperature, when examination by tlc showed the presence of a major product having R_F 0.57 in chloroform–methanol (5:1) (which appeared as two incompletely resolved spots on the chromatogram) as well as unchanged citronellol (R_F 0.8); in solvent A the product had R_F 0.8 and citronellol R_F 0.9. Tlc also indicated that almost no compound **1** was remaining in the reaction mixture after this time, but a small amount of material, apparently the acetylated di-P^1,P^2-α-D-mannopyranosyl pyrophosphate running faster on tlc, was observed. The reaction mixture was diluted to 5 ml with pyridine, and after the addition of water (2 ml) the precipitate of DCC and *N*,*N*′-dicyclohexylurea was filtered off. Evaporation, followed by three additions and evaporations of toluene, gave a crude product (0.27 g) which was chromatographed on a column of silica gel (9 g). Elution was started with chloroform and continued with chloroform–methanol (50:1) and chloroform–methanol (20:1). By using these two solvents, all the residual DCC, *N*,*N*′-dicyclohexylurea, and citronellol were removed from the column. The desired product (R_F 0.8, solvent A) was eluted with chloroform–methanol (5:1). The purest fractions (tlc) were combined to give crude 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl citronellyl phosphate (**4**, 55 mg). The product was de-O-acetylated with a solution of 1% sodium methoxide in methanol (1 ml). Examination of the solution by tlc showed that product **4** had R_F 0.18 (solvent A) and remained at the origin in chloroform–methanol (5:1). However, a considerable amount of material having the mobility of the starting material remained, and this was unaffected by either a prolonged reaction time, or the addition of more sodium methoxide. This material appeared as two close-running spots in chloroform–methanol (5:1) (R_F 0.53 and R_F 0.57) giving

a positive reaction with the phosphate specific spray reagent, and a violet-blue color with the anisaldehyde spray, the latter being characteristic of citronellol derivatives. We have noticed, however, that this phosphate spray gives blue spots with derivatives of DCC even if they do not contain phosphate, and therefore the identity of these by-products remains uncertain. A small portion of the reaction mixture was treated with a cation-exchange resin (pyridinium form) to remove excess sodium ions. Examination of the solution by tlc (solvent A) now gave an ill-defined streaky spot instead of the previous concise spot of R_F 0.18 for the phosphate diester **4**. Therefore, purification by preparative tlc was performed directly on the reaction mixture, with concomitant removal of excess sodium methoxide, the sodium ions remaining at the origin of the chromatogram. The reaction mixture was concentrated to *ca.* 0.5 ml and applied to a 20 cm × 10 cm plate, which was eluted with solvent B. After the plate was dried in air, the required band was located by spraying an area, *ca.* 0.3 cm wide on each side of the plate, 1–2 cm from the edge, with the phosphate specific spray reagent. After the position of the band had been marked, the sprayed (blue) areas were carefully discarded before the silica gel was removed from the plate and extracted by overnight stirring with solvent C. After filtration through Celite and several washings with solvent C, the combined filtrate and washings were evaporated to dryness. The residue was dissolved in chloroform–methanol (2:1) and after filtration through a cotton-wool plug and evaporation, followed by trituration with hexane, α-D-mannopyranosyl citronellyl sodium phosphate (**5**) was obtained as an amorphous solid (17 mg): mp 150–153°; $[\alpha]_D^{20}$ +25° (*c* 0.85, methanol); ir spectrum ν_{max}^{KBr} 3360 (OH), 2960 (CH_3 stretching), 2930 and 2860 (CH_2 stretching), 1600 (C=C stretching), 1465 ($-CH_2$), 1370 (CCH_3), 1290 (CHOH), 1220 (P=O), 1105 (CHOH), 1070 (CO, adjacent to a saturated residue), 1005, 985, 925, 885, and 805 cm^{-1} (peaks associated with $=CH_2$ and trisubstituted olefin, but without definite assignment). Elemental analysis of the product indicated that it probably contained silica gel, but the product was pure according to tlc (R_F 0.18, solvent A; 0.4, solvent B; 0.2, solvent D; and 0.7, solvent E) using the anisaldehyde, potassium permanganate, and phosphate sprays.

(b) The reaction of **1** with citronellol and DCC was performed as described in (a), but after the 20-hr stirring, the solution was evaporated, and toluene was added and evaporated three times. The residue was treated with 1% sodium methoxide in methanol (1 ml) for 30 min at room temperature (no further reaction was detected on tlc). A white precipitate of DCC and *N,N'*-dicyclohexylurea was filtered off and washed with methanol, and the combined filtrate and washings were treated with a cation-exchange resin (pyridinium form) until the pH of the solution was *ca.* 7. The resin was filtered off, the solution evaporated, and the residue extracted with hexane to remove unreacted citronellol. The remaining material, consisting of crude **4** (100 mg), was purified by preparative tlc as described in method (a), after the addition of sodium methoxide in methanol (1%, 0.5 ml, to facilitate the chromatographic resolution) on a 20 cm × 20 cm plate. Extraction of the plate as in (a) gave **4** as an amorphous solid (12 mg). Tlc indicated the presence of a small quantity of D-mannose, R_F 0.27 (solvent B) and R_F 0.32 (solvent E). Since the phosphate specific spray indicated that no inorganic phosphate was present, D-mannose was not formed by dephosphorylation of α-D-mannopyranosyl phosphate or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate during the work-up or chromatographic procedures. Therefore 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose must have been present as a minor product of the DCC mediated reaction.

In an attempt to investigate further the conditions for the formation of by-products in this reaction, two control experiments were carried out. In the first, DCC (100 mg) was stirred with **1** (25 mg) at room temperature for 20 hr. Examination of the mixture by tlc showed that most of **1** remained unchanged, except for a limited amount of conversion into a product which was probably di-P^1,P^2-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl) pyrophosphate (R_F 0.6, solvent B). In the second experiment, DCC (100 mg) was stirred with **2** for 20 hr at room temperature; tlc indicated that no reaction had occurred.

(c) 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (**1**, 200 mg), citronellol (**2**, 100 mg), and iPr_3PhsCl (60 mg, The Aldrich Chemical Co., Milwaukee, Wis. 07927) were dried thoroughly by four additions and evaporations of toluene. The residue was dissolved in dry pyridine (2.5 ml) and kept for 72 hr at room temperature. Methanol (5 ml) was added, and the reaction mixture was kept at room temperature for a further 2 hr. Examination by tlc showed the nearly complete reaction of **1** and the formation of **4**, which appeared as two incompletely resolved spots of R_F 0.51 (chloroform–methanol, 5:1) and 0.7 (solvent A) and as a single spot of R_F 0.89 (solvent B). A considerable amount of the product of R_F 0.6 (solvent B) and 0.26 (solvent A), presumably acetylated di-P^1,P^2-α-D-mannopyranosyl pyrophosphate derived from **1**, was also formed. Tlc also showed the presence of a minor by-product derived from **2** (R_F 0.56, solvent A) and a large amount of unchanged **2**. The pyridine was evaporated, the residue dissolved in chloroform (20 ml), and the solution extracted three times with water to remove the acetylated mannopyranosyl phosphate derivatives. The solution was evaporated, the residue dissolved in methanol, and the solution stirred for 48 hr with a large excess of a cation-exchange resin (sodium form). After removal of the resin, water (1 ml) was added, and the solution was extracted three times with hexane. Evaporation of the hexane extracts gave **2** (50 mg). Evaporation of the aqueous methanol solution gave crude **4** (0.18 g), which was purified by preparative tlc in solvent A on two 20 cm × 20 cm plates. The required band was located by spraying with the potassium permanganate spray, otherwise the procedure was the same as described in method (a) for the preparation of the de-O-acetylated compound **5**. After trituration with hexane, 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl citronellyl sodium phosphate (**4**) was obtained as an amorphous solid (0.1 g): mp 113–117°; $[\alpha]_D^{20}$ +35° (*c* 1, chloroform); ir spectrum ν_{max}^{KBr} 2960 (CH_3, stretching), 2930 and 2860 (CH_2, stretching), 1745 ($COCH_3$), 1375 (CCH_3), 1245 (broad, C=O, stretching, and P=O), 1150 (CH_3CCH_3), 1070 (CO, adjacent to a saturated residue) 980, and 875 cm^{-1} (peaks associated with $=CH_2$ and trisubstituted olefin, but without definite assignment). *Anal.* Calcd for $C_{24}H_{38}NaO_{13}P$: C, 48.89; H, 6.68; P, 5.25. Found: C, 48.95; H, 6.60; P, 5.18. The compound was pure according to tlc in three solvent systems using the anisaldehyde, potassium permanganate, and phosphate specific spray reagents: R_F 0.3 (chloroform–methanol, 5:1); R_F 0.68 (solvent A); and R_F 0.59 (solvent D). In the two less polar solvents, two incompletely resolved spots due to the isomers which comprise citronellol could be observed; when an aqueous solution of **4** was applied to the tlc plate and irrigated with chloroform–methanol (5:1), the two spots were clearly separated.

The acetylated phosphate diester **4** (60 mg) was treated with 1% sodium methoxide in methanol (1 ml) and the solu-

tion kept for 30 min at room temperature, when tlc in solvent A showed that **4** had been converted into citronellyl α-D-mannopyranosyl phosphate (**5**, R_F 0.18). The solution was diluted with methanol (4 ml) and treated with a small amount of cation-exchange resin (pyridinium form) to give pH 7. After removal of the resin, the solution was stirred at room temperature with a large excess of a cation-exchange resin (sodium form) for 48 hr. The resin was filtered off and washed with an excess of methanol, and the combined filtrate and washings were evaporated to dryness. The residue was triturated with hexane to give **5** as an amorphous solid (36 mg): mp 163–165° (with preliminary softening at 150°); $[\alpha]_D^{20}$ −29.4° (*c* 0.72, methanol). *Anal.* Calcd for $C_{16}H_{30}NaO_9P \cdot H_2O$: C, 43.83; H, 7.37; P, 7.06. Found: C, 43.77; H, 7.04; P, 7.04. The ir spectrum and tlc results were identical with those obtained with the product prepared by method (a). For the nmr spectrum, **5** (20 mg) was converted into the pyridinium form by passage of the solution in methanol slowly through a small column (0.5 × 2 cm) of a cation-exchange resin (pyridinium form). The resin was washed with methanol and the combined eluate evaporated. After three additions and evaporations of toluene, the sample was kept *in vacuo* over phosphorus pentaoxide for 20 hr. The dried sample was dissolved in [$^2H_5$]-pyridine (0.5 ml) and the solvent evaporated. This was repeated twice, and then a final solution was made in "Silanor P" (0.4 ml) and filtered into the nmr tube. The main peaks were at δ 0.83, 0.92 (doublet, CH_3 protons of $CH_3CH<$), 1.2–1.5 [broad complex peak, CH_2 and CH protons of $-CH_2-CH(CH_3)CH_2$], 1.57, 1.66 [intense doublet, CH_3 protons of $(CH_3)_2C=$], 1.9, 2.02 [poorly resolved doublet, CH_2 protons of $CH_2CH=C$ and $CH_2C(CH_3)=CH_2$], 4.35, 4.44 (broad, OH and C-H protons, C-2, C-3, and C-4 of α-D-mannopyranosyl residue), 4.68 (HDO), 7.12, 7.26, and 7.64 ppm (aromatic ring protons of pyridinium residue).

Catalytic Hydrogenation of **5**. A small sample of **5** (0.5 mg) in (a) methanol (1.5 ml) or (b) chloroform–methanol (2:1, 1.5 ml) was hydrogenated at room temperature and 1 atm pressure with a selection of different catalysts. The reaction mixture was examined by tlc after 4 and 24 hr in solvents B and E. The compound derived from **5** by saturation of the double bonds had the same R_F as the starting material, 0.4 (solvent B) and 0.7 (solvent E); however, the color of the spot was green with the anisaldehyde spray in contrast to the intense violet-blue color obtained with **5**. In solvent B, the R_F values for α-D-mannopyranosyl phosphate, D-mannose, and methyl α-D-mannopyranoside, were 0, 0.27, and 0.55, respectively. In solvent E, the R_F values for the same substances were 0.19, 0.32, and 0.61, respectively, and all spots were green. The results obtained with individual catalysts were as follows.

Platinum 5% on charcoal (Matheson Coleman and Bell, Norwood, Ohio, and E. Rutherford, N. J. 07073) and platinum dioxide (Englehard, Chemical Division, Newark, N. J.) gave in solution (a), after 4 hr, only reduced starting material; after 24 hr, this was still the main product with a trace of α-D-mannopyranosyl phosphate. In solution (b) platinum 5% on charcoal gave, after 4 hr, the reduced starting material accompanied by a trace of D-mannose and, probably, methyl D-mannoside. After 24 hr the proportions of these by-products had increased slightly.

Reduced chloroplatinic acid (H_2PtCl_6 supplied by Ventron-Alfa Inorganics, Beverly, Mass. 01915) (Wright *et al.*, 1967) gave in solutions (a) or (b), after 4 hr, mainly reduced starting material with a trace of D-mannose; after 24 hr a small amount of (probably) methyl D-mannoside was also present.

Palladium (10%) on charcoal (Fluka AG, Buchs S.G., Switzerland) gave in solution (a) after 24 hr the reduced starting material with a considerable amount of D-mannopyranosyl phosphate (*ca.* 5%) and a trace of a methyl D-mannoside.

It should be noted that the reaction mixtures after hydrogenation were in every case acidic, especially those containing chloroform. A similar observation was made when the mannolipid was absent from the hydrogenation mixture.

Dilute Acid Hydrolysis. A solution of **5** and α-D-mannopyranosyl phosphate (1 mg) in 0.2 M hydrochloric acid–methanol was kept at 100° for 5 min; in both cases tlc showed that D-mannose (R_F 0.27, solvent B; 0.32, solvent E) had been formed together with a trace of (presumably) methyl mannoside (R_F 0.61, solvent B; 0.44, solvent E). No α-D-mannopyranosyl phosphate had been formed. The only other major product, in the hydrolysate of **5**, was a phosphorylated derivative of citronellol, different from citronellyl phosphate (R_F 0.54, solvent B; 0.61, solvent E) and similar to the compound obtained by dilute acid hydrolysis of citronellyl phosphate (R_F 0.37, solvent B; R_F 0.52, solvent E), but complete identity of the product was not established. (This unexpected reaction was not encountered in the dilute acid hydrolysis of **7**, which gave dolichyl phosphate.)

Citronellyl Phosphate. Citronellol (**2**, 0.1 g) in *p*-dioxane (freshly distilled in the presence of sodium metal and kept over calcium hydride, 2 ml) containing 2,6-dimethylpyridine (80 mg) was stirred at 0° and treated with *o*-phenylene phosphorochloridate (0.14 g), prepared by the method of Khwaja *et al.* (1970), in *p*-dioxane (2 ml). The temperature was allowed to rise to 20° and, after 10 min, tlc showed that nearly all of compound **2** (R_F 0.85, solvent A) had been converted into a phosphorylated product (R_F 0.43). The precipitate of 2,6-dimethylpyridinium chloride was filtered off and washed with *p*-dioxane (2 ml). The filtrate was treated with 2,6-dimethylpyridine (80 mg) and water (a few drops, sufficient to give a clear solution) and, after 5 min, the mixture was evaporated. After two additions and evaporations of toluene, small amounts of 2,6-dimethylpyridinium chloride were removed by dissolution of the residue in *p*-dioxane and filtration. Evaporation gave the syrupy *o*-hydroxyphenyl phosphate, which was dissolved in *p*-dioxane (5 ml) and treated with lead tetraacetate (0.7 g, containing 10% acetic acid, Alfa Inorganics-Ventron). The dark-brown solution was stirred for 30 min, and then treated with 0.1 M potassium hydroxide in methanol, until the pH reached 12 and a heavy, brown precipitate had formed. After 30 min, a slight excess of glacial acetic acid was added. Evaporation of the solvents, followed by two additions and evaporations of toluene, gave a brown residue, which was vigorously stirred with solvent C until a clear solution was obtained (2–3 hr). The solution was treated with a cation-exchange resin (pyridinium form) and stirred overnight. The resin was filtered off, and the filtrate was treated with cyclohexylamine until a basic solution was obtained, showing that an excess of the amine was present. Evaporation, followed by two additions and evaporations of toluene, gave a solid which was triturated with ether–methanol (10:1, 25 ml). The solid was filtered off and washed with ether, and evaporation of the filtrate gave a brown syrup (0.15 g). The product was freed from contaminants (including **2**) by dissolution in water, filtration, and extraction with hexane. Evaporation of the aqueous solution gave citronellyl phosphate, a mixture of the mono- and dicyclohexylammonium salts, as a semisolid (0.12 g), pure according to tlc (R_F 0.39, solvent A). In order to obtain crystals, a solution in water (5 ml) was filtered through Celite, concentrated to 2 ml, and cooled to

0° to give a low yield (13 mg) of needles: mp 124–128°, $[\alpha]_D^{25}$ +1.3° (*c* 1, in 1:1 water–methanol); ir spectrum ν_{max}^{KBr} 2940, 2860 (CH_2, stretching), 1625 (C=C, stretching), 1450 (–CH_2, –CH_3), 1390 (CH_3CH), 1150 (CH_3CCH_3), 1070 (CO, stretching, adjacent to a saturated residue), 980 (CH_2=C<), and 805 cm^{-1} ($(CH_3)_2C$=CH–). *Anal.* Calcd for $C_{16}H_{34}NO_4P$: C, 57.28; H, 10.54; N, 4.18; P, 9.23; for $C_{22}H_{47}N_2O_4P$: C, 60.77; H, 10.91; N, 6.45; P, 7.12; for a 1:1 mixture, C, 59.02; H, 10.72; N, 5.31; P, 8.18. Found: C, 59.01; H, 10.85; N, 5.66; P, 7.24.

Dolichyl α-D-Mannopyranosyl Sodium Phosphate (**7**). (a) A mixture of pig liver dolichol (**3**, 28 mg), DCC (20 mg), and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (pyridinium form **1**, 4 mg) was completely dried by repeated addition and evaporation of toluene. After the mixture had been stirred for 24 hr in anhydrous pyridine (1 ml), examination by tlc showed the formation of a major product (R_F 0.75, chloroform–methanol, 5:1; 0.15, chloroform–methanol, 20:1) in the presence of a great proportion of unchanged **3** (R_F 0.85, chloroform–methanol, 20:1; 1.00, chloroform–methanol, 5:1). No compound **1** remained, but a small amount of the symmetrical pyrophosphate derived from it (R_F 0.26, solvent A) had been formed. Therefore, additional portions of DCC (20 mg) and **1** (4 mg) were added, and the mixture was dried as just described, and stirred for a further 24 hr in dry pyridine (1 ml). This procedure was repeated once more with a further portion of DCC and of **1**, so that total **1** added to the reaction was 12 mg. Finally, after more than 50% of **3** had undergone reaction, the pyridine was evaporated, the residue dissolved in chloroform (1 ml), and the solution chromatographed on a column of silica gel (3.0 g). Elution with chloroform was followed by elution with chloroform–methanol (50:1) which gave, initially, unreacted **3**, together with small amounts of DCC and *N,N'*-dicyclohexylurea (total 25 mg). The remainder of these two compounds was removed from the column by a prolonged elution with the same solvent, followed by a brief elution with chloroform–methanol (40:1). The desired product, 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl dolichyl phosphate (**6**), was eluted with chloroform–methanol 20:1, followed by 10:1, and 5:1 giving only impure material. The purest fractions (tlc) were combined to give **6** (21 mg) in the pyridinium form. The product was contaminated with small amounts of DCC and *N,N'*-dicyclohexylurea and was not characterized further.

The dolichol (**3**, 25 mg) obtained from the column was purified by preparative tlc in benzene–methanol (49:1) on a 20 cm × 5 cm plate. The band containing **3** was located by spraying the edges with the potassium permanganate spray. The silica gel was removed from the plate and extracted with chloroform–methanol (40:1) by stirring overnight. After filtration through Celite, and washing the solid several times with chloroform, the filtrate was evaporated to give dolichol (5 mg) identical by tlc with the original material.

The crude compound **6** (21 mg) was dissolved in chloroform (0.5 ml) and treated with 1% sodium methoxide in methanol (0.5 ml). After 30 min, tlc (solvent A) showed that the starting material (R_F 0.8) had been converted into a new product (R_F 0.54), together with a trace of a D-mannose-containing contaminant (R_F 0.35). As in the preparation of the citronellol derivative **5**, a substance having the same mobility as that of the acetylated phosphate diester (appearing as two close-running spots on the chromatogram) was presumably a contaminant, as it was not affected by a prolonged treatment with base. The reaction mixture was evaporated to a small volume under a stream of nitrogen. Chloroform was added until a clear solution resulted which was applied to a preparative thin-layer chromatogram (20 cm × 5 cm) and eluted with solvent B, which gave a better separation than did solvent A. The band containing compound **7** was located by spraying a narrow area near the center of the plate with (a) the phosphate spray and (b) the potassium permanganate spray. The silica gel was removed from the plate and extracted with solvent C by an overnight stirring. The solution was filtered through Celite, and the residue was washed several times with solvent C. The combined filtrates were evaporated to dryness, the residue was dissolved in chloroform, and the solution was filtered. Evaporation gave **7** as a syrup (12 mg), pure by tlc (solvents A, B, and D) except for a minor contaminant (R_F 0.92, chloroform–methanol, 5:1), which reacted with the phosphate spray. Also, the ir spectrum, apart from the expected peaks, had a peak at 1560 cm^{-1} (NH) which could be derived from *N,N'*-dicyclohexylurea, the carbonyl absorption being obscured by the peak at 1660 cm^{-1} (C=C, stretching). Therefore, a final preparative tlc was performed in solvent A, the location and extraction of the correct band being carried out as just described. This gave dolichyl α-D-mannopyranosyl sodium phosphate (**7**) as a syrup (6 mg): $[\alpha]_D^{20}$ +3.5° (*c* 0.8, in chloroform–methanol, 5:1); ir spectrum ν_{max}^{film} 3350 (OH), 2965 (CH_3, stretching), 2930 and 2860 (CH_2, stretching), 1730 (unassigned; see absorption at 1750 cm^{-1} of polyprenols from *Aspergillus niger*, Barr and Hemming, 1972), 1660 (C=C, stretching), 1450 (–CH_2, –CH_3), 1376 (CCH_3), 1220 (P=O), 1070 (CO, stretching, adjacent to saturated residue), 975, and 835 cm^{-1} (–CH=C<). Compound **7** gave only one spot on tlc in the following solvent systems after detection with the anisaldehyde, potassium permanganate, and phosphate specific sprays: R_F 0.54 (solvent A), 0.83 (solvent B), 0.67 (solvent D), and 0.83 (with tailing, solvent E).

(b) A mixture of **3** (12 mg) with **1** (9 mg) and iPr_3PhsCl (5.4 mg) was thoroughly dried by four additions and evaporations of toluene. Anhydrous pyridine (0.2 ml) was added, and the solution was kept at room temperature for 72 hr; tlc showed that at least 50% of **3** had been converted into a product having R_F 0.45 (chloroform–methanol, 5:1) and 0.8 (solvent A). Tlc also indicated that side reactions involving the lipid were minimal, but that the other major reaction product was the symmetrical pyrophosphate (R_F 0.26, solvent A) derived from **1** in the presence of iPr_3PhsCl. The reaction was stopped by the addition of methanol (1 ml) and after 2–3 hr the solvents were evaporated. The residue was dissolved in chloroform (5 ml) and the solution extracted three times with water (5 ml) to remove derivatives of **1**. The chloroform solution was evaporated to dryness. The residue, dissolved in chloroform (0.5 ml), was applied to a column of silica gel (1.5 g) and fractions (1 ml) were analyzed by tlc. Chloroform eluted two fractions containing unreacted **3** (4 mg). After further fractions had been eluted with chloroform–methanol (50:1) and (25:1), compound **6** was obtained with chloroform–methanol (15:1). The elution was completed with chloroform–methanol (10:1), which gave a small amount of **6**, contaminated with traces of other substances (tlc). The fractions containing **6** were combined and evaporated to yield a syrup (10 mg), pure by tlc, except for a trace of material running just ahead of the main spot (chloroform–methanol, 5:1).

Compound **6** (10 mg) in chloroform (0.5 ml) was treated with 1% sodium methoxide in methanol until the pH of the solution was strongly basic, indicating that an excess of the reagent was present. After 30 min, all the starting material

(R_F 0.8, solvent A) had been converted into a new product (R_F 0.54). The solution was treated with a cation-exchange resin (pyridinium form) until the pH was neutral, then methanol was added to the point of turbidity. After the addition of a few drops of chloroform to clarify the solution, it was stirred with a large excess of a cation-exchange resin (sodium form) for 48 hr at room temperature. The resin was filtered off, and the solution was evaporated to leave a residue which was dissolved in chloroform–methanol (5:1). The solution was filtered and evaporated to give dolichyl α-D-mannopyranosyl sodium phosphate (**7**) as a syrup (7 mg), containing only traces of contaminants. The ir spectrum was almost identical with that of the product obtained by method (a). *Anal.* Calcd for $C_{101}H_{166}NaO_9P \cdot 3.5H_2O$: C, 73.90; H, 10.62. Found: C, 73.91; H, 10.33.

Dilute Acid Hydrolysis of **7**. A sample of **7** (1 mg) was dissolved in 0.5 ml of a mixture of 1 M hydrochloric acid and methanol (1:10) and chloroform (2 drops, sufficient to obtain a clear solution). The mixture was heated in a water bath at 100° for 5 min, then chloroform–methanol (1:1) was added to give the original volume of solution. The hydrolysate contained one dolichol-containing product, which cochromatographed with dolichyl phosphate (Wedgwood and Warren, 1973) (R_F 0.63, solvent A; 0.76, solvent D). Four D-mannose-containing spots (green color with the anisaldehyde spray) were also observed: D-mannose (R_F 0.27) and methyl D-mannosides (R_F 0.43, 0.55, and 0.61, solvent B). A standard sample of methyl α-D-mannopyranoside had R_F 0.55. In solvent E, the mobilities were: D-mannose, R_F 0.32; methyl D-mannosides, R_F 0.44 and 0.61, the latter corresponding to methyl α-D-mannopyranoside. The solvents were evaporated under a stream of nitrogen gas, and the residue was extracted first with chloroform, then with water. All the dolichyl phosphate was in the chloroform extract, and no free dolichol was formed during the hydrolysis (tlc in benzene–methanol, 49:1). All the substances derived from the D-mannosyl moiety were in the water extract, but no α-D-mannopyranosyl phosphate was formed (tlc in solvent E). The mixture of substances present in the water extract was further examined by tlc on cellulose, in 1-propanol–ethyl acetate–water (7:1:2). Detection with the periodate–benzidine spray (Mowery, 1957) showed three spots (R_F 0.25, 0.32, and 0.55), the slowest moving one corresponding to D-mannose.

For comparison, samples of D-mannose (1 mg) and α-D-mannopyranosyl phosphate (1 mg) were heated for 5 min to 100° with (a) 1 ml of a mixture of 1 M HCl and methanol (1:10) and (b) 1 ml of a mixture of 0.2 M HCl and methanol (1:1). Under conditions (a) some of the D-mannose was converted into methyl α-D-mannopyranoside, together with a large proportion of a compound having R_F 0.61 (solvent B); α-D-mannopyranosyl phosphate gave D-mannose, methyl α-D-mannopyranoside, and a compound having R_F 0.43 (solvent B). Under conditions (b), D-mannose was, as expected, the main component, together with small amounts of mannosides (R_F 0.43, and 0.55, solvent B).

Reaction of **1** *with DCC*. In the preparation of **4** and **6** by the DCC method, acetylated di-P^1,P^2-D-mannopyranosyl pyrophosphate was considered to be a minor by-product, and the same substance was a probable major by-product in the preparation of **4** and **6** by the iPr_3PhsCl method. According to Cawley and Letters (1971), this compound was the main product when tetra-*O*-acetyl-D-mannopyranosyl phosphate was condensed with an alcohol in the presence of 1 equiv of DCC. Therefore, a mixture of **1** (20 mg) and DCC (10 mg) was dried by repeated additions and evaporations of toluene, then stirred in dry pyridine (0.4 ml) for 20 hr. Most of the starting material **1** (R_F 0.45, solvent B) was converted into a new product (R_F 0.6) which cochromatographed with the by-product just described (R_F 0.26, solvent A). After evaporation of the pyridine, the mixture was deacetylated by the addition of 1% sodium methoxide in methanol (0.5 ml). The solution was kept at room temperature for 30 min, and water (2 drops) was added to give a clear solution. Tlc on cellulose in 1 M ammonium acetate–ethanol (5:2) showed a single spot (R_F 0.9), detected with the periodate–benzidine spray reagent (Mowery, 1957) and gave an intense violet color with the phosphate spray, which was indicative of a pyrophosphate group (Warren *et al.*, 1973). In contrast, α-D-mannopyranosyl phosphate gave a faint blue spot on a white background (R_F 0.75), which later turned into a faint white spot on a blue background, indicative of a monophosphate group.

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Physical Characterization and Simultaneous Purification of Bacteriophage T_4 Induced Polynucleotide Kinase, Polynucleotide Ligase, and Deoxyribonucleic Acid Polymerase†

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ABSTRACT: The initial steps in the existing procedures for the purification of the bacteriophage T_4 induced polynucleotide kinase, ligase, and the DNA polymerase have been modified so as to enable their simultaneous preparation from one batch of the infected *Escherichia coli* cells. Procedures were devised to remove an exonuclease which often contaminated the preparations of the kinase and ligase. The kinase was shown to be homogeneous by gel electrophoresis. Its molecular weight under native conditions using gel filtration was found to be 140,000 ± 10% daltons. Under denaturing conditions employing sodium dodecyl sulfate electrophoresis the mol wt was estimated to be 33,000 ± 5% daltons. The ligase also gave a single band upon gel electrophoresis and the mol wt under denaturing conditions was 63,000 ± 5% daltons. Gel filtration of the latter enzyme in the absence of denaturing agents gave a mol wt of 68,000 ± 10% daltons.

The bacteriophage T_4 induced enzymes, polynucleotide kinase, polynucleotide ligase, and DNA polymerase, have proved to be important and useful in chemical and biological studies of DNA (Richardson, 1965; Weiss and Richardson, 1967a,b; Goulian *et al.*, 1968). The kinase and ligase form parts of the methodology for the laboratory synthesis of bihelical DNA of specific nucleotide sequence (Khorana *et al.*, 1972).

While satisfactory methods are available for the separate purification of each one of the three enzymes (Goulian *et al.*, 1968; Weiss *et al.*, 1968; Richardson, 1972), considerable saving in time and material could be achieved if a procedure could be developed which enables simultaneous preparation of the three enzymes from one batch of the T_4-infected *Escherichia coli* cells. Such a procedure was especially desirable for the work on the total synthesis of the transfer RNA genes where relatively large amounts of the enzymes are continually required (Khorana *et al.*, 1972).

In this paper we first describe a procedure which modifies the existing procedures to a single procedure for preparation of the enzymes. Procedures have also been developed for removing or completely inhibiting an exonuclease which sometimes is present after the final step in the purification of the kinase. Further physical characterizations of the kinase and the ligase are reported. The kinase is concluded to have a mol wt of ~140,000 daltons with subunits of ~33,000 daltons. The ligase appears to be a single polypeptide chain of mol wt ~68,000 daltons.

Experimental Section

Materials

T_4-Infected E. coli Cells. E. coli strain B62 was grown in 3XD medium (Fraser and Jerrel, 1953) to a cell density of 5×10^8/ml and then infected with T_4 amN82 phage at a multiplicity of 5:8. Thirty minutes after infection, the culture was quickly chilled on ice and the cells were harvested by centrifugation in a continuous flow centrifuge (DeLaval).

Ion exchangers, DEAE-cellulose (DE-23) and phosphocellulose (P11), were purchased from Whatman and DEAE-Sephadex A-50 was obtained from Sigma Chemical Co. Hydroxylapatite, Bio-Gel HTP, was the product of Bio-Rad Laboratories and Alumina 350 was obtained from Sigma Chemical Co.

[γ-^{32}P]ATP was prepared according to the published procedure (Glynn and Chappell, 1964). [α-^{32}P]ATP and [^{14}C]dATP were purchased from New England Nuclear and [^{3}H]ATP

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