Biochem. J. (1969) **111**, 325
Printed in Great Britain

Synthesis of 10,11-Dihydrofarnesyl Pyrophosphate from 6,7-Dihydrogeranyl Pyrophosphate by Prenyltransferase

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(*Received* 9 *September* 1968)

The syntheses of 6,7-dihydrogeraniol and of its pyrophosphate are described. It is shown that this analogue of geranyl pyrophosphate is a substrate for liver prenyltransferase and that the product synthesized by this enzyme from it and isopentenyl pyrophosphate is 10,11-dihydrofarnesyl pyrophosphate. The K_m value for 6,7-dihydrogeranyl pyrophosphate was determined to be $1{\cdot}11 \pm 0{\cdot}19\,\mu$M as compared with $4{\cdot}34 \pm 1{\cdot}71\,\mu$M for geranyl pyrophosphate. The maximum reaction velocity with the artificial substrate was, however, only about one-fourth of that observed with geranyl pyrophosphate. The binding of isopentenyl pyrophosphate to the enzyme was not affected by the artificial substrate.

It was observed during a study of prenyltransferase (EC 2.5.1.1) that, among several analogues of geranyl pyrophosphate designed as inhibitors of this enzyme (Popják, Holloway, Bond & Roberts, 1969), 6,7-dihydrogeranyl pyrophosphate was an alternative substrate to geranyl pyrophosphate for prenyltransferase. We record here experiments showing that prenyltransferase synthesizes 10,11-dihydrofarnesyl pyrophosphate by the condensation of 6,7-dihydrogeranyl pyrophosphate and isopentenyl pyrophosphate.

MATERIALS AND METHODS

Prenyltransferase. Two preparations of this enzyme, made from pig liver, were used: one had a specific activity of 100 and the other of 24, the specific activity being defined as the number of mμmoles of geranyl pyrophosphate converted, with isopentenyl pyrophosphate, into farnesyl pyrophosphate/min./mg. of protein (Holloway & Popják, 1967). Both preparations were free of isopentenyl pyrophosphate isomerase.

Product of reaction between 6,7-dihydrogeranyl pyrophosphate and isopentenyl pyrophosphate. Two experiments were made for the analysis and identification of the product formed from 6,7-dihydrogeranyl pyrophosphate and [^{14}C]isopentenyl pyrophosphate. In one of these (Expt. *A*) four incubations were made; these contained, in 1·0ml., 20μmoles of tris–HCl buffer, pH 7·9, 5μmoles of $MgCl_2$, 0·9mμmole of [4-^{14}C]isopentenyl pyrophosphate (10000 disintegrations/min.) and 50μg. (5 units) of prenyltransferase. To two of these solutions were added 10mμmoles of geranyl pyrophosphate, and to the other two 10mμmoles of 6,7-dihydrogeranyl pyrophosphate. The four solutions were incubated at 37° for 5min., then heated at 90° for 30sec. and cooled in ice. In one incubation containing geranyl pyrophosphate and in another containing the dihydro analogue, the alcohols were liberated by acid, and in the remaining two incubations they were liberated by hydrolysis for 18hr. at room temperature with intestinal alkaline phosphatase (0·5mg.) after the pH of the solutions had been adjusted to 9·5 with tris base. A mixture of linalool, geraniol, nerolidol and farnesol (about 1mg. of each) was added to each incubation before extraction of the reaction mixtures with ethyl chloride at 0°. A few microlitres of benzene were added to the extracts and then the ethyl chloride was evaporated at room temperature; the alcohols were analysed by gas–liquid radiochromatography.

In the second experiment (Expt. *B*) an incubation (13ml.) containing buffer and $MgCl_2$ in the usual concentrations was set up with 96 units (4mg.) of prenyltransferase and 2·8μmoles of dihydrogeranyl pyrophosphate plus 2·8μmoles of [1-^{14}C]isopentenyl pyrophosphate (specific radioactivity 0·069μc/μmole). After 3hr. incubation at 37°, the pH of the solution was adjusted to 9·5 and 4mg. of intestinal alkaline phosphatase, dissolved in 0·4ml. of 0·02M-$KHCO_3$, was added. After a further 3hr. incubation at 37°, ethanol was added to a concentration of 25% and the mixture was extracted five times with *n*-pentane. The combined extracts were evaporated to a few millilitres on a rotary evaporator. The pentane solution was then dried with a few lumps of $CaSO_4$ and made up to a known volume: one sample of this was counted for ^{14}C content and another analysed by gas–liquid radiochromatography. The remainder was concentrated to about 50μl. and applied in one spot on to a 5cm.×20cm. plate with a 300μ-thick layer of silica gel ('Kieselgel GF_{254} nach Stahl'; E. Merck A.-G., Darmstadt, Germany), the plate having previously been 'washed' with redistilled benzene, chloroform and ethanol. This thin-layer chromatogram was developed with

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10% ethyl acetate in benzene; it was scanned for radioactivity and inspected under ultraviolet light. The radioactive area (R_F 0·21) was scraped off the plate, the substance was eluted from the gel with diethyl ether and rechromatographed twice more in an identical manner. After the third chromatography, the eluted material was examined in the mass spectrometer.

Determination of K_m for 6,7-dihydrogeranyl pyrophosphate and of $V_{max.}$ of prenyltransferase with artificial substrate. For the determination of the value of K_m for dihydrogeranyl pyrophosphate, the assay for prenyltransferase was used (Holloway & Popják, 1967); standard incubations were set up containing, in 1 ml., 20 μmoles of tris–HCl buffer, pH 7·9, 5 μmoles of $MgCl_2$,; 25 μg. of prenyltransferase (specific activity 100) and various final concentrations of 6,7-dihydrogeranyl pyrophosphate (1·04, 1·95, 3·00 and 5·85 μM) and of [^{14}C]isopentenyl pyrophosphate (specific radioactivity 950 disintegrations/min./mμmole; 1·14, 1·9, 3·0 and 5·7 μM). The amount of new [^{14}C]allyl pyrophosphate formed was determined after 2 min. incubation at 37°, as described for the determination of farnesyl pyrophosphate in the normal prenyltransferase assay (Holloway & Popják, 1967), and was used as a measure of initial reaction velocity.

Geranyl pyrophosphate and [^{14}C]isopentenyl pyrophosphate. These compounds were those described previously (Holloway & Popják, 1967; Donninger & Popják, 1967).

Three specimens of [^{14}C]isopentenyl pyrophosphate were used: (i) a preparation labelled at C-4 (specific radioactivity 11 110 disintegrations/min./mμmole), generated enzymically from [2-^{14}C]mevalonate; (ii) a synthetic preparation labelled at C-1 (specific radioactivity 0·069 μC/μmole); (iii) a mixture of (i) and (ii) with a specific radioactivity of 950 disintegrations/min./mμmole (cf. Popják *et al.* 1969).

6,7-Dihydrogeranyl pyrophosphate. This was made by the phosphorylation of 1 m-mole (156 mg.) of 6,7-dihydrogeraniol by the method of Cramer & Böhm (1959) as modified by Popják, Cornforth, Cornforth, Ryhage & Goodman (1962) for the synthesis of farnesyl pyrophosphate. The products of phosphorylation were purified by the method of Holloway & Popják (1967), except that in the separation of the mono- and pyro-phosphoryl derivatives by chromatography on a column (8 cm. × 1 cm.) of DEAE-cellulose (Whatman DE-11 cellulose) aqueous, not methanolic, 80 mM-ammonium formate was the solvent. The purification was followed by thin-layer chromatography on Eastman Kodak Co. Chromatoplates (type K301, R2; reclassified as type 6061) with either propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (6:3:1, by vol.) or ethanol–aq. NH_3 (sp.gr. 0·88)–water (15:4:3, by vol.). The R_F values of the mono- and pyro-phosphoryl derivatives of 6,7-dihydrogeraniol were respectively 0·42 and 0·35 in the first system and 0·72 and 0·63 in the second. Based on analysis for acid-labile pyrophosphate (Goodman & Popják, 1960), the yield of dihydrogeranyl pyrophosphate was 5%. The preparation, which was free of the monophosphate and contained no inorganic phosphate, was also examined after its hydrolysis with intestinal alkaline phosphatase; the alcohol liberated had the same retention volume in gas–liquid chromatography as the alcohol used in the phosphorylation.

6,7-Dihydrogeraniol. This was prepared by the reduction with $LiAlH_4$ of ethyl *trans*-3,7-dimethyloct-2-enoate synthesized by a Wittig reaction (see below). From 198 mg. of the ester 159 mg. of the alcohol was obtained; gas–liquid chromatography of the preparation at 135° showed a single component with a retention volume (R_v) of 0·66 relative to that of geraniol.

A specimen of *cis*-6,7-dihydrogeraniol (dihydronerol; R_v 0·58 at 135°, relative to geraniol) was obtained similarly from the corresponding ester of the *cis* acid, but was not phosphorylated.

Dihydrogeraniol and dihydronerol were also obtained by the direct reduction with $LiAlH_4$ of the products of Wittig synthesis (see below). A sample (0·8 g.) of the crude products from the Wittig synthesis gave, after reduction with $LiAlH_4$ in the usual way, 0·6 g. of a pale-yellow oil, which was resolved by preparative gas–liquid chromatography at 120° into four components. The retention volumes of the three faster-moving fractions relative to the most slowly moving fourth component (*d*; R_v 1·0; 35%) were: (*a*) 0·166 (42%); (*b*) 0·64 (9·0%); (*c*) 0·83 (14%). These fractions were identified by their mass spectra and nuclear-magnetic-resonance (n.m.r.) spectra as 6-methylheptan-2-ol (*a*), 6,7-dihydronerol (*c*) and 6,7-dihydrogeraniol (*d*). Component (*b*) was not identified definitely; it had R_v in gas–liquid chromatography identical with that of tetrahydrogeraniol. Fractions (*c*) and (*d*) were identical with the alcohols obtained by the reduction of the isolated *cis*- and *trans*-3,7-dimethyloct-2-enoates.

Ethyl (cis *and* trans)*-3,7-dimethyloct-2-enoate.* This was synthesized by a Wittig reaction (Pommer, 1960*a*,*b*; Trippett & Walker, 1961; Wadsworth & Emmons, 1961) from 6-methylheptan-2-one and the phosphonate, $(CH_3 \cdot CH_2 \cdot O)_2PO \cdot CH_2 \cdot CO_2 \cdot CH_2 \cdot CH_3$ (prepared from triethyl phosphite and ethyl 2-bromoacetate; Kosolapoff, 1950). Sodium hydride, as a 50% suspension in oil (528 mg., about 11 m-moles), was put into a 50 ml. flask; the oil was extracted with light petroleum (b.p. 40–60°) under N_2 and the residual light petroleum was removed with a stream of dry N_2 and under vacuum. Dry tetrahydrofuran (15 ml.) was then added, followed by the phosphonate (10 m-moles, 2·25 g.). After the evolution of H_2 ceased, 6-methylheptan-2-one (10 m-moles, 1·28 g.) was added. The mixture was stirred for 3 days at room temperature, then poured into 150 ml. of water and extracted three times with diethyl ether. The combined ether extracts were washed with saturated aq. NaCl solution and the ethereal solution was dried further over $MgSO_4$. Evaporation of the ether left a pale-yellow oil (1·8 g.), which on examination by gas–liquid chromatography at 125° gave three components: (i) unchanged 6-methylheptan-2-one (25%); (ii) a smaller component (23%) with R_v 3·81 relative to that of (i); (iii) a larger component (52%) with R_v 5·36. Fractions (ii) and (iii) were assumed to be the ethyl esters of *cis*- and of *trans*-3,7-dimethyloct-2-enoic acid respectively, as they were formed in the proportions expected in similar syntheses. After their separation by preparative gas–liquid chromatography at 120° the n.m.r. spectra were compatible with the assumed structures. There is an advantage in separating the *cis* and *trans* isomers of the esters before reduction with $LiAlH_4$ in that the separation factor between the geometric isomers of the esters is 1·4, whereas the separation factor between the corresponding alcohols is only 1·2. Even after the application of 150 μl. of the products of the Wittig synthesis on to the gas–liquid-chromatography column a satisfactory resolution of the *cis* and *trans* esters was achieved. In contrast, only 50 μl. of the alcohols could be resolved without an overlap between the isomers.

6-Methylheptan-2-one. 6-Methylhept-5-en-2-one (3·79g.; supplied by Koch–Light Laboratories Ltd., Colnbrook, Bucks.) in methanol was hydrogenated with palladized charcoal (5% Pd on charcoal, 149mg.); the specimen absorbed the theoretical volume of H_2. The catalyst was filtered off through two layers of Whatman no. 42 paper and washed with methanol. Evaporation of the solvent left a colourless oil (3·84g.). Analysis of the product by gas–liquid chromatography at 104° gave a single component with R_v 0·614 relative to that of the starting material, which was also homogeneous on gas–liquid chromatography. The n.m.r. spectrum of the product compared with that of the starting material showed the disappearance of the olefinic proton (τ 4·97, triplet, in the spectrum of methylheptenone), the shift of the olefinic methyl protons (τ 8·37 in methylheptenone) to τ 9·13 (doublet, J 6cyc./sec.), the appearance of absorption lines (complex multiplet) of five protons attributable to those in the $>CH\cdot CH_2\cdot CH_2\cdot CO-$ group (centred at about τ 8·67). Mass spectrometry gave a molecular weight of 128 as compared with 126 for the starting material.

Gas–liquid chromatography. All the gas–liquid chromatography, analytical and preparative, was done on columns packed with Celite (100–120 mesh) coated with 10% Carbowax 20M (Union Carbide Corp., New York, N.Y., U.S.A.). In the analytical instrument, coupled with a radioactivity detector (Popják, Lowe & Moore, 1962), a 9ft. long × 4mm. diam. column was used. The preparative instrument (Varian Aerograph, Autoprep model A700; Varian Associates, Palo Alto, Calif., U.S.A.) was fitted with a 12ft. long × 6·25mm. diam. column; the effluent vapours were condensed at −40° with the aid of a home-made electrostatic precipitator at 6·5kv. The efficiency of collection was at least 90%. The temperatures at which the analyses or fractionations were done varied according to the nature of the specimens and are given in appropriate sections of this paper.

Assay of ^{14}C. ^{14}C was measured in a Packard Tri-Carb scintillation spectrometer as described by Holloway & Popják (1967). Radioactivity on thin-layer chromatograms was detected with a Packard chromatogram scanner (Packard Instrument Co., La Grange, Ill., U.S.A.).

Mass spectrometry. The mass spectra were taken in an MS-9 instrument (A.E.I. Ltd., Manchester). The samples were introduced on a probe directly into the ion chamber at ion-source temperatures of 50–100°; the spectra were taken at an ionization potential of 70ev. The advantages of using a probe for sample introduction are that small specimens (5–10μg.) are sufficient for the work and particularly that even alcohols give molecular ions. When, for instance, 6,7-dihydrogeraniol was introduced into the mass spectrometer through the heated gallium-inlet system, the molecular ions could not be recorded; instead the $[M-18]^+$ ions appeared at the highest m/e value of the spectrum. In contrast, from the probe 6,7-dihydrogeraniol gave molecular ions of good intensity.

Nuclear-magnetic-resonance spectrometry. The n.m.r. spectra were taken with a Varian A60 instrument (Varian Associates) on samples dissolved in $CDCl_3$.

RESULTS

6,7-Dihydrogeraniol. The identification of the 6,7-dihydrogeraniol used in the phosphorylation as the *trans* isomer rests mainly on the rule, so far unbroken, that the *trans* isomers in the prenol series have, in gas–liquid chromatography on polar stationary phases of the polyester type, a larger retention volume than the *cis* isomers (Popják &

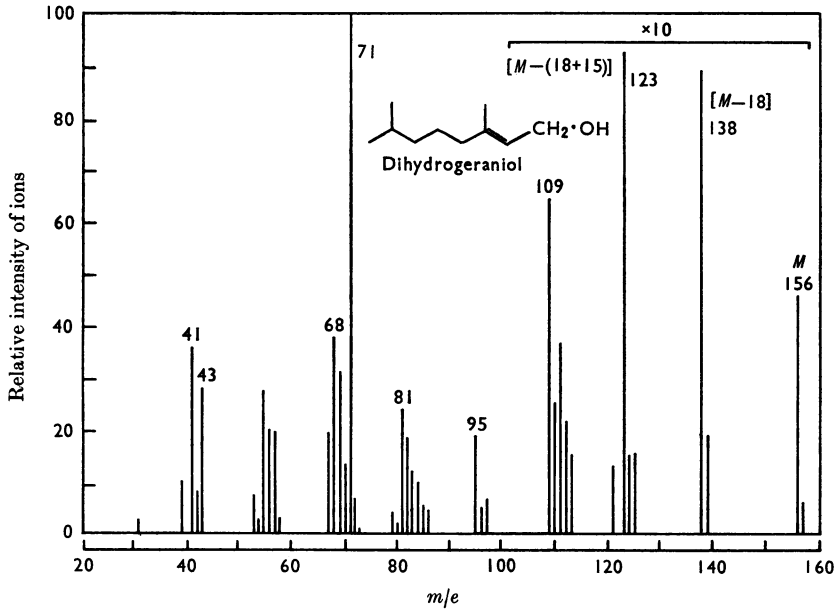


Fig. 1. Mass spectrum of 6,7-dihydrogeraniol. The intensity of ions above m/e 100 has been multiplied by 10.

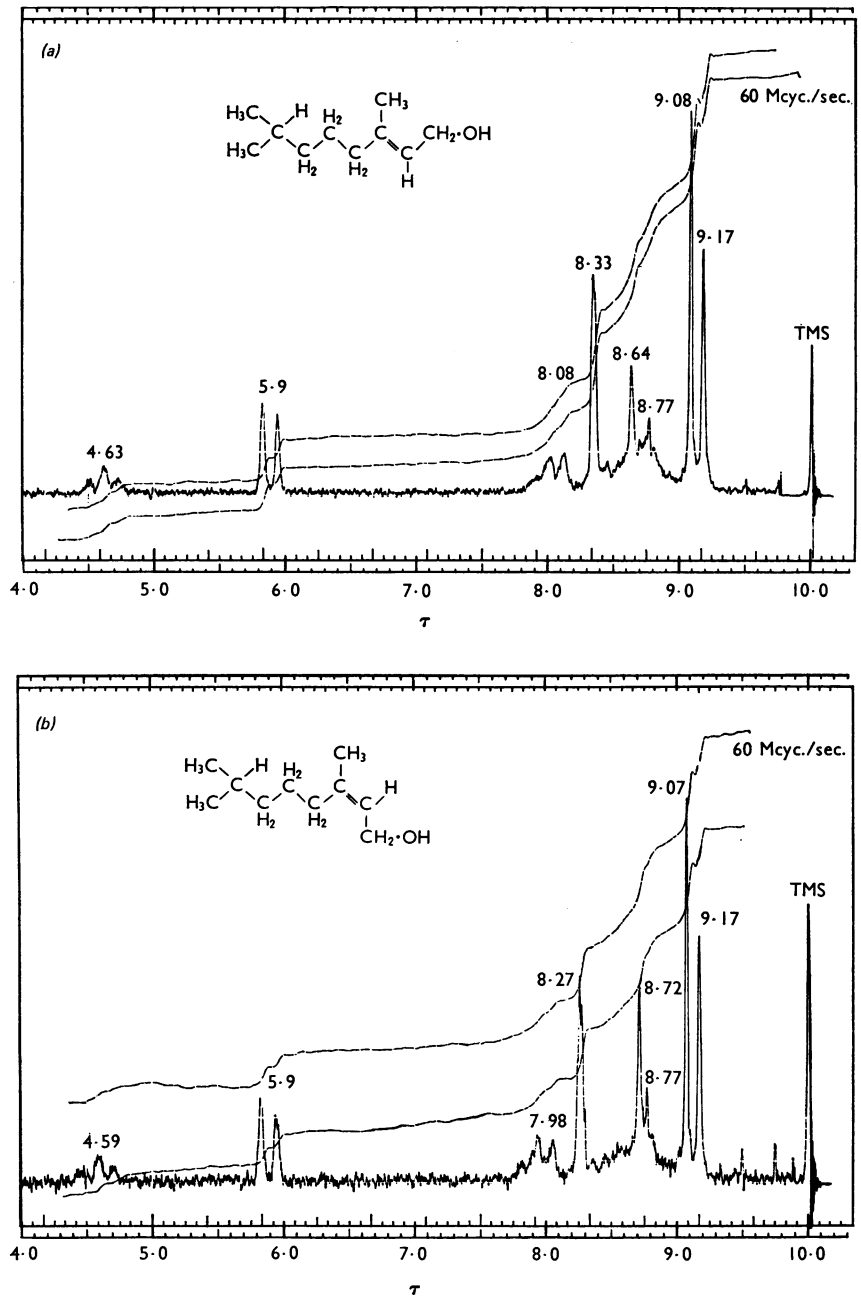


Fig. 2. N.m.r. spectra of (*a*) 6,7-dihydrogeraniol and (*b*) 6,7-dihydronerol. The chemical shifts shown are τ values relative to the absorption line of tetramethylsilane (TMS).

Cornforth, 1960; see also Cornforth, Cornforth, Popják & Yengoyan, 1966). The mass spectra of the two isomers are nearly identical. Fig. 1 shows the mass spectrum of *trans*-6,7-dihydrogeraniol; the main features of this are the finding of the molecular ion at *m/e* 156, the presence of fragments at *m/e* 138, 125 and 31 ($[M-18]^+$, $[M-31]^+$ and $[CH_2{\cdot}OH]^+$, ions characteristic of a primary alcohol), and the

base peak at *m/e* 71 instead of at *m/e* 69 as found, for instance, in geraniol and farnesol or, indeed, in any polyprenyl substance containing one double bond in each prenyl unit. Also, a peculiar feature of the spectrum of the dihydrogeraniol is the relatively intense peak at *m/e* 123, which, judging from the presence of a metastable ion at *m/e* 109·6, must have arisen from the $[M-18]^+$ ion by the loss of a methyl group. Fragments of this nature are of very weak intensity in the spectra of either geraniol or farnesol.

Figs. 2(*a*) and 2(*b*) are the n.m.r. spectra of 6,7-dihydrogeraniol and of 6,7-dihydronerol; these spectra are consistent with the assigned structures and show only the small differences expected in the chemical shifts of the allylic methylene (τ 8·27 in the *cis* and 8·33 in the *trans* isomer) and olefinic methyl protons (τ 8·72 in the *cis* and 8·64 in the *trans* isomer), and of the olefinic proton (τ 4·59 in the *cis* and τ 4·63 in the *trans* isomer).

Table 1. *6,7-Dihydrogeranyl pyrophosphate as substrate for prenyltransferase*

The incubations (1 ml.) contained: tris–HCl buffer, pH 7·9, 20 μmoles; $MgCl_2$, 5 μmoles; [^{14}C]isopentenyl pyrophosphate (11 000 disintegrations/min./mμmole), 1·14 mμmoles; prenyltransferase, 25 μg. (0·6 unit). The allyl pyrophosphates were added as shown. After 2 min. at 37° the reaction mixtures were analysed as in the standard prenyltransferase assay (Holloway & Popják, 1967).

Allyl pyrophosphate added and concn. (μM)	Observed reaction rate (mμmoles/min./mg.)
None	0·0
Geranyl pyrophosphate (3·0)	21·5
6,7-Dihydrogeranyl pyrophosphate (5·2)	9·7

6,7-Dihydrogeranyl pyrophosphate as an alternative substrate for prenyltransferase. Several analogues of geranyl pyrophosphate, such as geranyl monophosphate and the pyrophosphates of nerol, citronellol and of 6,7-dihydrogeraniol (Popják *et al.* 1969), were tested in the presence of [^{14}C]isopentenyl pyrophosphate as alternative substrates for prenyltransferase. We noted the formation of an allyl pyrophosphate (in the absence of geranyl pyrophosphate) only from dihydrogeranyl pyrophosphate. Table 1 records one of the earliest experiments. Since the enzyme preparation was free of isopentenyl pyrophosphate isomerase, the

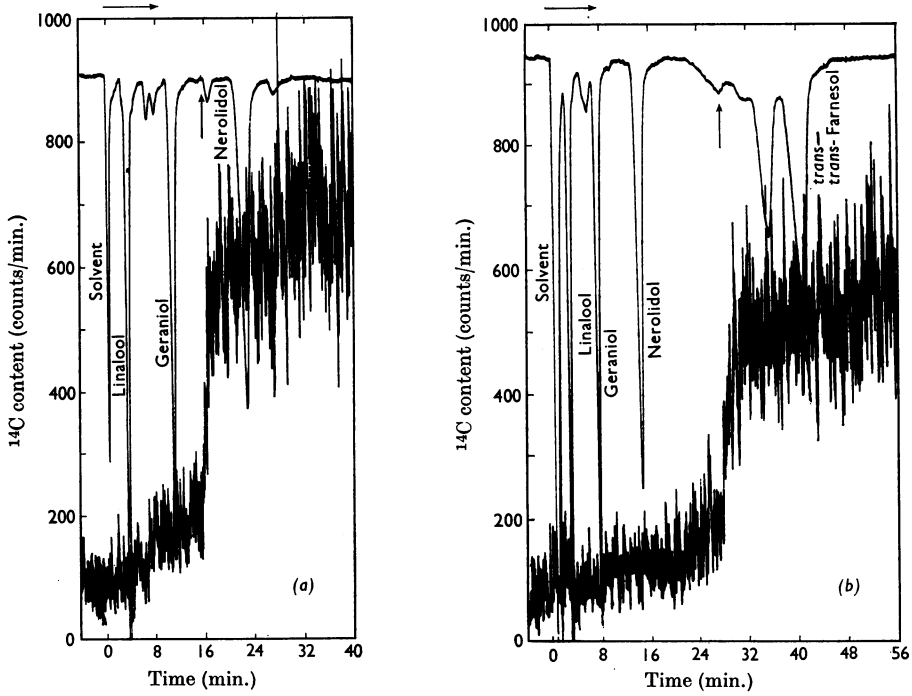


Fig. 3. Gas–liquid radiochromatograms of the ^{14}C-labelled substance released (*a*) by acid and (*b*) by alkaline phosphatase from the product of the reaction between 6,7-dihydrogeranyl pyrophosphate and [^{14}C] isopentenyl pyrophosphate. The experimental sample was chromatographed together with a mixture of prenols. The radioactive fraction (indicated by ↑) is thought to be 10,11-dihydronerolidol in (*a*) and 10,11-dihydrofarnesol in (*b*).

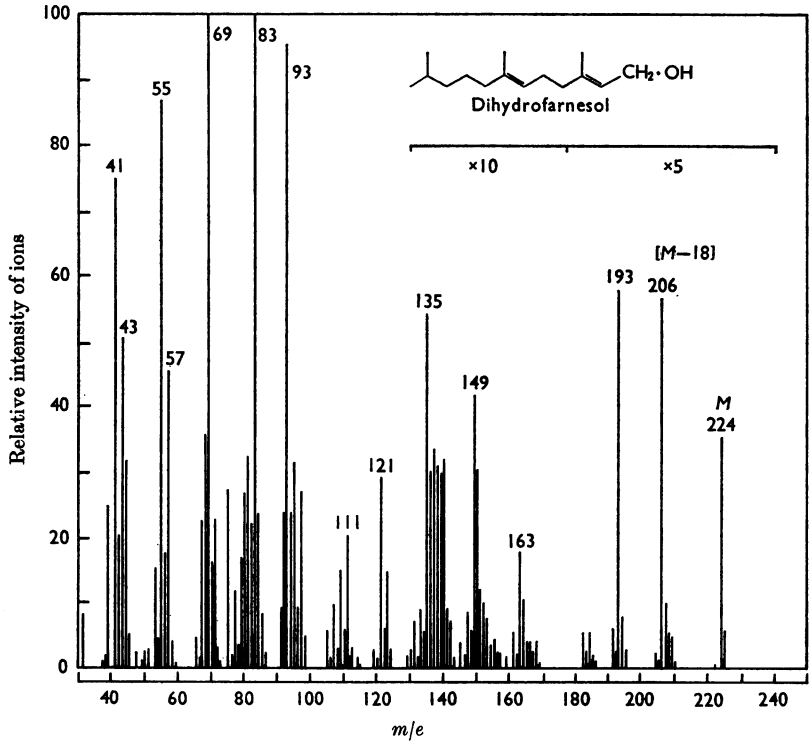


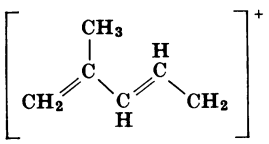
Fig. 4. Mass spectrum of 10,11-dihydrofarnesol. The intensity of ions between *m/e* 129 and 175 has been multiplied by 10 and of those above *m/e* 175 by 5.

finding of radioactivity in the light-petroleum extract of the acidified incubation mixture containing dihydrogeranyl pyrophosphate and [^{14}C]-isopentenyl pyrophosphate indicated that dihydrogeranyl pyrophosphate must have acted as an alternative substrate for the enzyme.

Product of the transferase reaction with 6,7-dihydrogeranyl pyrophosphate as substrate. The product of the reaction between 6,7-dihydrogeranyl pyrophosphate and [4-^{14}C]isopentenyl pyrophosphate, after hydrolysis either by acid or by alkaline phosphatase (see the Materials and Methods section; Expt. *A*) was analysed by gas–liquid radiochromatography (Figs. 3*a* and 3*b*). The radioactive product extracted after acid hydrolysis had a retention volume of 0·7 relative to that of nerolidol (Fig. 3*a*) and the one extracted after hydrolysis with phosphatase had a retention volume of 0·7 relative to that of *trans–trans*-farnesol (Fig. 3*b*). The corresponding radioactive products from the parallel incubations with geranyl pyrophosphate had a retention volume, as expected, coinciding exactly with nerolidol and with *trans–trans*-farnesol respectively (see Goodman & Popják, 1960).

Since we knew already that the retention volume of dihydrogeraniol was less than that of geraniol, it was a reasonable assumption to make that the product of the condensation of 6,7-dihydrogeranyl pyrophosphate with isopentenyl pyrophosphate was 10,11-dihydrofarnesyl pyrophosphate, which gave after acid hydrolysis, by allylic rearrangement (Popják, 1959), 10,11-dihydronerolidol, and after hydrolysis by alkaline phosphatase 10,11-dihydrofarnesol. This was proved by Expt. *B* (cf. the Materials and Methods section). The amount of ^{14}C extracted with *n*-pentane from the incubation mixture of Expt. *B*, after hydrolysis with phosphatase, 138000 disintegrations/min., was equivalent to 0·9 μmole of isopentenyl pyrophosphate. Gas–liquid-chromatographic analysis of the pentane extract showed only one radioactive component with a retention volume of 0·7 relative to that of *trans–trans*-farnesol; [^{14}C]isopentenol was absent. Thin-layer chromatography of the extract (see the Materials and Methods section) showed also only one radioactive component (R_F 0·21), but several fluorescing substances as well, which were separated completely from the radioactive material by repeating the chromatography twice. Based on the

radioactivity of the specimen eluted from the third thin-layer-chromatography step, only 0·3 μmole was left for further analysis. The mass spectrum of this substance is shown in Fig. 4; it is compatible with the structure of 10,11-dihydrofarnesol. The molecular ion was found at *m/e* 224 (in the spectrum of farnesol this is at *m/e* 222) with fragments at $[M-18]^+$ and $[M-31]^+$. The $[M-(18+15)]^+$ ion, which is very pronounced in the spectrum of 6,7-dihydrogeraniol, is weak (though recognizable) in this spectrum. The ion at *m/e* 135 arises from the allylic cleavage of the terminal saturated prenyl unit from the $[M-18]^+$ ion, i.e. $[M-(18+71)]$. Correspondingly, there is a fairly intense ion found at *m/e* 71 which is very weak in the spectrum of farnesol. Two fragments of identical intensity, at *m/e* 69 and 83, form the base peaks of the spectrum. The peak at *m/e* 69 is the characteristic base peak of prenyl substances containing more than one unsaturated prenyl unit. In the unsaturated polyprenols and polyprenyls, such as geraniol, farnesol or squalene, this base peak is associated with an intense ion at *m/e* 81; in this spectrum this is found at *m/e* 83. In the absence of information from the analysis of farnesol and of other polyprenyl substances labelled with deuterium in selected positions we are unable to offer concrete evidence about the nature of the fragment found at *m/e* 81 in unsaturated polyprenyls and at *m/e* 83 in the substance under investigation. Only the structure (I) of a methylpentadiene ion seems compatible with the fragment at *m/e* 81; correspondingly a methylpentene ion could account for the peak at *m/e* 83. Although there are some uncertainties in the interpretation of this spectrum, there is very little doubt that the substance isolated is 10,11-dihydrofarnesol.



(I)

Kinetic experiments with 6,7-dihydrogeranyl pyrophosphate. The experiment designed for the determination of the value of K_m for dihydrogeranyl pyrophosphate and the $V_{max.}$ for the artificial substrate, described in the Materials and Methods section, gave the results shown in Table 2. These data, analysed as described by Holloway & Popják (1967) for prenyltransferase, gave K_m values (means ± S.E.M.) of 0·97 ± 0·28 μM for isopentenyl pyrophosphate and 1·11 ± 0·19 μM for 6,7-dihydrogeranyl pyrophosphate. The $V_{max.}$ calculated for the reaction between dihydrogeranyl pyrophosphate and isopentenyl pyrophosphate was 26·0 ± 1·5 mμmoles/min./mg. compared with 98 ± 3 mμmoles/min./mg. determined with geranyl pyrophosphate for the same enzyme.

DISCUSSION

In the next paper (Popják *et al.* 1969) the effects of analogues of geranyl pyrophosphate on the prenyltransferase reaction are recorded. Some of these analogues, as well as the 6,7-dihydrogeranyl pyrophosphate, were tested as possible alternative substrates of prenyltransferase. The failure of these, in contrast with 6,7-dihydrogeranyl pyrophosphate, to react with isopentenyl pyrophosphate in the presence of liver prenyltransferase, established that both the allylic pyrophosphate structure and the *trans* configuration of the allylic bond were essential features of an effective substrate for this enzyme.

Since we have never observed the synthesis of a polyprenyl substance other than farnesyl pyrophosphate from the normal substrates with the purified liver prenyltransferase, the question arose: what factor(s) terminate the synthesis at the triprenyl stage? It was possible, for example, that the enzyme might be 'coded' by the number and position of double bonds in the alkyl chain; if such

Table 2. *Results of a kinetic experiment with 6,7-dihydrogeranyl pyrophosphate for the determination of K_m and V_{max}.*

The horizontal rows give values of initial reaction rates (v) with four fixed concentrations of dihydrogeranyl pyrophosphate; the four right-hand columns, when read from the top downwards, give values of v with four fixed concentrations of isopentenyl pyrophosphate and varied concentrations of dihydrogeranyl pyrophosphate. The data were analysed by the computer programme of Cleland (1963).

	Observed v (mμmoles/min./mg.)			
Concn. of isopentenyl pyrophosphate (μM)	1·14	1·90	3·00	5·70
Concn. of dihydrogeranyl pyrophosphate (μM)				
1·04	3·4	5·7	7·2	8·6
1·95	5·1	8·5	11·1	12·7
3·00	7·1	10·4	12·4	14·4
5·85	9·1	11·6	14·6	17·3

were the case the dihydrogeranyl pyrophosphate might have led to the synthesis not only of dihydrotriprenyl but also of dihydrotetraprenyl pyrophosphate. There is little doubt that the hydrolysis of dihydrofarnesyl pyrophosphate by acid resulted in the formation of dihydronerolidol (Fig. 3*a*) by allylic rearrangement just as nerolidol is formed from farnesyl pyrophosphate (Popják, 1959). If dihydrotetraprenyl pyrophosphate had been synthesized, by the further elongation of the dihydrofarnesyl pyrophosphate, its hydrolysis by acid should have given dihydrogeranyl-linalool, which would have been detectable on our gas–liquid radiochromatograms at a retention volume (R_v) approximately five times that of dihydronerolidol (cf. Popják & Cornforth, 1960). We found no radioactivity in the analysis of the acid-hydrolysis product synthesized from dihydrogeranyl pyrophosphate and [^{14}C]isopentenyl pyrophosphate at up to six times the R_v of the radioactive fraction appearing before nerolidol, the fraction attributed to dihydronerolidol. Hence it is not the number of double bonds but the number of prenyl units in the allyl pyrophosphate that determines the chain length of the polyprenyl residue synthesized. This restriction might be imposed by the size and conformation of lipophilic groups on the enzyme that bind the prenyl residue of the allyl pyrophosphate.

The value of K_m for dihydrogeranyl pyrophosphate, $1{\cdot}11 \pm 0{\cdot}19\,\mu$M, is significantly lower than that for geranyl pyrophosphate, $4{\cdot}34 \pm 1{\cdot}71\,\mu$M. However, the $V_{max.}$ is only about one-fourth of that observed with the natural substrate. The binding of isopentenyl pyrophosphate does not seem to be affected by the unnatural substrate, as the value of K_m for isopentenyl pyrophosphate determined here, $0{\cdot}97 \pm 0{\cdot}28\,\mu$M, does not differ significantly from the previously determined value of $1{\cdot}45 \pm 0{\cdot}12\,\mu$M.

We are indebted to Mrs R. H. Cornforth for her advice on the Wittig synthesis, to Mr R. Mallaby for a gift of the diethyl ethoxycarbonylmethyl phosphonate, to Mr V. Williams for the mass spectra and to Mr D. Green for the n.m.r. spectra.

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