The Purification of 3,3-Dimethylallyl- and Geranyl-Transferase and of Isopentenyl Pyrophosphate Isomerase from Pig Liver

By P. W. HOLLOWAY AND G. POPJÁK

'Shell' Research Ltd., Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent

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The enzyme catalysing the synthesis of farnesyl pyrophosphate from dimethylallyl pyrophosphate and isopentenyl pyrophosphate, or from geranyl pyrophosphate and isopentenyl pyrophosphate, has been purified 100-fold from homogenates of pig liver. The enzyme has optimum pH 7.9 and requires Mg^{2+} as activator in preference to Mn^{2+} ; it is inhibited by iodoacetamide, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate and phosphate ions in addition to the products of the reaction, inorganic pyrophosphate and farnesyl pyrophosphate. From product-inhibition studies of the geranyltransferase reaction, the order of addition of substrates to and release of products from the enzyme has been deduced: geranyl pyrophosphate combines with the enzyme first, followed by isopentenyl pyrophosphate. Farnesyl pyrophosphate dissociates from the enzyme before inorganic pyrophosphate. The existence of isopentenyl pyrophosphate isomerase in liver is confirmed. Methods for the preparation of the pyrophosphate esters of isopentenol, 3,3-dimethylallyl alcohol, geraniol and farnesol are also described.

It was shown by Cornforth, Cornforth, Donniger & Popják (1966a) that the condensation of a prenyl pyrophosphate, such as 3,3-dimethylallyl pyrophosphate or geranyl pyrophosphate, with isopentenyl pyrophosphate (3-methylbut-3-en-1-yl pyrophosphate) by a soluble enzyme system from liver is a bimolecular nucleophilic substitution, an S_N2 reaction, as it occurs with an inversion of configuration at the pyrophosphate-bearing carbon atom of the prenyl pyrophosphate. Farnesyl pyrophosphate is synthesized by two such consecutive reactions catalysed respectively by dimethylallyltransferase and geranyltransferase, which are called here collectively prenyltransferases.

It was shown further that the addition of the prenyl residue of dimethylallyl pyrophosphate or geranyl pyrophosphate to the double bond of isopentenyl pyrophosphate was most probably a two-step reaction involving first a *trans*-addition of the prenyl residue and of a nucleophile, X^- , to the double bond, followed by a *trans*-elimination of X^- and of the *R*-hydrogen from C-2 of the intermediary product of synthesis (Cornforth, Cornforth, Popják & Yengoyan, 1966b).

The chemistry of these biosynthetic reactions having been defined, it was decided to elucidate further their mechanism by enzyme kinetic studies; this was the main objective of the work described below.

It was known at the start of our work that the high-speed supernatant from liver homogenates contained all the enzymes needed for the conversion of mevalonate into farnesyl pyrophosphate, and that all these enzymes were precipitated by ammonium sulphate between 30 and 60% saturation (Popják, 1959; Goodman & Popják, 1960). For the kinetic experiments with the prenyltransferases it was necessary to remove interfering enzymes, especially isopentenyl pyrophosphate isomerase, which converts isopentenyl pyrophosphate into 3,3-dimethylallyl pyrophosphate (Agranoff, Eggerer, Henning & Lynen, 1960). The transferases were recognized first in yeast autolysates and purified about 20-fold by Lynen, Agranoff, Eggerer, Henning & Möslin (1959), who named the enzyme(s) 'farnesyl pyrophosphate synthetase'. While our work was in progress, Shah, Cleland & Porter (1965) described the isolation of isopentenyl pyrophosphate isomerase, and Benedict, Kett & Porter (1965) the isolation of geranyltransferase from pig liver, which was the source of the enzymes in our work also. Our results have been reported briefly (Holloway & Popják, 1966).

MATERIALS AND METHODS

High-voltage paper electrophoresis. All high-voltage paper-electrophoretic analyses of prenyl phosphates were

carried out on an apparatus manufactured by the Locarte Co., London, W. 14, with a voltage gradient of 72 v/cm. and pyridine-acetic acid-water (25:2:600, by vol.) buffer, pH 6.1. The paper strips used were either Whatman no. 1 or 3MM as specified. The prenyl monophosphates and prenyl pyrophosphates were detected on the paper by spraying the strips first with the acid molybdate reagent of Hanes & Isherwood (1949). The effect of acid at pH 2 and below on the prenyl monophosphates and prenyl pyrophosphates is the rapid liberation of inorganic orthophosphate and inorganic pyrophosphate respectively. The former gives an immediate yellow colour with molybdate and turns blue after exposure to u.v. light; inorganic pyrophosphate, on the other hand, does not give an immediate colour with the molybdate reagent, but turns slowly blue during exposure to u.v. light. Thus a prenyl monophosphate may be readily distinguished from a prenyl pyrophosphate. When the R_F values of the prenyl monophosphates and prenyl pyrophosphates on paper (or on thin-layer chromatograms) had been established by the above procedure, the compounds were detected as a routine by spraying with aq. 5% (w/v) ascorbic acid solution after the molybdate reagent.

[4- 14 C]isopentenyl pyrophosphate. This was made enzymically from [2- 14 C]mevalonic acid (specific activity 5.03 μ C/ μ mole; supplied by The Radiochemical Centre, Amersham, Bucks.) by the F_3^{80} -enzyme preparation from liver described by Goodman & Popják (1960) for the synthesis of farnesyl pyrophosphate. In this enzyme system, in the presence of 5 mm-iodoacetamide, the reaction of mevalonate proceeds no further than isopentenyl pyrophosphate on account of the inhibition of isopentenyl pyrophosphate isomerase (Agranoff *et al.* 1960; cf. also Cornforth *et al.* 1966b). The reaction mixture for this preparation contained in a final volume of 2 ml.: 18 mg. of F_3^{80} -protein from pig liver, tris-HCl buffer, pH 7.4 (50 mm), ATP (7 mm), $MgCl_2$ (5 mm), $MnCl_2$ (1 mm), RS -[2- 14 C]-mevalonate (3 mm, i.e. a total of 6 μ moles; 30.2 μ C), NaF (10 mm) and iodoacetamide (5 mm). After 2 hr. at 37° 2 ml. of ice-cold 10% (w/v) $HClO_4$ was added and the precipitated protein was centrifuged down. The supernatant was neutralized with N -KOH and the $KClO_4$ removed by centrifuging. The supernatant was freeze-dried and the residue, dissolved in 0.6 ml. of aq. 0.1 N - NH_3 , applied to six strips of Whatman 3MM paper for the separation of the isopentenyl pyrophosphate by high-voltage electrophoresis. The strips were analysed for radioactivity by a Packard model 7200 radiochromatogram scanner (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.): each gave three radioactive spots with electrophoretic mobilities of (a) 0.109, (b) 0.127 and (c) 0.146 mm. min^{-1} vcm $^{-1}$. Component (c), which comprised 18.5% of the total radioactivity (37% of the utilizable mevalonate), was identified as isopentenyl pyrophosphate and was eluted from the appropriate portions of the strips with aq. 0.1 N - NH_3 . The yield based on the radioactivity eluted from the paper was 0.38 μ mole (1.9 μ C), the elution being inefficient.

[1- 14 C]isopentenyl pyrophosphate. [1- 14 C]isopentenol was prepared by the reduction with $LiAlH_4$ of 3-methyl[1- 14 C]-but-3-enoic acid synthesized by the carbonation with $^{14}CO_2$ of methylallylmagnesium chloride in tetrahydrofuran (Kharasch & Fuchs, 1944; Wagner, 1949; Yuan & Bloch, 1959; Eggerer & Lynen, 1960). The labelled alcohol had a specific activity of 1 μ C/ μ mole and was diluted with un-

labelled material to a specific activity of 0.069 μ C/ μ mole from which the [1- 14 C]isopentenyl pyrophosphate (as the trilitium salt) was prepared by our colleague Dr C. Donninger. Briefly, the labelled alcohol was first phosphorylated with $POCl_3$ and pyridine (Foote & Wold, 1963); the resulting monophosphoric acid ester was then converted by the method of Moffatt & Khorana (1961), through the phosphoromorpholidate as intermediate, into the pyrophosphate. High-voltage electrophoresis, as described for the biosynthetic isopentenyl pyrophosphate, showed that the preparation contained 80% of isopentenyl pyrophosphate and 20% of another component that had a mobility of 1.12 relative to isopentenyl pyrophosphate. The preparation did not contain inorganic orthophosphate. On thin-layer (0.25 mm.) plates of silica gel H with propan-1-ol-aq. NH_3 (sp.gr. 0.88)-aq. 1% (w/v) EDTA (6:3:1, by vol.) as developing solvent (Plieninger & Immel, 1965), the specimen showed three ^{14}C -labelled components: isopentenyl pyrophosphate (80%; R_F 0.08), isopentenyl monophosphate (12%; R_F 0.19) and an unidentified substance (8%; R_F 0.25). The preparation was used without further purification as we had no evidence to show that the impurities present acted as enzyme inhibitors. An allowance was made in the calculation of substrate concentrations for the presence of the enzymically unreactive species in the [1- 14 C]isopentenyl pyrophosphate.

Dimethylallyl pyrophosphate. This was made by the phosphorylation of 3,3-dimethylallyl alcohol by the method of Cramer & Böhm (1959) as modified by Popják, Cornforth, Cornforth, Ryhage & Goodman (1962) for the phosphorylation of farnesol. The products from the phosphorylation of 4 m-moles of 3,3-dimethylallyl alcohol were extracted from the acetonitrile solvent into aq. 0.1 N - NH_3 as described by Popják *et al.* (1962). This solution was concentrated on a rotary evaporator to a few millilitres and applied to a column (60 cm. $\times$ 1.3 cm.) of silica gel (Whatman SG31) previously equilibrated with propan-1-ol-aq. NH_3 (sp.gr. 0.88)-aq. 1% (w/v) EDTA (6:3:1, by vol.) (Plieninger & Immel, 1965). The column was eluted with the same solvent system; 3 ml. fractions were collected and analysed for dimethylallyl monophosphate and dimethylallyl pyrophosphate by high-voltage electrophoresis on Whatman no. 1 paper. The fractions containing dimethylallyl pyrophosphate (nos. 35-44, eluted after the monophosphate) were combined and concentrated to 4 ml. Addition of acetone (4 ml.) and ethanol (0.5 ml.) precipitated a crystalline white solid, which was redissolved in 1 ml. of aq. 0.1 N - NH_3 and was recrystallized by the addition of 1 ml. of acetone; the crystallization was repeated once more. The final product, dried over silica gel *in vacuo*, weighed 179 mg.; calculated as the triammonium salt, this corresponds to 0.6 m-mole (15% yield). A sample was analysed for acid-labile phosphate (Goodman & Popják, 1960): it contained 5.78 μ moles/mg., which is 86% of the theoretical required by triammonium dimethylallyl pyrophosphate.

Geranyl pyrophosphate. This was made by the same process as described for dimethylallyl pyrophosphate: 4 m-moles (616 mg.) of geraniol (pure *trans*-isomer; Meranol brand of A. Boake, Roberts and Co. Ltd., London, E. 15) gave 189 mg. of twice-recrystallized triammonium geranyl pyrophosphate (0.52 m-mole; yield 13%). The specimen contained 5.4 μ moles of acid-labile phosphate/mg. (98% of the theoretical required by triammonium geranyl pyrophosphate).

Farnesyl pyrophosphate. *trans-trans*-Farnesol (60mg.; 270 μ moles) was phosphorylated as described by Popják *et al.* (1962), except for the isolation of the products of phosphorylation. The previously described procedure was followed up to the stage of extracting the products from the acetonitrile solvent into dilute aq. NH_3 . Thereafter the isolation of the farnesyl pyrophosphate (see below) was followed by thin-layer chromatography on silica-gel plates (Eastman-Kodak chromatogram sheet type K301, R2) with propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) as developing solvent. On these precoated plates farnesyl monophosphate and farnesyl pyrophosphate have, with the above solvent system, R_F values 0.64 and 0.54 respectively, whereas inorganic orthophosphate and pyrophosphate have R_F less than 0.1. The inorganic salts and organic phosphates from the aq.-ammoniacal extract of the phosphorylation mixture were separated by adsorption and differential elution from Amberlite XAD-2 polystyrene resin (Rohm and Haas, Philadelphia 5, Pa., U.S.A.). The Amberlite XAD-2 resin was prepared by washing it several times alternately with methanolic 0.01N- NH_3 and with aq. 0.01N- NH_3 , the last washing being with the latter. A suspension of the resin in aq. 0.01N- NH_3 was added in portions to the aqueous extract of the products of phosphorylation until, according to analysis on the thin-layer sheets, all the phosphorylated farnesol became adsorbed on the resin; the supernatant was then discarded. The inorganic salts were first removed from the resin by repeated washing with aq. 0.01N- NH_3 ; the farnesyl derivatives were then eluted with methanolic 0.01N- NH_3 , the elution being followed by thin-layer chromatography. The combined methanol extracts (about 1ml.) were applied directly on to a column (65mm. $\times$ 4mm.) of DEAE-cellulose (Whatman DE-11 cellulose) equilibrated with methanol containing ammonium formate (80mm) for the separation of farnesyl monophosphate and farnesyl pyrophosphate (Rilling, 1966). Farnesyl monophosphate was eluted with methanol-80mm-ammonium formate (2ml.) and farnesyl pyrophosphate with methanol-80mm-ammonium formate-0.2N- NH_3 (2ml.). The eluate of farnesyl pyrophosphate was evaporated to dryness *in vacuo* (water pump). The dry residue was dissolved in 0.2ml. of aq. 0.1N- NH_3 and evaporated again; the process was repeated twice more.

The final product was dissolved in 1ml. of 10mm-tris-HCl buffer, pH 8.5. According to analysis for organic acid-labile pyrophosphate (Goodman & Popják, 1960), the preparation contained 20 μ moles of farnesyl pyrophosphate; it was free of inorganic phosphate and farnesyl monophosphate and showed only a single spot (R_F 0.54) on chromatography on the Eastman-Kodak thin-layer sheets as above.

Purification of prenyltransferase(s). Fresh pig liver, chilled in ice, was cut into small pieces and homogenized in 100g. lots in a Waring Blendor for 30sec. with 200ml. of a 0.25M-sucrose solution containing KHCO_3 (25mm) and EDTA (1mm). The homogenate was centrifuged successively at 600g for 15min., at 9800g for 20min. and finally at 45000g for 3hr., the sediment after each centrifuging being discarded. The last supernatant (S_{45}), filtered through cheesecloth to remove floating fat, was the source of the prenyltransferases and of isopentenyl pyrophosphate isomerase.

The S_{45} fraction was fractionated with $(\text{NH}_4)_2\text{SO}_4$ between the following limits of saturation: 0-40, 40-50,

50-60, 60-70, 70-80 and 80-100%. The pH was kept at 7.5 by the addition of aq. NH_3 ; the precipitated protein was collected by centrifuging. Each fraction was dissolved in a small volume of 10mm-tris-HCl buffer, pH 7.7, and dialysed against the same buffer for 3hr.

The protein precipitated between 40 and 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ (F_{50} fraction) was substantially free of the isomerase but contained about one-third of the total transferase units of the original S_{45} fraction. This fraction was further treated for the purification of the transferases as follows.

The pH of the dialysed F_{50} solution was adjusted to 5 with N-acetic acid; after 5min. a copious white precipitate was removed by centrifuging at 2000g for 15min. and the pH of the supernatant brought to 7.5 with N-KOH. A further small precipitate, formed during neutralization, was removed by centrifuging at 105000g for 30min. The supernatant (FH) retained its enzymic activity for several months when kept at -20° .

Samples of FH fraction (approx. 8ml. containing 400mg. of protein) were applied to a column (360mm. $\times$ 25mm.) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated in 10mm-tris-HCl buffer, pH 7.7, and eluted with the same buffer. The column had a void volume of 67ml. as determined with blue dextran (Pharmacia). The transferase had an elution volume of 130ml., the same as that of crystalline bovine serum albumin. The enzyme eluted by the subsequent 16ml. of buffer had the highest specific activity (G-200-1 fraction) and was precipitated by adding the solution slowly to 32ml. of vigorously stirred saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 7.7. After 30min. the precipitate was collected by centrifuging at 5700g for 20min.; the resulting pellet was kept at -20° . The precipitate was dissolved in a small volume of 10mm-tris-HCl buffer, pH 7.7, and dialysed against the same buffer for 3hr. This solution (G-200-1 fraction), free of isomerase, was used for the kinetic and other studies; it retained its enzymic activity for several months when kept at -20° .

The transferase(s) from the G-200-1 fraction were further purified by chromatography on a DEAE-cellulose (Whatman DE-11) column. A sample of G-200-1 fraction (1ml.; 2.8mg. of protein/ml.) was applied to a column (100mm. $\times$ 15mm.) of DEAE-cellulose previously equilibrated with 10mm-tris-HCl buffer, pH 7.7, containing 2-mercaptoethanol (1mm). The column was eluted with the same buffer with successive stepwise increases in NaCl content of 0, 100mm, 150mm and 300mm. Approx. 40ml. of each buffer was run through the column, the changes being made when a protein peak had been completely eluted. The elution of protein from Sephadex and DEAE-cellulose columns was followed with an LKB Uvicord instrument (type 4701A; LKB Produkter AB, Stockholm, Sweden). The protein eluted with buffered 150mm-NaCl had the highest transferase activity ('DEAE' fraction; cf. Table 2). Protein was assayed by the biuret method of Gornall, Bardawill & David (1949).

Assay of dimethylallyl- and geranyl-transferases (EC 2.5.1.1). The product of the reaction catalysed by these enzymes is [^{14}C]farnesyl pyrophosphate, irrespective of whether 3,3-dimethylallyl pyrophosphate + [^{14}C]isopentenyl pyrophosphate or geranyl pyrophosphate + [^{14}C]isopentenyl pyrophosphate are used as substrates. The assay of the enzymes was therefore based on the hydrolysis of farnesyl pyrophosphate in acid at 37° to nerolidol and

farnesol, and the solubility of these alcohols in light petroleum (Lynen *et al.* 1959; Popják, 1959; Goodman & Popják, 1960); ^{14}C was assayed in the light-petroleum extract. In contrast, [^{14}C]isopentenyl pyrophosphate is stable to acid at 37° and, further, even if [^{14}C]isopentenol were liberated in the incubation mixture from the substrate, it is not extracted from an aqueous-ethanolic solution by light petroleum.

During the purification of the enzymes, the geranyltransferase was assayed in 1 ml. incubation mixtures containing 100 μmoles of tris-HCl buffer, pH 7.9, 5 μmoles of MgCl_2 , 50 μmoles of geranyl pyrophosphate, 50 μmoles (7500 disintegrations/min.) of [^{14}C]isopentenyl pyrophosphate and 0.1 mg., or less, of protein. After 5 min. at 37° the reaction was stopped by the addition of 1 ml. of 2N-HCl in 80% (v/v) ethanol. Geraniol, nerolidol and farnesol (1 mg. of each) were added to the mixture, which, after 30 min. at 37° , was made alkaline with 10N-NaOH and extracted three times with light petroleum (b.p. $30-40^\circ$). The combined extracts were dried over anhydrous MgSO_4 and made up to 5 ml. with the solvent, and a sample was assayed for radioactivity.

During the isolation of geranyltransferase, all protein fractions were assayed also for dimethylallyltransferase by the procedure just described for geranyltransferase, except that dimethylallyl pyrophosphate, instead of geranyl pyrophosphate, was used as condensing partner with [^{14}C]isopentenyl pyrophosphate.

One unit of the transferases is defined as that amount of enzyme which, in the presence of dimethylallyl pyrophosphate or geranyl pyrophosphate, converts 1 μmole of [^{14}C]isopentenyl pyrophosphate into farnesyl pyrophosphate/min. All the reaction rates with these enzymes have been calculated in terms of μmoles of isopentenyl pyrophosphate converted into farnesyl pyrophosphate/mg. of protein/min. The specific activity of the enzymes is defined as enzyme units/mg. of protein.

In the kinetic and product-inhibition studies of geranyltransferase, with the G-200-1 enzyme fraction, the routine assays were modified: the 1 ml. incubation mixtures contained 20 μmoles of tris-HCl buffer, pH 7.9, 5 μmoles of MgCl_2 , 12.5 $\mu\text{g.}$ of protein (specific activity 100), and geranyl pyrophosphate and a mixture of [^{14}C] and [^3H]isopentenyl pyrophosphate (800 disintegrations/min./ μmole) in amounts dictated by the nature of the experiment. (The [^{14}C]isopentenyl pyrophosphate was added to increase the specific radioactivity of the substrate in the incubations and hence the accuracy of measurements when very low concentrations of substrate had to be used.) After 1 or 2 min. at 37° the reaction was stopped and the amount of farnesyl pyrophosphate formed was determined as above. During such short incubation times and with the small amounts of enzyme used, less than one-half of the substrates was utilized and the reaction rate remained essentially linear with respect to time; therefore the amount of product formed during the incubation period was taken as a measure of the initial reaction velocity.

The products of the prenyltransferase reaction were identified from larger incubations which contained in 4 ml. 400 μmoles of tris-HCl buffer, pH 7.9, 20 μmoles of MgCl_2 , 4 mg. of protein (specific activity 100), 0.5 μmole of either 3,3-dimethylallyl pyrophosphate or geranyl pyrophosphate and 0.5 μmole (7500 disintegrations/min.) of [^{14}C]isopentenyl pyrophosphate. After 10 min. at 37° the pH

of the mixture was adjusted to 9.5 with N-NaOH and 2 mg. of intestinal alkaline phosphatase (EC 3.1.3.1; supplied by British Drug Houses Ltd., Poole, Dorset), contained in 0.2 ml. of 0.05M-KHCO₃, was added. After 4 hr. at 37° linalool, geraniol, nerolidol and farnesol (2 mg. of each) were added to the incubations, which were then extracted three times with light petroleum (b.p. $30-40^\circ$). The combined extracts were dried over anhydrous MgSO_4 and concentrated under N_2 to a small volume; portions of the extracts were then analysed by gas-liquid radiochromatography (Popják, Lowe & Moore, 1962). The products of the prenyltransferase reaction were also analysed by gas-liquid radiochromatography after acid hydrolysis of the [^{14}C]prenyl pyrophosphate(s) formed, as described for the routine assay of the enzyme. The column used in gas-liquid radiochromatography was 9 ft. long $\times$ 4 mm. diam. packed with Celite (100-120 mesh) coated with 10% Carbowax 20M (Union Carbide Corp., New York, N.Y., U.S.A.).

Assay of isopentenyl pyrophosphate isomerase (EC 5.3.3.2). The activity of the isomerase was measured in all protein fractions by a method identical with the routine assay for the transferases, except that [^{14}C]isopentenyl pyrophosphate was the sole substrate for the reaction. Any radioactivity extractable with light petroleum after acidification of the reaction mixture was a measure of the amount of the substrate isomerized to dimethyl[^{14}C]allyl pyrophosphate, which, in the presence of excess of [^{14}C]isopentenyl pyrophosphate and the prenyltransferases, was converted into farnesyl pyrophosphate. Crude enzyme preparations (S_{45} , F_{50} and F_{60} ; cf. Table 1) contained the transferases in amounts sufficient for this assay, but purified fractions (F_{70} and F_{80} ; cf. Table 1) had to be fortified with the prenyltransferases for the assay to be successful. As a routine 10 units of prenyltransferase were added for each unit of isomerase assayed. The activity of the isomerase is expressed as one-third of the μmoles of [^{14}C]isopentenyl pyrophosphate converted into farnesyl pyrophosphate/mg. of protein/min. in the presence of an excess of prenyltransferases, since only 1 molecule of dimethylallyl pyrophosphate is incorporated into each molecule of farnesyl pyrophosphate.

The identity of the product of the isomerization was established by the following experiment. A 2 ml. reaction mixture containing 200 μmoles of tris-HCl buffer, pH 7.4, 50 μmoles (7500 disintegrations/min.) of [^{14}C]isopentenyl pyrophosphate and 30 mg. of protein (dialysed preparation, F_{70} fraction; cf. Table 1) was used. After 30 min. at 37° the pH was adjusted to 9.5 with N-KOH, and 2 mg. of intestinal alkaline phosphatase, contained in 0.2 ml. of 0.05M-KHCO₃, and 10 μmoles of MgCl_2 (0.1 ml.) were added. After 4 hr. at 37° 2.5 g. of $(\text{NH}_4)_2\text{SO}_4$ was added and the mixture carefully distilled from a micro-distillation apparatus (cf. Cornforth *et al.* 1966b). The distillate (1.5 ml.) was collected in a tube cooled in an ice-salt mixture. A portion of the distillate was assayed for ^{14}C : all the radioactivity added to the incubation was accounted for in the distillate. The remainder of the distillate was saturated with $(\text{NH}_4)_2\text{SO}_4$ and, after addition of 1 mg. each of isopentenol and dimethylallyl alcohol, was extracted at ice temperature three times with 1 ml. of ethyl chloride. Over 90% of the radioactivity added to the original incubation as [^{14}C]isopentenyl pyrophosphate was accounted for in the ethyl chloride extract. After addition of 20 $\mu\text{l.}$ of

benzene, the ethyl chloride was evaporated off at room temperature and a sample of the remaining benzene solution was analysed by gas-liquid radiochromatography. The efficiency of measuring ^{14}C in conjunction with the gas-liquid chromatography was 40%.

Assay of ^{14}C . A Packard Tri-Carb model 314 EX scintillation spectrometer (Packard Instrument Co. Inc.) was used as a routine for assays of ^{14}C with an efficiency of approx. 70%. The standard scintillator solution contained 0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene. For assays of ^{14}C in water the scintillator solution was diluted with ethanol in the ratio 7:3 (v/v); 10 ml. of this diluted scintillator could accept up to 0.2 ml. of an aqueous solution and give a 40% efficiency of counting. The counting efficiencies with both scintillators were checked with $[^{14}\text{C}]$ toluene of known specific activity. The standard error of counting never exceeded $\pm 3\%$.

RESULTS

Purification of prenyltransferases and isopentenyl pyrophosphate isomerase. The results shown in Table 1 illustrate the separation of the transferases and isopentenyl pyrophosphate isomerase by ammonium sulphate fractionation of the S_{45}

supernatant of pig-liver homogenates. Although the F_{50} and F_{60} fractions contained the bulk of the transferases, only the former was used for further purification of these enzymes because the latter contained much of the isomerase as well. The transferase activity of the F_{70} fractions from various preparations was so feeble that its isomerase content could be assayed by the method described only after addition of purified transferase.

The results of the further purification of the F_{50} fraction, as outlined in the Materials and Methods section, are shown in Table 2. Neither the G-200-1 fraction nor the protein after DEAE-cellulose chromatography contained any isomerase. The overall purification achieved was 100-fold.

Although the purification procedure was designed for the geranyltransferase, it was found that the preparations were as active with dimethylallyl pyrophosphate as with geranyl pyrophosphate at all stages of purification (Table 3). The possible significance of this observation is discussed below.

Products of reactions catalysed by isopentenyl pyrophosphate isomerase and by prenyltransferase.

Table 1. *Isolation of prenyltransferase(s) and isopentenyl pyrophosphate isomerase from extracts of pig liver by ammonium sulphate fractionation*

The starting material for the fractionation was the supernatant of homogenates centrifuged at 45000g for 3 hr. (S_{45}). The fractions shown in the left-hand column were those obtained between the specified percentage saturations with ammonium sulphate. The conditions of assays were those described in the Materials and Methods section, except that the 1 ml. incubation mixtures contained 50 μmoles each of dimethylallyl pyrophosphate and geranyl pyrophosphate as well as of $[^{14}\text{C}]$ isopentenyl pyrophosphate.

Ammonium sulphate fraction	Total protein (mg.)	Prenyltransferase		Isomerase	
		Specific activity	Total enzyme units	Specific activity	Total enzyme units
Unfractionated S_{45}	730	3.5	2550	0.3	219
0-40% (F_{40})	136	1.0	136	0.0	0
40-50% (F_{50})	108	9.0	970	0.3	32
50-60% (F_{60})	128	8.5	1090	0.8	102
60-70% (F_{70})	84	1.0	84	1.2	101
70-80%	36	0.5	18	0.5	18
80-100%	24	0.0	0	0.0	0

Table 2. *Purification of geranyltransferase*

The details of the purification procedures are described in the Materials and Methods section, where the symbols for the fractions are also defined.

Fraction	Total protein (mg.)	Specific activity	Total enzyme units
S_{45}	20800	1.5	31200
F_{50}	1710	7.5	12800
FH	1450	7.5	10900
G-200-1	100	39.0	3900
'DEAE'	11	152.0	1670

Table 3. *Dimethylallyl- and geranyl-transferase activities of prenyltransferase preparations during various stages of purification*

The fractions used in Expt. 1 were obtained as described in the Materials and Methods section (cf. also Table 2). The 'DEAE' fraction used in Expt. 2 was the protein eluted from a DEAE-cellulose column with 10mm-tris-HCl buffer, pH 7.7, containing NaCl (200mm, not 150mm as described in the Materials and Methods section) and 2-mercaptoethanol (1mm). Dimethylallyltransferase was assayed with dimethylallyl pyrophosphate + [1-¹⁴C]-isopentenyl pyrophosphate; geranyltransferase was assayed with geranyl pyrophosphate + [1-¹⁴C]isopentenyl pyrophosphate.

Expt. no.	Fraction	Dimethylallyl-transferase specific activity	Geranyl-transferase specific activity
1	S ₄₅	1.5	1.5
	F ₅₀	6.0	6.0
	FH	7.0	6.0
	G-200-1	20.0	20.0
2	FH	35.0	46.0
	'DEAE'	74.0	97.0

Fig. 1. Gas-liquid-radiochromatographic analysis of product of isopentenyl pyrophosphate isomerase. The details of the experiment are described in the Materials and Methods section. The analysis was done at 85°. The simultaneous recording of the response of the mass detector and of the scintillation counter was taken with a multi-channel recorder, the base lines of the two galvanometers being on opposite sides of the chart. The appearance of radioactivity coincided with the mass peaks of isopentenol and dimethylallyl alcohol.

The experiment designed to demonstrate the product of isopentenyl pyrophosphate-isomerase catalysis was described in the Materials and Methods section. Here it is shown (Fig. 1) that in the particular experiment about one-half of the [1-¹⁴C]isopentenyl pyrophosphate added to the incubation mixture was converted into dimethylallyl pyrophosphate. This follows from the observations that (a) all the radioactivity added to the reaction mixture as [1-¹⁴C]isopentenyl pyrophosphate was found in the distillate after treatment of the enzyme incubation with phosphatase, and (b) that no other radioactive substance but isopentenol and 3,3-dimethylallyl alcohol was found in the preparation. Thus we can confirm the existence of isopentenyl pyrophosphate isomerase in liver, shown by Shah *et al.* (1965), as in yeast (Agranoff *et al.* 1960).

The nature of the product of the reaction catalysed by the prenyltransferase was established by previously published procedures (Popják, 1959; Goodman & Popják, 1960). Gas-liquid radiochromatography of the alcohols formed when incubation mixtures of prenyltransferase, containing at the start dimethylallyl pyrophosphate or geranyl pyrophosphate and [1-¹⁴C]isopentenyl pyrophosphate, were treated (a) with acid or (b) with alkaline phosphatase, revealed in (a) mostly radioactive nerolidol and a little farnesol, and in (b) radioactive *trans-trans*-farnesol, all the radioactivity of the preparations, except for a small unidentified component emerging between linalool and geraniol, being accounted for in farnesol. Fig. 2 shows the analysis of the products of prenyltransferase reaction, after hydrolysis with phosphatase, when dimethylallyl pyrophosphate and [1-¹⁴C]isopentenyl pyrophosphate were the substrates; there was no detectable trace of [1-¹⁴C]-geraniol. Records identical with this were obtained when geranyl pyrophosphate replaced dimethylallyl pyrophosphate in the reaction. The nature of

Fig. 2. Gas-liquid-radiochromatographic analysis of the products of the prenyltransferase reaction when dimethylallyl pyrophosphate and [^{14}C]isopentenyl pyrophosphate were the substrates. The experimental details are described in the Materials and Methods section. The analysis was done at 195° . After the emergence of the first radioactive fraction between linalool and geraniol, the range of the radioactivity detector was changed to lower sensitivity. About 90% of the radioactivity coincided with the mass peak of *trans-trans*-farnesol.

the radioactive fraction (or possibly two unresolved fractions) emerging between linalool and geraniol is not known, but the same component was seen after the action of phosphatase on pure [^{14}C]-farnesyl pyrophosphate.

General properties of prenyltransferase. Although all our transferase preparations were as active with dimethylallyl pyrophosphate as with geranyl pyrophosphate, the detailed studies of the enzyme (general properties, kinetics) were made only with geranyl pyrophosphate as joint substrate with [^{14}C]isopentenyl pyrophosphate. For this reason we refer below to 'geranyltransferase' alone.

The pH optimum of the transferase was 7.9–8.0 as measured in four buffer systems (Fig. 3), although in 100mM-tris-hydrochloric acid buffers nearly identical reaction rates were found between pH 7.5 and 8.5. Phosphate buffers were unsuitable for the study of this enzyme: 100mM-phosphate buffer

caused a 90% inhibition even near the pH optimum of the enzyme (cf. Fig. 3). Geranyltransferase has an absolute requirement for Mg^{2+} , Mn^{2+} being an inferior substitute (Fig. 4).

In addition to orthophosphate and inorganic pyrophosphate (see below), thiol reagents inhibited the enzyme also: 80% inhibition was observed after preincubation of the enzyme at 37° for 5 min. with 2mM-iodoacetamide, 5 μM -*N*-ethylmaleimide or 0.5 μM -*p*-hydroxymercuribenzoate. The inhibition caused by the last reagent was completely reversed by subsequent addition of 2-mercaptoethanol (1mM).

Kinetic studies with geranyltransferase. (a) Determination of K_m values. The K_m values for geranyl pyrophosphate (substrate A) and isopentenyl pyrophosphate (substrate B) were determined by extrapolation procedures essentially by the method of Florini & Vestling (1957). For this

Fig. 3. Effect of pH on the activity of geranyltransferase. Each 1 ml. incubation mixture contained buffer (100 mM), MgCl_2 (10 mM), geranyl pyrophosphate (100 μM), [^{14}C]isopentenyl pyrophosphate (100 μM) and 50 μg . of protein (fraction G-200-1; specific activity 40). After 5 min. at 37° the incubations were analysed for [^{14}C]farnesyl pyrophosphate. The buffers used were: ●, sodium maleate; ○, tris maleate; □, tris-HCl; △, $\text{NH}_3\text{-NH}_4\text{Cl}$; ▽, $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$.

purpose initial reaction velocities of the geranyltransferase reaction were determined at four different fixed concentrations of each substrate, while the concentration of the other was varied. With the aid of the results so obtained and a computer programme kindly provided by Professor W. W. Cleland (cf. Cleland, 1963a), the maximum reaction velocities, V , belonging to each fixed concentration of, e.g., substrate B were calculated from the Michaelis equation (1), where K'_a is the apparent K for substrate A at one concentration of substrate B, $[\text{S}_1]$ is the concentration of the varied substrate A and v is the observed initial reaction velocity. The four values of V were then fitted by another computer programme to eqn. (2), where $[\text{S}_2]$ is the concentration of substrate B. The ratio of the two constants a and c in eqn. (2) gives the value of K_m for substrate B. The value of K_m for substrate A was found similarly.

$$v = \frac{V[\text{S}_1]}{K'_a + [\text{S}_1]} \quad (1)$$

$$\frac{1}{V} = \frac{a}{[\text{S}_2]} + c \quad (2)$$

Although computer programmes are not essential for the evaluation of these data, they have the advantage that personal bias of plotted data is eliminated and that the standard error of the computations becomes readily available.

The primary data fed into the computer, values

Fig. 4. Effects of Mg^{2+} and Mn^{2+} on the activity of geranyltransferase. Each 1 ml. incubation mixture contained tris-HCl buffer, pH 7.9 (10 mM), geranyl pyrophosphate (50 μM), [^{14}C]isopentenyl pyrophosphate (50 μM), 25 μg . of protein (specific activity 80) and either MgCl_2 (○) or MnCl_2 (□), in the concentrations shown. The incubations were made at 37° for 5 min.

of initial reaction velocities, v , observed at four fixed concentrations of substrates B and A, and four varied concentrations of substrates A and B respectively, pertaining to each fixed concentration of the other substrate, are shown in Table 4.

The values of K_m obtained from these experimental data, together with their standard errors, were $4.34 \pm 1.71 \mu\text{M}$ for geranyl pyrophosphate and $1.45 \pm 0.117 \mu\text{M}$ for isopentenyl pyrophosphate.

(b) Analysis of reaction mechanism of geranyltransferase by product-inhibition studies. Cleland has discussed in detail the theory of inhibition of enzyme-catalysed reactions by their products (Cleland, 1963c) and how the patterns of product inhibition may be used to deduce the mechanism of reactions (Cleland, 1963b,d).

We have carried out four sets of experiments in which inorganic pyrophosphate, shown by Lynen *et al.* (1959) to be one of the products of the geranyltransferase reaction, and farnesyl pyrophosphate were tested as inhibitors. Initial reaction velocities were measured, in the presence of one or other product at various concentrations, with one of the two substrates of geranyltransferase kept at a fixed concentration while the concentration of the other was being varied. The experiments and their results are set out in Figs. 5-7, in which the reciprocals of the observed initial velocities are plotted

Table 4. Data used with a computer programme of Cleland (1963a) for the calculation of the values of K_m of geranyl pyrophosphate and isopentenyl pyrophosphate in the geranyltransferase reaction

Initial reaction rates, v , were determined in 1 min. incubations as described in the Materials and Methods section. The horizontal lines of the Table give values of v pertaining to four fixed concentrations of geranyl pyrophosphate and varied concentrations of isopentenyl pyrophosphate; the four right-hand columns, when read from the top downwards, give the values of v related to four fixed concentrations of isopentenyl pyrophosphate and varied concentrations of geranyl pyrophosphate.

Concn. of substrate A (geranyl pyrophosphate) (μM)	Concn. of substrate B (isopentenyl pyrophosphate) (μM)			
	0.86	1.43	2.28	4.29
	Observed v ($\text{m}\mu\text{moles/min.}$)			
(i) 3.0	24.5	31.9	34.0	39.1
(ii) 4.0	28.0	34.0	39.3	43.1
(iii) 5.0	28.6	36.9	43.7	48.5
(iv) 6.5	30.4	39.2	43.0	52.4

Fig. 5. Effects of farnesyl pyrophosphate on initial velocities, v , of the geranyltransferase reaction. In (a) the concentration of geranyl pyrophosphate was varied and that of [^{14}C]isopentenyl pyrophosphate (specific radioactivity 800 disintegrations/min./m-mole) kept constant at $0.9\mu\text{M}$. In (b) the concentration of geranyl pyrophosphate was constant at $3\mu\text{M}$, the concentration of [^{14}C]isopentenyl pyrophosphate being varied. In both experiments the concentrations of added farnesyl pyrophosphate were: $\circ$, none; $\square$, $2\mu\text{M}$; ∇ , $4\mu\text{M}$; $\bullet$, $8\mu\text{M}$. The other conditions of the 1 ml. incubation mixtures are described in the Materials and Methods section. The incubations were made at 37° for 2 min.

against the reciprocals of the concentrations of the varied substrate. As discussed in more detail below, we interpret the results as indicating that farnesyl pyrophosphate behaved as a non-competitive inhibitor whether geranyl pyrophosphate or isopentenyl pyrophosphate was the varied substrate (Figs. 5a and 5b). Inorganic pyrophosphate, on the other hand, was a competitive inhibitor with respect to geranyl pyrophosphate (Figs. 6 and 7a) and a non-competitive inhibitor with respect to isopentenyl pyrophosphate (Fig. 7b).

DISCUSSION

The main object of our work was the study of the mechanism of the geranyltransferase reaction.

Kinetic experiments alone can distinguish between various enzyme mechanisms involving more than one substrate and product, but for such analyses data of higher precision than our assays permitted are needed. We had to rely on the determination of the products formed in brief incubations by chemical manipulations, followed by assay of ^{14}C , as a measure of initial reaction rates. Such a procedure is less accurate than, e.g., a continuous spectrophotometric assay; its errors are reflected in the standard error of the K_m values for the two substrates (about $\pm 30\%$ for the K_m of geranyl pyrophosphate and about $\pm 10\%$ for that of isopentenyl pyrophosphate). Because of the limitations of the assays of enzymic activity we relied on product-inhibition studies for deducing the mechanism of

Fig. 6. Effects of inorganic pyrophosphate on initial velocities, v , of the geranyltransferase reaction at varying concentrations of geranyl pyrophosphate and a fixed concentration ($1.0 \mu\text{M}$) of $[^{14}\text{C}]$ isopentenyl pyrophosphate. Owing to the very low concentrations of geranyl pyrophosphate used, and hence low reaction rates in this experiment, 5 ml. incubation mixtures were set up to provide amounts of product sufficient for assay. The reaction mixtures contained, in addition to the substrates: $20 \mu\text{g}$. of protein/ml. (specific activity 75), tris-HCl buffer, pH 7.9 (100 mm), and MgCl_2 (4 mm). The concentrations of added inorganic pyrophosphate were: $\circ$, none; $\square$, 0.5 mM ; $\triangle$, 1.0 mM . The incubations were made at 37° for 2 min.

the geranyltransferase reaction. Cleland (1963b,c,d) has pointed out that the pattern of inhibition of enzymic reactions by their products is characteristic of the mechanism. Figs. 5–7 show that both farnesyl pyrophosphate and inorganic pyrophosphate inhibited the geranyltransferase reaction. When farnesyl pyrophosphate was the inhibitor, the vertical intercepts as well as the slopes of the lines representing the plot of $1/v$ against $1/[\text{S}]$ were affected, irrespective of whether geranyl pyrophosphate or isopentenyl pyrophosphate was the varied substrate; the lines representing these plots intersected, within experimental error, at a point to the left of the ordinate and above the abscissa (Figs. 5a and 5b). The plots of the vertical intercepts, or of the slopes of these lines, against the inhibitor (farnesyl pyrophosphate) concentrations were found to be linear. Hence we conclude that farnesyl pyrophosphate acted as a non-competitive inhibitor with respect to both substrates of geranyltransferase.

When inorganic pyrophosphate was the inhibitor and isopentenyl pyrophosphate the varied substrate (Fig. 7b), again both the vertical intercepts and the slopes of the lines of the $1/v$ versus $1/[\text{S}]$ plots were affected; thus the inhibition in this instance is also non-competitive.

As is seen from Figs. 6 and 7(a), inorganic pyrophosphate behaved differently when geranyl pyrophosphate was the varied substrate. In this instance the lines of the $1/v$ versus $1/[\text{S}]$ plots

Fig. 7. Effects of inorganic pyrophosphate on initial velocities, v , of the geranyltransferase reaction. In (a) the experiment was similar to that of Fig. 6, except that the concentration of $[^{14}\text{C}]$ isopentenyl pyrophosphate was kept constant at $1.8 \mu\text{M}$ and that the concentrations of geranyl pyrophosphate were varied at a higher level than in the experiment of Fig. 6. In (b) $[^{14}\text{C}]$ isopentenyl pyrophosphate was the varied substrate, the concentration of geranyl pyrophosphate being kept constant at $3 \mu\text{M}$. The 1 ml. incubation mixtures had the same general composition as that of the experiments of Figs. 5(a) and 5(b). The concentrations of added inorganic pyrophosphate were: $\circ$, none; $\square$, 0.5 mM ; ∇ , 0.7 mM ; $\triangle$, 1.0 mM ; $\bullet$, 1.5 mM .

Scheme 1. Mechanism I.

intersected, within experimental error, on the ordinate and only the slopes of the lines were markedly affected, a condition characteristic of competitive inhibition.

The pattern of product inhibition observed is compatible with only two of the eight mechanisms listed by Cleland for enzymic reactions with two substrates and two products (cf. Table II in Cleland, 1963b). One of these, called 'Iso Ping Pong Bi Bi' by Cleland (1963b), would require, apart from an isomerization of the enzyme, that the substrate which combines first with the enzyme should be isopentenyl pyrophosphate and that the pyrophosphate released in the geranyltransferase reaction is derived from isopentenyl pyrophosphate and not from geranyl pyrophosphate. The latter requirement conflicts with the known chemistry of the reaction (cf. Cornforth *et al.* 1966b). Thus we are led to consider in detail only one mechanism for the geranyltransferase reaction, which is in accord, not only with the pattern of product inhibition, but also with the chemistry of the reaction. This mechanism (I), called 'Ordered Bi Bi' by Cleland (1963b), is shown in Scheme 1, where E is the enzyme, S_1 and S_2 are the two substrates and P and Q are the products of the reaction; the k values are velocity constants.

We are proposing the formation of a central complex (ES_1S_2-EPQ), which is required by the previous chemical studies of the prenyltransferase reaction (Cornforth *et al.* 1966b). This central complex represents the postulated intermediate of the condensation product of a prenyl pyrophosphate with isopentenyl pyrophosphate and stabilized by the addition of a nucleophilic group, X^- , to C-3 as shown in formula (I).

(I: R = dimethylallyl, or geranyl)

Our conclusion is that S_1 is geranyl pyrophosphate, S_2 is isopentenyl pyrophosphate, P is farnesyl pyrophosphate and Q is inorganic pyrophosphate.

These conclusions follow from a consideration of the rate equation for mechanism I (cf. Cleland, 1963b).

In eqns. (3), (4), (5) and (6) below, the symbols are:

$$f_1 = \frac{K_{ia}}{K_a}, f_2 = \frac{K_{ip}}{K_p}, f_3 = \frac{K_{iq}}{K_q}$$

$[S_1]$ and $[S_2]$ are the concentrations of the varied substrate; $\sigma_1 = K_a/[S_1]$ when S_1 is the fixed substrate and $\sigma_2 = K_s/[S_2]$ when S_2 is the fixed substrate. $[P]$ and $[Q]$ are the concentrations of the first and second product respectively dissociating from the enzyme. K_a, K_s, K_p, K_q are Michaelis constants and K_{ia}, K_{ib}, K_{ip} and K_{iq} are 'inhibition constants' defined by Cleland (1963b). In terms of the velocity constants of mechanism I:

$$\begin{aligned} K_a &= \frac{k_5 k_7}{k_1 (k_5 + k_7)}, & K_s &= \frac{(k_4 + k_5) k_7}{(k_5 + k_7) k_3}, \\ K_p &= \frac{(k_4 + k_5) k_2}{(k_2 + k_4) k_6}, & K_q &= \frac{k_2 k_4}{(k_2 + k_4) k_8}, \\ K_{ia} &= \frac{k_2}{k_1}, & K_{ib} &= \frac{k_2 + k_4}{k_3}, \\ K_{ip} &= \frac{k_5 + k_4}{k_6} & \text{and} & \quad K_{iq} = \frac{k_7}{k_8} \end{aligned}$$

It can be shown that the reciprocals of the initial reaction velocities, v , for mechanism I, when the product, P, dissociating first from the enzyme is used as inhibitor and S_1 and S_2 are the varied substrates in turn, are given by eqns. (3) and (4) respectively:

$$\frac{1}{v} = \frac{K_a}{V} \left(1 + f_1 \sigma_2 + \frac{[P]/f_3 K_p}{f_1 \sigma_2} \right) \frac{1}{[S_1]} + \frac{1}{V} \left(1 + \sigma_2 + \frac{[P]/(f_3 + f_2 \sigma_2)}{f_2 f_3 K_p} \right) \quad (3)$$

$$\frac{1}{v} = \frac{K_b}{V} (1 + f_1 \sigma_1) \left(1 + \frac{[P]}{K_p f_3} \right) \frac{1}{[S_2]} + \frac{1}{V} \left(1 + \sigma_1 + \frac{[P]}{f_2 K_p} \right) \quad (4)$$

Both the vertical intercept (when $1/[S_1]$ or $1/[S_2] = 0$) and the slopes of eqns. (3) and (4) are a linear function of $[P]$, the product we have equated with farnesyl pyrophosphate. The experimental observations are in accord with the deductions that follow from eqns. (3) and (4), i.e. that product P should act as a non-competitive inhibitor whether S_1 or S_2 is the substrate the concentration of which is being varied.

When Q, the last product to dissociate from the enzyme, is used as inhibitor, and S_1 or S_2 are the varied substrates, $1/v$ is represented by eqns. (5) and (6):

$$\frac{1}{v} = \frac{K_s}{V} (1 + f_1 \sigma_2) \left(1 + \frac{[Q]}{K_{iq}} \right) \frac{1}{[S_1]} + \frac{1}{V} (1 + \sigma_2) \quad (5)$$

$$\frac{1}{v} = \frac{K_b}{V} \left(1 + f_1 \sigma_1 + \frac{[Q] f_1 \sigma_1}{K_{iq}} \right) \frac{1}{[S_2]} + \frac{1}{V} \left(1 + \sigma_1 + \frac{[Q] \sigma_1}{K_{iq}} \right) \quad (6)$$

Eqns. (5) and (6) predict that product Q will act as a competitive inhibitor when S_1 is the varied substrate, since the vertical intercept of the plot of $1/v$ against $1/[S_1]$ is independent of the concentration of Q, but that it will behave as a non-competitive inhibitor when S_2 is the varied substrate at non-saturating (fixed) concentrations of S_1 , i.e. when $\sigma_1 = K_a/[S_1] > 0$. The observations made with inorganic pyrophosphate as an inhibitor are broadly in accord with these predictions, as it behaved as a competitive inhibitor when geranyl pyrophosphate was the varied substrate and as a non-competitive inhibitor when the concentration of isopentenyl pyrophosphate was varied.

In eqn. (5) the slope of the plot of $1/v$ against $1/[S_1]$ and in eqn. (6) both the slope and intercept are linear functions of $[Q]$. However, the plots of the slopes of the lines in Figs. 6 and 7(a) and of the slopes and intercepts of the lines in Fig. 7(b) against the concentrations of inorganic pyrophosphate turned out to be parabolic functions of inorganic pyrophosphate concentration. Therefore, if inorganic pyrophosphate is Q, in the expressions for the slope in eqn. (5) and for both the slope and intercept in eqn. (6), a term of the type $(a + b[Q] + c[Q]^2)$ should appear. This kind of relationship is expected (cf. Cleland, 1963c,d) when the inhibitor reacts with more than one form of the enzyme. It is relevant, though, that the inhibitory concentrations of inorganic pyrophosphate used were very large in comparison with substrate concentrations. The experimental results suggest that the 'parabolic' nature of inhibition by inorganic pyrophosphate would not have been apparent at concentrations below 1 mM.

An alternative sequential mechanism II (Scheme 2) for the geranyltransferase reaction, named 'Ping

Pong Bi Bi' by Cleland (1963b), which might have been considered for the geranyltransferase reaction, is excluded, not only by the product inhibition pattern, but also by previous chemical studies of the reaction between a prenyl pyrophosphate and isopentenyl pyrophosphate.

The rate equations for mechanism II (not presented) predict that product P should behave as a non-competitive inhibitor when S_1 is the varied substrate at non-saturating (fixed) concentrations of S_2 , and as a competitive inhibitor when S_2 is the varied substrate at all (fixed) concentrations of S_1 . Product Q, on the other hand, should be a competitive inhibitor when S_1 is the varied substrate at all (fixed) concentrations of S_2 and a non-competitive inhibitor when S_2 is the varied substrate at unsaturating concentrations of S_1 . This pattern of inhibition is different from that observed.

Mechanism II differs from mechanism I in that one of the two products of the reaction is formed and released from the enzyme before a modified form (F) of the enzyme combines with the second substrate. With geranyltransferase, this could have meant (a) the initial alkylation of the enzyme with the geranyl residue of geranyl pyrophosphate and release of inorganic pyrophosphate, followed by (b) the reaction of the alkylated form of the enzyme with isopentenyl pyrophosphate. Such a mechanism was suggested by Bloch (1965) in his Nobel Lecture. Reactions (a) and (b) would necessarily be nucleophilic substitutions of the S_N2 type, each resulting in an inversion of configuration at C-1 (the pyrophosphate-bearing carbon atom) of the geranyl residue; in the final product the configuration of the hydrogen atoms (distinguishable by isotopic labelling) at this carbon centre should then be the same as at C-1 of the starting geranyl pyrophosphate. It was shown (Cornforth *et al.* 1966a) that in farnesyl pyrophosphate the configuration at the carbon atom derived from C-1 of geranyl pyrophosphate was epimeric with the configuration at C-1 of the geranyl pyrophosphate used in the reaction as substrate. We are thus satisfied that the deductions based on product-inhibition studies of the geranyltransferase reaction are in harmony with the conclusions of the previous chemical investigations of this reaction.

It is difficult to comment on some of the differences between our observations and those of Benedict *et al.* (1965) on the general properties of geranyltransferase, since these workers have done

Scheme 2. Mechanism II.

Scheme 3. Scheme of binding sites of substrates on prenyl transferase (see the Discussion section).

all their measurements in a phosphate buffer (50mM), not realizing that phosphate ions inhibited the reaction. This may account for Benedict *et al.* (1965) finding Mn^{2+} rather than Mg^{2+} as the preferred cation in the reaction, and also that their preparations, purified only about 15-fold, were inactive with dimethylallyl pyrophosphate. In our preparations, even after 100-fold purification, the dimethylallyltransferase/geranyltransferase activity ratio remained unaltered. Lynen *et al.* (1959) have also found their preparations of 'farnesyl pyrophosphate synthetase' made from yeast autolysates to be equally active with dimethylallyl pyrophosphate and geranyl pyrophosphate.

Previous chemical studies of the stereochemistry of farnesyl pyrophosphate biosynthesis (Cornforth *et al.* 1966a,b) have shown that the mechanism of the carbon-to-carbon bond formation was the same for the synthesis of the diprenyl pyrophosphate as well as for the triprenyl pyrophosphate. This fact, taken together with our inability to separate the geranyltransferase from dimethylallyltransferase even after 100-fold purification of the former, suggests the attractive hypothesis that these two catalytic activities are the properties of one protein. This idea requires that the enzyme can bind either one molecule of dimethylallyl pyrophosphate and two of isopentenyl pyrophosphate, or one molecule of geranyl pyrophosphate and one of isopentenyl pyrophosphate. A linear, and schematic, representation of these ideas is given in Scheme 3. Since it was shown (Cornforth *et al.* 1966b) that the addition of the prenyl residue (dimethylallyl or geranyl) to the double bond of isopentenyl pyrophosphate occurs from a plane on which the $-CH_2 \cdot CH_2 \cdot O \cdot PO_3H \cdot PO_3H_2$, $-CH_3$ and $=CH_2$ groups of isopentenyl pyrophosphate appear in a clockwise order, we imagine that the binding of dimethylallyl pyrophosphate (or of geranyl pyrophosphate) and isopentenyl pyrophosphate may occur on two sides of a narrow groove in the

Fig. 8. Alignment of dimethylallyl pyrophosphate and isopentenyl pyrophosphate required for linear transition state during their condensation by an S_N2 reaction. The molecular models are viewed from the direction of the pyrophosphate groups, from above and slightly from the right.

enzyme. One side could be lipophilic and the other contain the binding sites for the polar groups of the prenyl pyrophosphate. Since, according to the product-inhibition studies, isopentenyl pyrophosphate is bound to the enzyme after geranyl pyrophosphate, it is plausible to suppose that the binding of the latter is assisted by the pyrophosphate group of the first substrate already bound to the enzyme. These ideas are supported by the examination of molecular models. The alignment of, e.g., dimethylallyl pyrophosphate and of

isopentenyl pyrophosphate required for the linear transition state in an S_N2 reaction between the two substrates is shown in Fig. 8; interaction between the pyrophosphate groups is clearly possible and may be enhanced by co-ordination by Mg^{2+} .

The inhibition of the geranyltransferase reaction by iodoacetamide, *N*-ethylmaleimide and *p*-hydroxymercuribenzoate suggests the involvement of a thiol group in the catalytic reaction. It is possible that the nucleophilic group, X^- , required for the stabilization of the intermediary product of condensation, is a thiol group of the enzyme. The inhibition of the liver geranyltransferase by iodoacetamide is in contrast with the resistance of the 'farnesyl pyrophosphate synthetase' from yeast to this reagent (Lynen *et al.* 1959).

At the beginning of this paper we used the term prenyl pyrophosphate to describe collectively dimethylallyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate, and also the term prenyltransferase for dimethylallyltransferase and geranyltransferase. This terminology follows a previous proposal (Popják & Cornforth, 1960) that the terpene alcohols should be called prenols with the prefix mono-, di-, tri- (or poly- etc.), according to the number of isoprenoid units they contain. For the liver enzyme studied here, the term 'mono-prenyl- and diprenyl-transferase A' is suggested, to distinguish it from bacterial or plant enzymes that synthesize polyprenyl pyrophosphates with a chain length longer than that of farnesyl pyrophosphate.

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