The Partial Purification, Properties, and Mechanism of Action of Pig Liver Isopentenyl Pyrophosphate Isomerase*

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Numerous reports on the intermediate reactions in the biosynthesis of terpenoids were published after the characterization of mevalonic acid (1, 2) and the establishment of its conversion to squalene (3-8), cholesterol (9, 10), o xoacetone (11-14), and rubber (15-17). These reports established the conversion of mevalonic acid to mevalonic 5-phosphate, mevalonic pyrophosphate, and $\Delta^2$-isopentenyl pyrophosphate (18-26). They also established isopentenyl pyrophosphate as the biologically active isoprene unit, as suggested by Ruzicka (27).

Proof of the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate has provided a substrate for the initial condensation reaction in the formation of terpenyl pyrophosphates (28). The condensation of these $C_8$ units yields geranyl pyrophosphate. Further condensations of terpenyl pyrophosphates then result in the formation of farnesyl and geranylgeranyl pyrophosphates.

The isopentenyl pyrophosphate isomerase of yeast has been isolated by Agranoff et al. (29), and some of the properties of this enzyme have been reported. A mechanism for the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate has also been proposed. However, experimental proof for this proposal is still lacking.

The present paper reports the partial purification of the isopentenyl pyrophosphate isomerase from a mammalian source (pig liver) and some of the properties of this enzyme. Experiments with tritiated water and $^{14}$C-labeled isopentenyl pyrophosphate are also reported. These experiments provide proof of the mechanism of the isomerization reaction. In this reaction a proton is accepted by carbon atom 4 of isopentenyl pyrophosphate and the resultant carbonium ion is then stabilized, possibly through reaction with a thiol group of the enzyme. Subsequent deprotonation at carbon atom 2, and breakage of the thioether bond, result in the formation of dimethylallyl pyrophosphate.

EXPERIMENTAL PROCEDURE

Chemicals—Pig livers were obtained as a gift from Oscar Mayer and Company, Madison, Wisconsin. $\text{dl-Mevalonic acid}$ was purchased from Mann Research Laboratories, and mevalonic acid-$2^{14}$C was obtained as the lactone from the Volk Chemical Company. Stock solutions of these compounds were prepared through treatment with excess KOH, followed by neutralization with acid to pH 8.0. ATP, glutathione, and $\text{p-hydroxymercuribenzoate}$ were purchased from the Sigma Chemical Company, and imidazole, $\beta$, $\beta$-dimethylallyl chloride, and 2-mercaptoethanol were products of Eastman Organic Chemicals. Iodoacetamide and trimethyl phosphate were supplied by K and K Laboratories. EDTA was purchased from Fisher Scientific Company, and N-ethylmaleimide was obtained from Schwarz Bio-Research, Inc. 3,3'-Dimethylacrylic acid was furnished by Aldrich Chemical Company, and tritium oxide was obtained from New England Nuclear Corporation. Calcium phosphate gel, for enzyme purification, was prepared according to the method of Keilin and Hartree (30), and DEAE-cellulose was purchased from Bio-Rad Laboratories. Snake venom diesterase (Naja naja) was obtained from Ross Allen's Reptile Institute, and alkaline phosphatase was secured from Worthington Biochemical Corporation.

Synthesis of Isopentenol and Dimethylallyl Alcohol—Isopentenoic acid was synthesized from methallyl chloride according to the method of Wagner (31). The resultant isopentenonic acid and commercial dimethylacrylic acid were then each reduced with LiAlH$_4$ (32) to yield isopentenol and dimethylallyl alcohol, respectively. Each alcohol was distilled under reduced pressure (32) and then each was analyzed for purity by gas-liquid chromatography. No impurities were found in either compound.

Preparation of Pig Liver Enzyme System—Pig liver was cut into small pieces and homogenized for 7 seconds in a Waring Blender with 2 volumes of 0.1 m phosphate buffer, pH 7.4, containing $\text{MgCl}_2$, 0.004 M; nicotinamide, 0.034 M; FAD, 0.001 M; and GSH, 0.0025 M. The homogenate was filtered through cheesecloth and then centrifuged successively at 600 $\times$ g for 20 minutes, 18,000 $\times$ g for 30 minutes, and 144,000 $\times$ g for 45 minutes. Protein of the supernatant solution was fractionated by neutralization with acid to pH 8.0. ATP, glutathione, and $\text{Fe}^{2+}$, $\text{Mg}^{2+}$, $\text{Ni}^{2+}$, and $\text{Zn}^{2+}$ were used for enzyme activity measurements.

Preparation of Enzyme for Biosynthesis of Isopentenyl Pyrophosphate—The dialyzed solution of pig liver soluble enzymes was supplemented with tritiated water and 14C-labeled isopentenyl pyrophosphate.
(35 to 65% fraction), obtained as described above, was treated
with calcium phosphate gel at a gel to protein ratio of 1:1. The
adsorbed protein was eluted with an equal volume of 0.05 M
phosphate buffer, pH 7.0, and the resultant solution was dialyzed
against 0.005 M phosphate buffer, pH 7.0, containing 0.001 M
2-mercaptoethanol. This protein was used for the biosynthesis
of isopentenyl pyrophosphate. The protein not adsorbed on
the calcium phosphate gel was purified further, as will be de-
scribed later, for isopentenyl pyrophosphate isomerase activity.
The protein concentrations of all solutions were determined
by the biuret method of Gornall, Bardawill, and David (33).

**Biosynthesis of Isopentenyl Pyrophosphate**—Biosynthesis of
isopentenyl pyrophosphate-4-14C was carried out by incubation
of dl-mevalonic acid-2-14C, 0.1 μmole (2.25 × 10^6 c.p.m. per
μmole); nonradioactive dl-mevalonic acid, 1.9 μmoles; ATP,
20 μmoles; MgCl₂, 12 μmoles; KF, 30 μmoles; iodoacetamide,
6.0 μmoles; imidazole buffer, pH 7.0, 50 μmoles; and enzyme, 2
mg of protein, in a volume of 1.0 ml at 37° for 3 hours. The
reaction was terminated by heating the mixture at 70° for 3
minutes. The heat-denatured protein was then removed by
centrifugation. EDTA, 20 μmoles, pH 7.0, was added to the
denatured protein and the mixture was incubated for 1 hour at
37°. The protein was again removed by centrifugation, and
the supernatant solution was combined with the solution ob-
tained on centrifugation of the initial incubation mixture. The
biosynthetic isopentenyl pyrophosphate was purified on a Dowex
1 formate column according to the method of Bloch et al. (24).
The fractions collected from the column were assayed for radio-
activity, either with a Nuclear-Chicago thin end window gas
scintillation spectrometer. The fractions containing isopentenyl pyro-
phosphate were combined and treated with Dowex 50W to remove
ammonium ions. Formic acid was removed by repeated lyoph-
ilization to dryness, and then the residual isopentenyl pyro-
phosphate was dissolved in water and assayed for purity. When
a higher specific radioactivity of isopentenyl pyrophosphate was
desired, nonradioactive mevalonic acid was omitted from the
reaction mixture.

Isopentenyl pyrophosphate was identified by paper chroma-
tography on acid, alkali, and EDTA-washed (34) Whatman
No. 3MM paper in a solvent system of 1-propanol, ammonia,
and water (60:30:10). Radioactivity was detected on the
chromatogram with a Vanguard automatic chromatogram scanner.
The Rf value of isopentenyl pyrophosphate was 0.44 to 0.49.
After isopentenyl pyrophosphate was eluted from the
paper, an aliquot was cleaved with snake venom diesterase (Naja
naja), 2 mg of protein, in 50 μmoles of Tris buffer, pH 9.2, by
incubation for 3 hours at 37°. The enzymatically liberated iso-
pentenol was extracted with methyl ether, then concentrated to
a small volume, and then identified by gas-liquid
chromatography. All of the radioactivity emerged from the
gas chromatogram with carrier isopentenol.

**Assay for Isopentenyl Pyrophosphate Isomerase Activity**—Isop-
entenyl pyrophosphate-4-14C, 150 μmoles (14,400 c.p.m.),
was incubated with Tris buffer, pH 8.0, 50 μmoles; MgCl₂, 8
μmoles; KF, 12 μmoles; GSH, 5.0 μmoles; and enzyme (25 μg to
2.0 mg of protein) for 10 minutes at 37°. When more purified
enzyme preparations were used, KF was omitted. The reaction
was terminated at the end of the incubation period by the addi-
tion of 0.2 ml of 1 N HCl. The reaction mixture was incubated
for 10 minutes at 37° to cleave the allylic pyrophosphate, and
the liberated terpenols were extracted with four 3-ml portions of
diethyl ether. The ether extracts were combined and then
dried over MgSO₄, and an aliquot was counted for radioactivity.
When more than half of the substrate was consumed, the assay
was repeated with less enzyme, more substrate, or a decreased
incubation time.

The dimethylallyl alcohol liberated by the above procedure
rearranges to the tertiary alcohol, dimethylvinylearbinol, in the
presence of acid. Whenever the unarranged product, dimeth-
yllial alcohol, was desired, the terpenyl pyrophosphate was
cleaved with alkaline phosphatase. In this case the reaction
was carried out as reported above, but at the end of the incuba-
tion period the pH was adjusted to 9.2 with Tris buffer. Then
2 mg of alkaline phosphatase were added, and the reaction mix-
ture was incubated at 37° for 1 hour. At the end of the incuba-
tion period the liberated alcohols were extracted with diethyl
ether, concentrated to a small volume, and separated on vapor
phase chromatography as described earlier (Fig. 1).

**Partial Purification of Isopentenyl Pyrophosphate Isomerase**—
The supernatant solution obtained by the previously described
gel treatment of the protein was dialyzed against 0.002 M
phosphate buffer, pH 6.5, containing 0.001 M mercaptoethanol, for
6 to 8 hours, with one change of buffer at 4 hours. The protein
was then added to a DEAE-cellulose column that had been
previously equilibrated with 0.002 M phosphate buffer, pH 6.5.
The chromatogram was washed successively with 0.005 M and
0.025 \text{ M} \text{ phosphate buffer, pH 6.5. Isopentenyl pyrophosphate isomerase activity was present in the last portion of protein eluted with 0.025 \text{ M} \text{ buffer (Fig. 2). This protein was routinely stored on Dry Ice under nitrogen.}

Biosynthesis and Isolation of Dimethylallyl Pyrophosphate—Dimethylallyl pyrophosphate was formed on incubation of isopentenyl pyrophosphate-4-^{14}C, 156 mmoles (14,400 c.p.m.), with Tris buffer, pH 8.0, 100 \mu \text{moles; MgCl}_2, 10 \mu \text{moles; and isopentenyl pyrophosphate isomerase, 1.6 mg of protein, at 37^\circ \text{C} for 1 hour. At the end of the incubation period the reaction mixture was lyophilized to dryness. The residue was extracted with 80\% \text{ alcohol containing 2\% ammonia, and the extracted pyrophosphates were chromatographed on Whatman No. 1 paper (34) with a solvent system of 1-propanol, ammonia, and water 60:20:20). Radioactivity was detected with a Vanguard chromatogram scanner (Fig. 3). The } R_f \text{ values obtained were .42 to 0.48 for isopentenyl pyrophosphate and 0.33 to 0.36 for dimethylallyl pyrophosphate. The } R_f \text{ values varied slightly with the lot of ammonia used.}

Identification of Dimethylallyl Alcohol—Dimethylallyl pyrophosphate, either isolated from paper as mentioned above or present in the incubation mixture, was cleaved with alkaline phosphatase. The liberated alcohol was removed by extraction with ethyl ether, the solution was concentrated, and the alcohol was subjected to gas chromatography. A retention time of 38 minutes was obtained (Fig. 1). The alcohol was trapped on ether-moistened glass wool in a T-tube in a Dry Ice-acetone bath. The alcohol was then eluted with ethyl ether, and a portion of the eluate was assayed for radioactivity. The radioactivity coincided with carrier dimethylallyl alcohol when chromatographically pure dimethylallyl pyrophosphate was used. However, when the contents of the incubation mixture were cleaved directly with alkaline phosphatase, two radioactive peaks, coinciding with isopentenyl and dimethylallyl alcohols, were observed. Carrier dimethylallyl alcohol, 82 mg, was added to the remaining portion of the dimethylallyl alcohol-4-^{14}C separated by gas liquid chromatography, and the 3,5-dinitrobenzoyl derivative was made (35). The crude ester was recrystallized from aqueous alcohol to constant specific radioactivity. The melting point of the ester was 69-71\degree (Table I).

Conversion of Dimethylallyl Pyrophosphate to Farnesyl Pyrophosphate—Dimethylallyl pyrophosphate-4-^{14}C, 43 \text{ mmoles (110,000 c.p.m.), isolated on paper as described earlier, was incubated with 4 mg of crude hog liver soluble protein (35 to 65\% fraction); Tris-buffer, pH 8.0, 50 \mu \text{moles; MgCl}_2, 5 \mu \text{moles;...}}
At the end of the incubation period the reaction mixture was lyophilized and the dry residue was extracted with 80% alcohol containing 2% ammonia. The alcoholic extract was placed on Whatman No. 3MM paper (34) and chromatographed in a solvent system containing 1-propanol, ammonia, and water (60:30:10). The paper was scanned for radioactivity with a Vanguard automatic chromatogram scanner.

**Incorporation of Tritium into Isopentenyl and Dimethylallyl Pyrophosphates on Incubation of Isopentenyl Pyrophosphate with Isomerase—**Isopentenyl pyrophosphate-4-14C, 255 mpmoles (1233 c.p.m. per mpmole); Tris buffer, pH 7.4, 10 μmoles; MgCl₂, 1.6 μmoles; GSH, 1.0 μmole; and isopentenyl pyrophosphate isomerase, 0.58 mg of protein, were lyophilized to dryness. Then 0.2 ml of tritiated water (4000 c.p.m. per μm-molar) was added, and the solution was incubated at 37°. At intervals of 5, 10, 15, 20, 30, 45, and 60 minutes, 0.025-ml aliquots were removed and lyophilized to dryness. The residue was repeatedly lyophilized after the addition of water to remove any traces of contaminating tritium oxide. Tris buffer, pH 9.2, 100 μmole; MgCl₂, 10 μmoles; and alkaline phosphatase, 1.0 mg of protein, were then added. The volume was made to 1.0 ml and the tubes were incubated at 37° for 3 hours. The enzymatically liberated isopentenol and dimethylallyl alcohols were extracted with ether, washed with water saturated with ether to remove exchangeable tritium, and dried over MgSO₄. The alcohols were separated by gas-liquid chromatography. The trapped alcohols were assayed for their content of 3H and 14C. The 3H:14C ratio was then calculated for each compound.

**Incorporation of Tritium into Isopentenyl and Dimethylallyl Pyrophosphates at Equilibrium—**The experimental conditions were the same as those described in the previous section, except that the reaction was allowed to reach equilibrium by incubation for 3 hours. Fresh enzyme was then added, and the incubation mixture was lyophilized to dryness. Tritiated water, 0.2 ml, was added, and 0.025-ml aliquots were withdrawn at the end of 5, 10, 15, 20, 30, 45, 60, 90, and 120-minute intervals. Tritiated water and exchangeable tritium were removed as described earlier. The samples withdrawn after 10, 30, 60, and 120 minutes were treated with alkaline phosphatase, and the terpenols were separated by gas chromatography as indicated in the previous experiment. Aliquots removed after 5, 15, 45, and 90 minutes were acid-hydrolyzed and then extracted with ethyl ether to remove dimethylvinylcarbinol. The ether extracts were washed with water saturated with ether and dried over MgSO₄, and aliquots were counted for 3H and 14C contents. The acid-stable isopentenyl pyrophosphate left in the aqueous phase was cleaved with alkaline phosphatase, and the alcohol was extracted, washed, and assayed as described earlier. The 3H:14C ratios were then determined for each compound.

**Determination of Location and Quantity of Tritium Incorporated into Isopentenyl and Dimethylallyl Pyrophosphates**—Isopentenyl pyrophosphate-4-14C, 604 mpmoles (825 c.p.m. per mpmole); imidazole buffer, pH 7.2, 50 μmoles; MgCl₂, 4 μmoles; GSH, 5 μmoles; and protein, 0.5 mg, were incubated at 37° in a volume of 0.5 ml for 4 hours. Then 0.5 ml of tritiated water (19,570 c.p.m. per μm-molar) was added, and 0.2-ml aliquots were removed at intervals of 2, 6, 10, 20, and 30 minutes. The aliquots were treated with alkaline phosphatase, and the liberated alcohols were subjected to gas chromatography, as mentioned earlier, to obtain isopentenol and dimethylallyl alcohol. An aliquot of each of these alcohols was assayed for radioactivity to determine the 3H:14C ratio. To each of the remaining radioactive alcohols, 82 mg of either carrier isopentenol or dimethylallyl alcohol were added. Each was then subjected to cleavage by ozonolysis according to the method of Knowles and Thompson (36) (Fig. 4).

The formaldehyde formed from isopentenol was distilled into a solution of 10% dioxane in alcohol (pH 4.5). The distillate was warmed in a steam bath for 5 minutes, and the excess of alcohol was removed with a gentle stream of air. The solution was allowed to stand overnight at 4° to complete the precipitation of the dioxane derivative. The crystals were filtered under vacuum and then recrystallized to constant specific radioactivity from aqueous alcohol. The melting point of the derivative was 189–191°. The 3H:14C ratio was then calculated for the formaldehyde molecule. The tritium content of the other half of the isopentenol molecule, 3-ketobutan-1-ol, was calculated by difference (3H:14C ratio in isopentenol minus 3H:14C ratio in formaldehyde). This calculation is valid because the hydrogen atoms of formaldehyde are not exchangeable (37).

The acetone formed through the reductive cleavage of the ozonide of dimethylallyl alcohol was distilled into an ethanol solution of 4-phenylsemicarbazide which contained a few drops of glacial acetic acid. The distillate was warmed for 5 minutes on a steam bath, and the excess of alcohol was removed with a gentle stream of air. The 4-phenylsemicarbazone needles of acetone (m.p. 168°) were recrystallized from aqueous alcohol, and the melting point of the derivative was 189–191°. The 3H:14C ratio was then calculated for the formaldehyde molecule. The tritium content of the other half of the isopentenol molecule, 3-ketobutan-1-ol, was calculated by difference (3H:14C ratio in isopentenol minus 3H:14C ratio in formaldehyde). This calculation is valid because the hydrogen atoms of formaldehyde are not exchangeable (37).

The acetone formed through the reductive cleavage of the ozonide of dimethylallyl alcohol was distilled into an ethanol solution of 4-phenylsemicarbazide which contained a few drops of glacial acetic acid. The distillate was warmed for 5 minutes on a steam bath, and the excess of alcohol was removed with a gentle stream of air. The 4-phenylsemicarbazone needles of acetone (m.p. 168°) were recrystallized from aqueous ethanol to constant specific radioactivity, and the 3H:14C ratio was calculated. The tritium bound to the other half of the dimethylallyl alcohol molecule was calculated by difference.
The precipitated protein was centrifuged and the precipitate was washed five times with 5-ml phosphate isomerase, 5.2 mg of protein, was added with stirring. 0.2 ml of perchloric acid. In another experiment, 7 seconds were allowed for the reaction. The acid-labile radioactivity was determined as indicated in the text.

Table II

<table>
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<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>Recovery</th>
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<td>0.09</td>
<td>2,790</td>
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<tr>
<td>Ammonium sulfate</td>
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<td>0.14</td>
<td>2,296</td>
<td>1.64</td>
<td>87</td>
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<tr>
<td>Supernatant solution (from calcium phosphate gel)</td>
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<td>0.20</td>
<td>1,700</td>
<td>2.36</td>
<td>65</td>
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<tr>
<td>Diethylaminoethyl cellulose</td>
<td>228</td>
<td>0.86</td>
<td>193</td>
<td>9.9</td>
<td>7</td>
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</tbody>
</table>

* A unit = 1.0 μmole of product formed per minute of incubation.

Fig. 5. The conversion of isopentenyl pyrophosphate-4-14C to acid-labile terpenyl pyrophosphates as a function of time. Isopentenyl pyrophosphate-4-14C, 156 nmoles (14,400 c.p.m.); Tris buffer, pH 8.0, 50 μmoles; MgCl2, 5 μmoles; KF, 12 μmoles; and crude pig liver soluble fraction (35 to 65%) (NH4)2SO4 fraction), 1 mg of protein, were incubated at 37° in a volume of 1 ml for the time intervals indicated. The acid-labile radioactivity was determined as indicated in the text.

and GSH, 10 μmoles; for 5 minutes at 37°. Isopentenyl pyrophosphate isomerase, 5.2 mg of protein, was added with stirring. After 5 seconds the reaction was terminated by the addition of 0.2 ml of perchloric acid. In another experiment, 7 seconds were allowed for the reaction. The precipitated protein was centrifuged and the precipitate was washed five times with 5-ml portions of 0.2 N acetic acid. The final washes were free of radioactivity. The protein was then dissolved in 0.1 ml of 1 N NaOH, and the solution was incubated at 37° for 15 minutes. An aliquot of the solution was taken for a determination of radioactivity and another aliquot was assayed for protein.

Detection of Radioactivity—All toluene-soluble samples were assayed for radioactivity in a diphenyloxazole-dimethyl-p-bis-2'-(5'-phenyloxazoyl)benzene-toluene solution in a Packard liquid scintillation spectrometer. Aqueous solutions of pyrophosphate esters and protein were assayed in a dioxane-phosphor solution (38). The efficiencies of counting for 14C were 68.0 and 72.5%, respectively. Counting efficiencies of 9.8 to 12% in dioxane-phosphor and 24.0% in diphenyloxazole-dimethyl-p-bis-2'-(5'-phenyloxazoyl)benzene-toluene solutions were obtained when tritium-containing samples were assayed. All of the crystalline derivatives prepared in the present study were soluble in toluene and were nonquenching in the amounts used for assay of radioactivity.

The paper chromatographs containing the radioactive compounds were scanned with a Vanguard automatic chromatogram scanner.

RESULTS

Partial Purification of Isopentenyl Pyrophosphate Isomerase—The isopentenyl pyrophosphate isomerase of pig liver was purified approximately 10-fold by the procedures reported in Table II. However, the purification was less than expected on the basis of the protein loss during chromatography on DEAE-cellulose. No explanation is immediately evident for the nonagreement of the purification factor in this step with that expected on the basis of protein loss, except for the possibility of partial enzyme destruction on DEAE-cellulose.

The protein separated on DEAE-cellulose behaved as a single band when subjected to disk gel electrophoresis. However, this preparation was not homogeneous in molecular weight. At least two different proteins appeared on ultracentrifugation.

Properties of Isopentenyl Pyrophosphate Isomerase—The relationship between synthesis of acid-labile terpenols and time is reported in Fig. 5. A linear response with time was obtained for 30 minutes. A linear response was also obtained when the protein concentration of the incubation mixture was varied.

Isopentenyl pyrophosphate isomerase is not completely inactivated in 3 minutes at a temperature of 70°. However, it is completely inactivated either at longer times or at higher temperatures. Isopentenyl pyrophosphate isomerase is inhibited slightly by phosphate ions at a concentration of 0.05 M, whereas acetate ions at 0.05 M appear to enhance it activity. The enzyme has a requirement for Mg++ ions. A thiol compound is not an absolute requirement for enzyme activity, but such a compound does increase the activity of the enzyme.

Equilibrium of Isomerization Reaction—Isopentenyl pyrophosphate was incubated with the isomerase and the required cofac-
tors to establish the equilibrium of the reaction. At equilibrium, 87% of the substrate, isopentenyl pyrophosphate, was converted to dimethylallyl pyrophosphate (Fig. 6).

**pH Optimum**—Isopentenyl pyrophosphate isomerase does not have a pronounced pH optimum. Its activity remains essentially unchanged between pH 4.0 and 8.3 (Fig. 7). A slight inhibition of the reaction was observed when phosphate buffers were used, whereas acetate buffers were slightly stimulatory.

**Metal Ion Requirement**—Mg**2+** ions appear to be a requirement for the isomerase reaction (Fig. 8). These ions are not replaced by Mn**2+**. The $K_m$ value obtained for Mg**2+** is approximately $1 \times 10^{-3}$ M.

![Fig. 7. The effect of pH on the synthesis of dimethylallyl pyrophosphate. Isopentenyl pyrophosphate-$4\text{C}$, 156 mmoles (14,400 c.p.m.); MgCl$_2$, 8 mmoles; GSH, 5 mmoles; and enzyme, 0.1 mg of protein, were incubated in 1 ml at 37° for 10 minutes with 50 mmoles of the various buffers. The acid-labile terpenols were extracted and assayed for radioactivity as described in the text.](image)

![Fig. 8. The effect of magnesium ion concentration on the isomerase reaction. Isopentenyl pyrophosphate-$4\text{C}$, 156 mmoles (14,400 c.p.m.); Tris buffer, pH 8.0, 50 mmoles; GSH, 5 mmoles; isomerase, 0.10 mg of protein; and MgCl$_2$, as indicated, were incubated at 37° in a volume of 1 ml for 10 minutes. The extraction and assay of acid-labile terpenols are described in the text.](image)

![Fig. 9. Affinity of isopentenyl pyrophosphate isomerase for isopentenyl pyrophosphate. The incubation mixture contained Tris buffer, pH 8.0, 50 mmoles; MgCl$_2$, 8 mmoles; GSH, 5 mmoles; protein, 25 µg; and isopentenyl pyrophosphate-$4\text{C}$, as indicated, in a volume of 1 ml. Samples were incubated for 5 minutes at 37°. The acid-labile terpenols were extracted and assayed as described in the text. Each line represents the results of a single experiment.](image)

### Table III

<table>
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<th>Inhibitor</th>
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<th>Inhibition after addition of 0.02 µGSH</th>
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<td>Iodoacetamide</td>
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<td>$5.0 \times 10^{-3}$</td>
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<td>%</td>
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<td></td>
<td>$1 \times 10^{-2}$</td>
<td>74</td>
<td>%</td>
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<tr>
<td></td>
<td>$2 \times 10^{-2}$</td>
<td>86</td>
<td>%</td>
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<td>p-Hydroxymercuribenzoate</td>
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<tr>
<td></td>
<td>$2.5 \times 10^{-6}$</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-5}$</td>
<td>94</td>
<td>63</td>
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</table>

**Michaelis Constant for Isopentenyl Pyrophosphate**—The Michaelis constant for isopentenyl pyrophosphate was obtained from a Lineweaver-Burk plot of $1/v$ against $1/S$ (Fig. 9). A $K_m$ value of $8.2 \times 10^{-8}$ M was obtained.

**Effect of Sulfhydryl-binding Agents**—Isomerase activity is stimulated by sulphydryl agents such as glutathione, and it is inhibited by various sulphydryl-binding agents such as p-hydroxymercuribenzoate, N-ethyImaleimide, and iodoacetamide (Table III). N-EthyImaleimide, at concentrations of $3 \times 10^{-8}$ and $4 \times 10^{-8}$ M, inhibited the reaction about 92 and 94%, respectively, whereas p-hydroxymercuribenzoate inhibited the isomerase reaction 94% at a concentration of $1 \times 10^{-4}$ M. The inhibition by p-hydroxymercuribenzoate was partially reversed by the addition of glutathione. Iodoacetamide inhibited the reaction 86% at a concentration of $2 \times 10^{-4}$ M.

**Reversibility of Isomerase Reaction**—An $R_p$ value of 0.78, which coincided with authentic radioactive farnesyl pyrophosphate, was observed when pure dimethylallyl pyrophosphate was incubated with isomerase plus farnesyl pyrophosphate synthetase and the reaction products were subjected to paper chromatography (Fig. 10). The formation of higher terpenyl pyrophosphate requires the presence of isopentenyl pyrophosphate for the condensation reaction (39). Therefore, isopentenyl pyrophosphate must have been formed from dimethylallyl pyrophos-
Mechanism of Isomerization Reaction

Incorporation of Tritium of Tritiated Water into Isopentenyl and Dimethylallyl Pyrophosphates—When the isomerase reaction was carried out in a medium containing tritium oxide, with isopentenyl pyrophosphate as the substrate, tritium was incorporated into the substrate and the product of the reaction (Fig. 11). In the initial phase of the reaction, the rate of incorporation of tritium into the substrate was much slower than the rate of incorporation into the product. However, at 30 minutes the two rates were equal, and subsequently the substrate incorporated tritium at a faster rate than the product.

Rate of Incorporation of Tritium into Isopentenyl and Dimethylallyl Pyrophosphates at Equilibrium—The rate of incorporation of tritium from the medium into isopentenyl and dimethylallyl pyrophosphates was studied in systems at equilibrium. In these studies tritium oxide was added to the medium after equilibrium was attained. The rates of tritium exchange are reported in Fig. 12.

Determination of Location and Quantity of Tritium Incorporated into Isopentenyl and Dimethylallyl Pyrophosphates—Isopentenyl pyrophosphate exchanged a proton at a much faster rate than dimethylallyl pyrophosphate at each of the times at which analyses were made (Fig. 12). These results indicated clearly that a proton is incorporated from the medium into substrate and product during the isomerization reaction. However, these results raised the following questions. Is tritium present on carbon atom 2 of isopentenyl pyrophosphate and carbon atom 4 of dimethylallyl pyrophosphate? Is the addition and removal of a proton at these positions stereospecific? Is a proton added at the methylene carbon atom of isopentenyl pyrophosphate to initiate the reaction, or is the reaction initiated by the removal of a proton at carbon atom 2? Or is the isomerization reaction a single step, concerted mechanism whereby the addition and removal of protons occur simultaneously? These possible mechanisms of the reaction are presented in Fig. 13.

To answer the above questions, a study of the location of tritium in isopentenyl and dimethylallyl pyrophosphates was undertaken. This study was extended to include the rate of binding of tritium on carbon atoms 2 and 4 of these compounds with time. The results of this experiment are reported in Fig. 14. Of the \(^{3}H\) and \(^{14}C\) contents of dimethylallyl alcohol, 95 to 100\% were found in the acetone molecule, thus indicating the
absence of tritium at carbon atom 2 of this molecule. This result clearly proves that the addition and the removal of a proton at carbon atom 2 is stereospecific.

The methyl carbon of the dimethylallyl alcohol has a free degree of rotation, and hence all 3 hydrogen atoms of this carbon are equivalent. One would therefore not expect any stereospecificity in the addition or removal of a proton on this carbon atom. The presence of tritium in the formaldehyde derivative obtained from isopentenol verifies this expectation.

Further analyses were made of the tritium content of the isopentenol molecule in an effort to secure additional information on the mechanism of the reaction. At the end of 2 minutes, almost all of the tritium was found attached to carbon atom 4 (the formaldehyde fragment). However, as time elapsed, 3-keto-

MECHANISM I
\[
\begin{align*}
\text{CH}_3 & \xrightarrow{H^+} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H^+} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP}
\end{align*}
\]

MECHANISM II
\[
\begin{align*}
\text{CH}_3 & \xrightarrow{H^+} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H^+} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP}
\end{align*}
\]

MECHANISM III
\[
\begin{align*}
\text{CH}_3 & \xrightarrow{H^+} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP} \\
\text{CH}_3 & \xrightarrow{H^+} \text{CH} = \text{C} = \text{CH} - \text{CH}_3 - \text{O} - \text{PP}
\end{align*}
\]

FIG. 13. Possible mechanisms for the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate.

**Discussion**

The isopentenyl pyrophosphate isomerase of pig liver has been purified to an activity of almost 1.0 µmole of product formed per minute per mg of protein. This enzyme is similar in many properties to the enzyme isolated from yeast (29). It has a \( K_m \) value of 8.2 \( \times 10^{-4} \) m for isopentenyl pyrophosphate, and an equilibrium ratio of 87:13 for dimethylallyl and isopentenyl pyrophosphates, respectively. This enzyme is readily inhibited by sulfhydryl inhibitors and is slightly inhibited by phosphate ions. The enzyme is slightly activated by acetate ions, and its activity is enhanced appreciably by thiol compounds. The enzyme appears to have a requirement for Mg++ ions. Mn++ ions are unable to substitute for Mg++. The enzyme has a broad pH optimum, 4.0 to 8.3. It has not been isolated as a pure protein, but it behaves as a single component on disk gel electrophoresis. Furthermore, its behavior on ultracentrifugation indicates that it has a molecular weight of approximately 60,000.

The results reported in this paper confirm the previous report (29) of the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate. Furthermore, the reversibility of the reaction is established in the present paper through the formation of farnesyl pyrophosphate from dimethylallyl pyrophosphate and through the incorporation of tritium of tritium oxide into both product and substrate during the reaction.

A suggestion of the mechanism of the isomerization of iso-
pentenyl pyrophosphate to dimethylallyl pyrophosphate is obtained through an analysis of the structure of the substrate molecule. Isopentenyl pyrophosphate contains a terminal C—C double bond. Four electrons participate in such a bond, and two of these electrons are readily available for reaction with an electrophilic species such as a proton or a Lewis acid. By an attack with such an electrophilic species, a carboxonium ion could be formed. This ion could then neutralize itself by the elimination of a proton from carbon atom 2 of isopentenyl pyrophosphate, with the formation of a carbanion, as the first step in the reaction. This ion would stabilize itself by the addition of a proton at carbon atom 4. A third possible mechanism would involve a concerted reaction (40). In this reaction a proton would be added and eliminated simultaneously.

Studies have been made to elucidate the mechanism of the isomerization of isopentenyl pyrophosphate in the presence of tritiated water. Since the equilibrium of the isomerization reaction lies far toward the formation of dimethylallyl pyrophosphate, it would be expected that isopentenyl pyrophosphate would be rapidly converted to dimethylallyl pyrophosphate and that tritium would therefore be incorporated much more rapidly into dimethylallyl pyrophosphate than into isopentenyl pyrophosphate in the initial phase of the reaction. This result was obtained (Fig. 11). However, it was also observed that isopentenyl pyrophosphate exchanges tritium at a much more rapid rate than dimethylallyl pyrophosphate after equilibrium is achieved. This observation was confirmed in studies of the exchange rates after equilibrium was established (Fig. 12).

The rate equations for two of the possible mechanisms for isopentenyl pyrophosphate isomerization were derived to aid in the establishment of the exact mechanism of this reaction (see "Appendix"). These equations were then applied to the change in quantity of tritium on particular carbon atoms of dimethylallyl alcohol and isopentenol as a function of time. It would be expected, from theoretical considerations, that tritium would be present in a minimum of three positions in both dimethylallyl and isopentenyl pyrophosphates when the substrate and the product are equilibrated in the presence of tritium oxide. Whether additional tritium would be bound to each of these molecules would depend upon whether tritium is added and removed stereospecifically at carbon atom 2.

During the initial phase of the exchange reaction, tritium appears almost exclusively in the methylene carbon of isopentenyl pyrophosphate (Fig. 14). The shape of the curve for the time course of labeling in the formaldehyde fragment of the molecule (Fig. 14) is hyperbolic rather than sigmoid as predicted by Mechanism II (carbanion formation) (Fig. 13). This result clearly rules out the possibility of the removal of a proton at carbon atom 2 of isopentenyl pyrophosphate, thus yielding trideuteriofarnesyl pyrophosphate, whereas 2-S-monodeuterioisopentenyl pyrophosphate would retain all the label in the formaldehyde fragment of isopentenyl pyrophosphate. This result also rules out the possibility of a one-step, concerted mechanism. It is likely that more than one intermediate exists in the isomerization reaction. However, theoretical considerations would still yield the same result in rates of exchange of tritium into the two ozonization fragments of isopentenol and dimethylallyl alcohol.

The carbonium ion mechanism may also be examined without a theoretical mathematical treatment. In order to secure label (tritium) at carbon atom 4 of dimethylallyl pyrophosphate during the isomerization of isopentenyl pyrophosphate, both Steps I and II must occur in sequence, as tritium is incorporated in Step I and the product dimethylallyl pyrophosphate is isolated after Step II. Likewise, to obtain label at carbon atom 2 of isopentenyl pyrophosphate (since carbon atom 2 of dimethylallyl pyrophosphate does not contain any tritium), both Steps II and I are necessary, since in this case tritium is incorporated during the Step II reaction. Thus, in each case (label at carbon atom 4 of dimethylallyl pyrophosphate and label at carbon atom 2 of isopentenyl pyrophosphate), both Steps I and II are necessary. However, the reactions proceed in opposite directions for the incorporation of tritium. Although the rates of Step I and Step II are different, as observed experimentally, the overall summation of the rate constants of Step I and Step II should be equal inasmuch as the over-all reaction was at equilibrium. Hence, the rates of exchange of tritium of 3-ketobutan-1-ol and that of the end carbon of dimethylallyl pyrophosphate should be equal.

Another significant result is evident on further examination of the data of Fig. 14. It was observed, following the degradation of dimethylallyl pyrophosphate, that almost 95 to 100% of the radioactivity of this compound was located in the 4-phenylsemicarbazone derivative of acetone. This result clearly indicates that the removal and addition of a proton at carbon atom 2 is stereospecific. Since the methyl carbon of dimethylallyl pyrophosphate is free to rotate, all of the hydrogens on this carbon atom are equivalent and hence tritium is not added and removed stereospecifically on this carbon atom.

The data reported in this paper are in agreement with the observation made by Popjak (41), who reported that 4-R-4 monodeuterioisovalerate gave trideuteriofarnesyl pyrophosphate, whereas 4-S-4 monodeuterioisovalerate gave unlabeled farnesyl pyrophosphate. Popjak concluded that the hydrogen atom originally attached to carbon atom 4 of mevalonate in a trans position to the carbon 3’ methyl group is eliminated in the conversion of mevalonic acid to farnesyl pyrophosphate. Since the removal of a proton from carbon atom 2 of isopentenyl pyrophosphate is stereospecific, it appears that 2-R-monodeuteriospentenyl pyrophosphate would retain all the label in farnesyl pyrophosphate, thus yielding trideuteriofarnesyl pyrophosphate, whereas 2-S-monodeuteriospentenyl pyrophosphate would yield unlabeled farnesyl pyrophosphate (Fig. 15).

Inhibition studies with various sulfhydryl binding agents indicate that a sulfhydryl group is involved at the active center. Several possibilities exist as to the exact role of this sulfhydryl group. (a) This group could cause a structural change of the protein molecule in the presence of isopentenyl pyrophosphate; (b) the sulfhydryl group of this molecule could add covalently to the carbonium ion; or (c) the sulfhydryl group could stabilize the carbonium ion through the formation of an ion pair.

The isolation of protein bound radioactivity (Table IV) is
consistent with the proposal that a thioether linkage is formed as reported for the Wildegrodt reaction. Thus, the possibility of a structural change of the protein or the existence of an ion pair is highly unlikely. These results are in agreement with the mechanism proposed by Agranoff et al. (29) for the isomerization reaction.

The above studies indicate a carbonium ion formation as the mechanism of isomerization of isopentenyl pyrophosphate (Fig. 16). In this mechanism, isopentenyl pyrophosphate, when incubated with isopentenyl pyrophosphate isomerase and triitated water, incorporates tritium at the methylene carbon atom. The resulting carbonium ion is neutralized, possibly through the nucleophilic addition of a sulfhydryl group of the enzyme. A covalent thioether linkage would then be formed. The proton from carbon atom 2 is then excreted into the medium, and dimethylallyl pyrophosphate is formed. In the reverse reaction, a proton is added stereospecifically at carbon atom 2 of dimethylallyl pyrophosphate.

![2-S-monodeuterio (tritio)-isopentenyl pyrophosphate](image)

![2-R-monodeuterio (tritio)-isopentenyl pyrophosphate](image)

**Fig. 15. R and S configurations of isopentenyl pyrophosphate.**

**Fig. 10. Mechanism of isomerization of isopentenyl pyrophosphate.** The starred atoms indicate the exchangeable hydrogens of isopentenyl and dimethylallyl pyrophosphates.

**SUMMARY**

1. The partial purification of isopentenyl pyrophosphate isomerase from pig liver has been achieved, and the properties of this enzyme have been reported.

2. When equilibrium of the isomerase reaction is reached, the ratio of dimethylallyl pyrophosphate to isopentenyl pyrophosphate is 87:13. The reversibility of the isomerase reaction also has been established through the conversion of dimethylallyl pyrophosphate to farnesyl pyrophosphate.

3. A stereospecific addition or removal of a proton at carbon atom 2 occurs in the isomerization reaction.

4. A mechanism of isomerization by isopentenyl pyrophosphate isomerase is presented. In this mechanism, the methylene carbon atom of isopentenyl pyrophosphate is first protonated, resulting in the formation of a carbonium ion. This ion is then neutralized, possibly by covalent bond formation with a sulfhydryl group of the protein. The loss of a proton from carbon atom 2 follows.

5. An enzyme-bound, acid-stable, radioactive compound was isolated. The isolation of this compound is in agreement with the suggestion that an intermediate in the isomerase reaction is bound covalently to a sulfhydryl group of the protein.

6. A theoretical mathematical analysis was made of the rates of exchange of a proton of the medium with isopentenyl and dimethylallyl pyrophosphates.

**APPENDIX**

**MECHANISM 1**

<table>
<thead>
<tr>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>kT</td>
<td>k</td>
</tr>
<tr>
<td><strong>Step I</strong></td>
<td><strong>Step I</strong></td>
</tr>
<tr>
<td>kT</td>
<td>k</td>
</tr>
<tr>
<td>X</td>
<td>X</td>
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<tr>
<td><strong>Step II</strong></td>
<td><strong>Step II</strong></td>
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<tr>
<td>kT</td>
<td>k</td>
</tr>
<tr>
<td>P</td>
<td>T</td>
</tr>
<tr>
<td>P</td>
<td>T</td>
</tr>
</tbody>
</table>

In these mechanisms, A = isopentenyl pyrophosphate; X = intermediate; P = dimethylallyl pyrophosphate; T = tritium from tritiated water; k1 and k2 are rate constants in the forward and backward directions of Step I; and k3 and k4 are rate constants in the forward and backward directions of Step II.

**Equations**

**Definitions**—X = label on the methylene carbon of the intermediate; X* = label on carbon 2 of the intermediate; *A = label on the methylene carbon of isopentenyl pyrophosphate; *A* = label on carbon 2 of isopentenyl pyrophosphate; *P = label on carbon 4 of the dimethylallyl pyrophosphate.

Consider Mechanism 1—At equilibrium,

\[ k(T)(A)(E) = k(T)(X) \]  
\[ k(T)(X) = k(T)(P)(E) \]

Assuming the steady state,

\[ \frac{d(X)}{dt} = (A)(E)k(T) - (k_2 + k_3)X = 0 \]  
\[ \frac{d(X^*)}{dt} = (P)(E)k(T) - (k_2 + k_3)X^* = 0 \]
the reaction mechanism presented in this paper.

Professor Howard W. Whitlock for his helpful discussions of

0.5 of the isopentenol molecule (3-ketobutan-1-01).

molecule) is equal to the rate of exchange of a proton in the right

phase of isopentenyl pyrophosphate to be zero in the initial phase of

WRIGHT, L. D., CRESSON, E. L., SKEGGS, H. R., MACRAE, G.

of isopentenyl pyrophosphate to be zero in the initial phase of

methylallyl pyrophosphate (the acetone fragment of the mole-

any label.

Acknowledgment—The authors wish to express their thanks to

the authors wish to express their thanks to

Professor Howard W. Whitlock for his helpful discussions of

Consider Mechanism 2—The intermediate X does not contain any label.

This mechanism predicts the label on the methylene carbon of

isopentenyl pyrophosphate to be zero in the initial phase of

the reaction exchange.

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Isopentenyl Pyrophosphate Isomerase

Damayanti H. Shah, W. W. Cleland and John W. Porter


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