On the Mechanism of Formation of Isopentenylpyrophosphate*

MARJORIE LINDBERG,† C. YUAN, A. DEWAARD,‡ and KONRAD BLOCH

From the James B. Conant Laboratories, Department of Chemistry, Harvard University, Cambridge, Massachusetts
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In the enzymatic decarboxylation of mevalonate-5-pyrophosphate to isopentenylpyrophosphate (3-methyl-3-butenyl-1-pyrophosphate) and CO_2, ATP is consumed and stoichiometric amounts of ADP and inorganic phosphate are formed. Attempts to isolate a phosphorylated intermediate have not been successful, but evidence has been obtained with the aid of O^{18} that the terminal phosphate of ATP interacts with the tertiary hydroxyl group of mevalonate-5-pyrophosphate during the decarboxylative β-elimination. 3-O^{18}-mevalonolactone was synthesized and converted enzymatically to 3-O^{18}-mevalonate-5-pyrophosphate, which was decarboxylated to isopentenylpyrophosphate. The inorganic phosphate isolated from the reaction mixture contained O^{18}, showing that oxygen had been transferred from the tertiary hydroxyl group of mevalonate-5-pyrophosphate to the terminal phosphate residue of ATP.

The isopentenyl structure of the condensing unit in terpene biogenesis and the general mechanism by which it is formed have been deduced from studies on squalene synthesis with deuteriomevalonic acid and with D_2O (Rilling *et al.*, 1958; Rilling and Bloch, 1959). These deductions were shown to be valid when isopentenylpyrophosphate (3-methyl-3-butenyl-1-pyrophosphate) was isolated and characterized first in this laboratory (Chaykin *et al.*, 1958; Bloch, 1958) and shortly afterwards by Lynen and his associates (1958), who also reported the first chemical synthesis of this key intermediate. The transformation of mevalonic acid to isopentenylpyrophosphate is effected by three ATP-dependent steps, catalyzed respectively by mevalonic kinase (Tchen, 1958; Lynen, 1958; Levy and Popják, 1960), mevalonic phosphate kinase (Bloch, 1958; Henning *et al.*, 1959; Bloch *et al.*, 1959b) and a decarboxylase (Bloch, 1958; Bloch *et al.*, 1959b; deWaard *et al.*, 1959; Agranoff *et al.*, 1960). The first two of these enzymes appear to be conventional kinases. The "decarboxylase" system is more complex and is characterized by several unusual features. This enzyme, which has been partially purified (Bloch *et al.*, 1959b), catalyzes an apparently bimolecular reaction between mevalonate-5-pyrophosphate and ATP to form the four reaction products isopentenylpyrophosphate, CO_2, ADP, and P_i. It has been suggested that 3-phospho-5-pyrophosphomevalonic acid is formed as a transitory intermediate which undergoes a decarboxylative β-elimination of phosphate to the isopentenyl derivative (deWaard *et al.*, 1959). With the aid of O^{18}, evidence for the temporary formation of an ester linkage between phosphate and the tertiary hydroxyl group of mevalonate-5-pyrophosphate has now been obtained.

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EXPERIMENTAL

Materials.—H_2O^{18} was obtained from the Weizmann Institute of Science, Israel, mevalonolactone from Fluka, A. G., Chemische Fabrik Buchs/SG, 2-C^{14}-mevalonic acid from Tracerlab, Inc., and cobra venom from the Ross Allen Reptile Institute. Avidin was purchased from Nutritional Biochemicals Corp., the nucleotides from Sigma Chemical Company, and 4-hydroxy-2-butanone from Eastman Kodak Company.

O^{18} analyses were carried out in an analytical mass spectrometer, type 21-103C, Consolidated Engineering Corp.

Enzyme Preparations.—Mevalonic kinase was prepared from dried baker's yeast (Fleischmann) according to Tchen (1958), and further purified on a 23 × 1 cm DEAE-cellulose column by elution with 0.001 M potassium phosphate buffer, pH 7.0. Mevalonic kinase activity appeared with the 7 to 26 ml eluate fractions, and had a specific activity of 14.7 μmoles of D,L-mevalonolactone converted/hour/mg protein.

Mevalonic phosphate kinase prepared according to Bloch *et al.* (1959b) had a specific activity of 3.5 μmoles of substrate converted/hour/mg protein.

Decarboxylase, prepared as described by Bloch *et al.* (1959b) (100-fold purification), was adjusted to pH 5.2 with 0.2 M sodium succinate buffer. The supernatant fluid was dialyzed against Tris-KCl buffer, 0.025 M with respect to Tris and KCl. The specific activity of the enzyme was 37 μmoles of substrate converted/hour/mg protein (140-fold purification).

Synthesis of 3-O^{18}-D,L-Mevalonolactone.—The procedures of Cornforth *et al.* (1958) and Hoffman *et al.* (1957) were followed except that the 4-acetoxy-2-butanone used in the Reformatsky reaction was labeled with O^{18}.

Redistilled unlabeled 4-acetoxy-2-butanone (2 g) was dissolved in 1 ml of H_2O^{18} (32.3 atom % excess O^{18} and 0.965 atom % excess O^{17}) containing 0.04 ml of 2 N H_2SO_4. The solution was allowed to stand at 25° for 6 hours (*c. f.* Bunton *et al.*, 1955).

The solution was then neutralized by addition of 0.08 mmole of sodium acetate, and the water was distilled off *in vacuo* and recovered in a receiver cooled by dry ice. The O^{18}-containing acetoxyketone remaining in the distilling flask was dried over anhydrous Na_2SO_4 and $CaSO_4$ and distilled *in vacuo* (b.p. 96°, 25 mm), yield 1.77 g. This product was condensed with ethyl bromoacetate (1.32 g) to 3-O^{18}-5-acetoxy mevalonic acid ethylester according to Cornforth *et al.* (1958). The ester was distilled (b.p. 82°, 0.1 mm) and saponified, and the aqueous solution of the sodium mevalonate was passed through a Dowex-50 column (H^+ form) in the cold room (2–4°). The aqueous eluate was lyophilized and the residue dried *in vacuo* over P_2O_5. On distillation (96–98°, 0.05 mm) there was obtained 0.744 g of O^{18}-mevalonolactone. When dissolved in ether and left at −5° for 24 hours the lactone crystallized; m.p. 23–28°. The infrared spectrum was identical with that of a commercial sample of mevalonolactone. The O^{18} content of the product (average of five analyses) was 5.63 atom % excess, or 16.89% for the labeled O atom at C-3 of mevalonolactone. In an enzymatic assay with mevalonic kinase the labeled racemic lactone was found to be 50% active.

AMP-P^{32} was prepared from AMP and P_i^{32} by the method of Lowenstein (1957) and separated from AMP and P_i by gradient elution on a Dowex-1 (formate) column (Hurlbert *et al.*, 1954).

Conversion of O^{18}-Mevalonic Acid to O^{18}-Mevalonate Phosphate.—The incubation mixture contained 0.01 M ATP, 0.01 M $MnSO_4$, 0.01 M KF, 0.045 M potassium phosphate buffer (pH 7.2), 400 μmoles 3-O^{18}-D,L-mevalonolactone (17 atom % excess), 20 × 10^6 cpm mevalonic acid-C^{14} (11 μmoles), and mevalonic kinase (260 mg protein), in a volume of 400 ml. The incubation was carried out in air at 30° for 3 hours. The reaction was stopped by immersion of the tubes in boiling water and the precipitated protein removed by centrifugation. Mevalonate phosphate was separated from unreacted mevalonic acid by gradient elution on a Dowex-1 (formate) column (42 cm × 2 cm diameter) with increasing concentrations of ammonium formate in 4 N formic acid (Hurlbert *et al.*, 1954). The elution was begun with 80 ml water in the mixing flask and 100 ml 4 N formic acid in the reservoir, followed by 500 ml 0.6 M ammonium formate in 4 N formic acid. Formic acid and water were evaporated *in vacuo* and the ammonium formate was removed by sublimation. The over-all yield was 10% (40 μmoles mevalonate-5-phosphate), determined by measurement of the radioactivity of the C^{14}-mevalonate-5-phosphate.

Conversion of O^{18}-Mevalonate Phosphate to O^{18}-Mevalonate Pyrophosphate.—The incubation mixture contained 0.011 M ATP, 0.026 M $MgCl_2$, 0.012 M potassium phosphate buffer, pH 7.2, 40 μmoles O^{18}-mevalonate-5-phosphate (containing 2 × 10^6 cpm C^{14}-mevalonate-5-phosphate) and mevalonic phosphate kinase (230 mg protein), in a volume of 75 ml. The mixture was incubated at 30° for 1 hour. The protein was denatured by heat in a boiling water bath and mevalonate-5-pyrophosphate separated from mevalonate-5-phosphate by elution on a Dowex-1 (formate) column (19 cm × 2 cm dia.); mevalonate-5-phosphate and ADP were eluted with 450 ml 0.1 M ammonium formate in 4 N formic acid, and mevalonate-5-pyrophosphate and ATP were eluted with 600 ml 0.6 M ammonium formate in 4 N formic acid. The optical density of the column fractions at 260 mμ was measured to locate the fractions containing ADP and ATP, and aliquots were counted to locate mevalonate-5-phosphate and mevalonate-5-pyrophosphate. The fractions containing mevalonate-5-pyrophosphate were combined and, after removal of formic acid and ammonium formate, the mevalonate-5-pyrophosphate was separated from ATP by chromatography on Whatman 3MM filter paper (57 × 46.3 cm) with the solvent system methanol, concd. NH_4OH, water (60:10:30 by volume). The paper and solvent were equilibrated in the chromatography tank for 45 minutes and chromatography allowed to proceed for $10^1/_2$ hours at room temperature. The paper was dried at room temperature and scanned with a UV lamp to locate the nucleotide area and with a monitor to locate the radioactive area. Mevalonate-5-pyrophosphate was eluted with water and an aliquot counted. The final product contained 1.5 × 10^6 cpm C^{14} or 30.2 μmoles O^{18}-mevalonate-5-pyrophosphate, a yield of 75% from mevalonate-5-phosphate. To check the effect of chromatography on a Dowex column on the stability of the O^{18} in mevalonic acid and its derivatives under the conditions used for isolation of mevalonate-5-phosphate and mevalonate-5-pyrophosphate, the unreacted O^{18}-mevalonic acid from the mevalonic kinase reaction was recovered from the Dowex-1 (formate) column by elution with formic acid–ammonium formate. The residue, after removal of formic acid and ammonium formate, was triturated with ether and the ether decanted. The ether-soluble residue was 97% pure mevalonic acid as determined by the specific radioactivity. The material (6 mg) was diluted with unlabeled mevalonic acid and converted to CO_2 by heating at 440° for $4^1/_2$ hours with mercuric chloride in a sealed tube (Rittenberg and Ponticorvo, 1956). The mevalonic acid contained 8.42 and 9.07 atom % excess O^{18} in duplicate analyses. The mevalonic acid had therefore lost approximately 50% of the O^{18} content by chemical exchange, probably because of the acidity of the solvents in the column chromatography used to separate mevalonic acid from mevalonate-5-phosphate.

To check for further losses of O^{18}, the unreacted O^{18}-mevalonate-5-phosphate recovered from the mevalonic phosphate kinase reaction was converted to mevalonic acid enzymatically with cobra venom phosphatase. The incubation mixture (5.4 ml) contained 8 μmoles mevalonate-5-phosphate, 0.0074 M magnesium acetate, 0.074 M Tris buffer (pH 9.0), and 10 mg cobra venom (*Naja naja*). The mixture was incubated at 30° for 1 hour. Mevalonic acid was separated from mevalonate-5-phosphate on a Dowex-1 (formate) column and further purified by chromatography on a Dowex 50 (H^+) column and on Whatman #1 paper with the solvent system methanol–concd. NH_4OH–water (60:10:30 by volume). The mevalonic acid was diluted 10-fold

and converted to the benzhydrylamide (Wolf *et al.*, 1957) and the derivative recrystallized from benzene; m.p. 92–94°. The amide was converted to CO_2 by combustion at 440° for 4½ hours in the presence of mercuric chloride in a sealed tube. O^{18} analysis of the CO_2 gave a value of 3.45 atom % excess O^{18} for the tertiary hydroxyl group of mevalonic acid. In a control experiment the synthetic mevalonic acid containing 17 atom % excess O^{18} was converted to the benzhydrylamide and analyzed for O^{18} under the same conditions as described above. The value obtained (17 atom % excess O^{18}) showed that no loss of O^{18} had occurred during formation of the derivative. Reisolation of mevalonate-5-phosphate had therefore led to a loss of an additional 60% of the O^{18} content, presumably as the result of acid-catalyzed exchange during the second chromatographic step, when mevalonate-5-phosphate was separated from mevalonate-5-pyrophosphate. From these results it is clear that the mevalonate-5-pyrophosphate used as substrate in the decarboxylase reaction could have retained only a fraction of the O^{18} originally present in mevalonolactone. Because of lack of sufficient material, the actual value could not be determined by direct analysis.

Conversion of O^{18}-Mevalonate-5-Pyrophosphate to Isopentenylpyrophosphate.—The incubation mixture for the decarboxylation reaction contained 0.003 M ATP, 0.01 M $MgCl_2$, 0.01 M KF, 0.03 M Tris buffer (pH 7.3), 24 μmoles O^{18}-mevalonate-5-pyrophosphate, and decarboxylase (3.6 mg protein) in a volume of 100 ml. The enzyme had been dialyzed against Tris-KCl buffer to remove inorganic phosphate. The mixture was incubated at 30° for 50 minutes and the reaction stopped by the addition of one-tenth volume perchloric acid. After the addition of 10 N KOH to the phenol red end-point, the precipitated $KClO_4$ was centrifuged off. To the supernatant fluid, adjusted to pH 4, was added 10 g acid-washed Norite A for separating the inorganic phosphate from the nucleotides and from organic phosphates. The inorganic phosphate was obtained by washing the charcoal with water; the solution was neutralized (pH 7), and lyophilized to a small volume. The phosphate was converted to $MgNH_4PO_4$ by adding 1.2 ml magnesia mixture (0.7 mmoles Mg^{++}, 2.2 mmoles NH_4^+). Approximately 0.5 ml of an aqueous suspension of Dowex 50 (H^+) resin was added to the crystalline $MgNH_4PO_4$, and the resin was washed with several portions of water until the washings were neutral. The solution, after adjustment of the pH to 4.4 with 1 N KOH, was lyophilized to a small volume. The KH_2PO_4 was crystallized from water-dioxane and analyzed for O^{18} by the method of Williams and Hager (1958). The results of five analyses for the same sample are shown in Table I.

One half of the charcoal was washed thoroughly with water to remove all remaining inorganic phosphate and then eluted with 15% aqueous pyridine. A one-tenth aliquot of the eluate was chromatographed on Whatman No. 1 paper in *tert*-butanol, formic acid, water (40:10:16 by volume). The paper strip was scanned with an actigraph (Nuclear-Chicago Corporation) and showed a single peak having an R_F value identical with that of authentic isopentenylpyrophosphate. There was no radioactivity in the area corresponding to the R_F value of mevalonate-5-pyrophosphate. In a separate experiment in which the same enzyme preparation was used under the same conditions, the decarboxylation of C^{14}-mevalonate-5-pyrophosphate was found to be quantitative as measured by the radioactivity of the recovered isopentenylpyrophosphate after separation by paper chromatography. The remainder of the charcoal was eluted with 1% NH_4OH in 50% ethanol. The eluates were chromatographed on Dowex-1 (formate) columns to separate ADP from ATP and from isopentenylpyrophosphate. The ADP was hydrolyzed in 2 N HCl, and the inorganic phosphate was purified as described above and analyzed for O^{18} content. The O^{18}-content was 0.003 atom % excess above natural abundance, showing that no oxygen had been transferred from the substrate to ADP during the decarboxylase reaction.

TABLE I

TRANSFER OF O^{18} FROM 3-O^{18}-MEVALONATE-5-PYROPHOSPHATE TO INORGANIC PHOSPHATE IN THE DECARBOXYLASE REACTION

Sample No.	Atom % Excess O^{18} in KH_2PO_4	Dilution	Atom % Excess Calculated for 4 Atoms of Phosphate Oxygen	Atom % Excess Calculated for 1 Atom of Phosphate Oxygen
1	0.040	15-fold	0.600	2.4
2	0.033	15	0.500	2.0
3	0.035	15	0.525	2.1
4	0.152	3.75	0.570	2.28
5	0.149	3.75	0.559	2.24

ADP-ATP Exchange.—The enzyme was incubated with ADP^{32} and nonradioactive ATP in both the absence and the presence of mevalonate-5-pyrophosphate. The incubation flasks contained 0.01 M $MgCl_2$, 0.01 M KF, 0.03 M potassium phosphate buffer (pH 7.0), 0.005 M ATP, 0.0036 M ADP^{32} (12×10^6 cpm), and, when present, 0.175 μmoles mevalonate pyrophosphate in a total volume of 0.2 ml. Inorganic phosphate, ADP, and ATP were separated by gradient elution on a Dowex-1 (formate) column. The ATP fraction was located by measurement of optical density at 260 mμ and the radioactivity counted. The results are shown in Table II.

TABLE II

ADP^{32}-ATP EXCHANGE

μMoles Mevalonate-5-pyrophosphate	cpm ADP^{32} Added	cpm in ATP^{32}	% of cpm in ATP
0	12×10^6	0.059×10^6	0.5
0.175	12×10^6	0.012×10^6	0.1

Effect of Inhibitors.—Mevalonate - 5 - pyrophosphate-C^{14} was incubated with decarboxylase in the presence of avidin. The incubation mixture contained 0.005 M ATP, 0.012 M $MgCl_2$, 0.012 M KF, 0.035 M Tris buffer (pH 7.2), 8000 cpm C^{14}-mevalonate-5-pyrophosphate, 40 μg avidin (0.1 unit), and decarboxylase (20 μg protein). The incubation was carried out in air at 30° for 50 minutes and the deproteinized reaction mixture was applied to a 1.5 in. wide strip of Whatman No. 1 paper and chroma-

tographed in the system containing *tert*-butanol, formic acid, water. There was no inhibition of conversion of mevalonate-5-pyrophosphate to isopentenylpyrophosphate in the presence of avidin (Table III).

TABLE III

EFFECT OF INHIBITORS ON THE DECARBOXYLASE REACTION

Additions	cpm in Isopentenylpyrophosphate Exp. 1	Exp. 2	Exp. 3
None	8000	8000	8000
0.1 unit avidin	8080	7860	9088
0.4 M NH_2OH	7710	7816	7416
0.8 M NH_2OH	8400	8672	7536

C^{14}-Mevalonate-5-pyrophosphate (8000 cpm) was incubated with decarboxylase in the presence of salt-free hydroxylamine (either 0.4 M or 0.8 M final concentration) (Beinert *et al.*, 1953). There was less than 5% inhibition of the conversion of mevalonate-5-pyrophosphate to isopentenylpyrophosphate (Table III) and no detectable formation of hydroxamate.

DISCUSSION

For the interaction between mevalonate-5-pyrophosphate and ATP catalyzed by the decarboxylase system, the most attractive reaction site is the tertiary hydroxyl group of the mevalonic acid derivative. If a phosphate ester linkage is formed, as the splitting of ATP to ADP and P_i indicates, then the oxygen of the tertiary hydroxyl group of mevalonic acid should be transferred to phosphate when elimination (C-O cleavage) takes place. Whether such a transfer occurs can be ascertained with the aid of O^{18}. Labeled oxygen is readily introduced into the tertiary hydroxyl group of mevalonic acid by acid-catalyzed exchange with H_2O^{18}. However, in acid solution the carboxyl oxygens also undergo exchange, and although the label in the carboxyl group can be removed by back-exchange in alkaline solution—conditions which do not remove O^{18} from the tertiary hydroxyl—it is difficult to prove that the mevalonic acid so prepared contains O^{18} exclusively at C-3. This method was therefore abandoned in favor of an unequivocal chemical synthesis. O^{18} was introduced by exchange into 4-acetoxy-2-butanone and the labeled ketone condensed with ethyl bromoacetate. The 3-O^{18}-mevalonic acid was converted enzymatically into mevalonate-5-phosphate and then into the 5-pyrophosphate. During isolation and separation from the adenine nucleotides by chromatography on Dowex, the two phosphate esters were exposed to the strong acidity of the formic acid–ammonium formate solvent system used for elution. As already mentioned, these conditions promote exchange of the oxygen from the tertiary hydroxy group and thus could cause substantial losses of O^{18} during the preparation of mevalonate-5-pyrophosphate. For these reasons it would have been desirable to determine the O^{18} content of the mevalonate-5-pyrophosphate used in the enzymatic transfer reaction. Because of the technical difficulties involved in isolating a sufficient quantity of this material, the O^{18} analyses were performed instead on both the unreacted mevalonic acid recovered from the mevalonic kinase reaction and on the mevalonic acid obtained by enzymatic hydrolysis of mevalonate-5-phosphate. Both samples had been in contact with acid during chromatography on a Dowex column. These treatments were found to reduce the original O^{18} content of mevalonic acid to less than 25% of the original. It is therefore a reasonable estimate that the O^{18} concentration was similarly reduced in the mevalonate-5-pyrophosphate which had been subjected to identical conditions during two successive separations by chromatography on a Dowex column, *i.e.*, it probably contained less than 5 atom % excess O^{18}.[1]

The inorganic phosphate isolated from the decarboxylase reaction contained 0.55 atom % excess O^{18}, or 2.2 atom % excess calculated for one of the four oxygen atoms (Table I). This result establishes an interaction between the substrate and the terminal phosphate of ATP, with transfer of the O^{18} from the tertiary hydroxyl group of mevalonate-5-pyrophosphate to phosphate when the C-O bond is broken. Although this value is only 17% of the theoretical value expected on the basis of the O^{18} content of the mevalonic acid, the observed O^{18} concentration in P_i is in line with the estimated value, allowing for losses of O^{18} during the preparation of mevalonate-5-pyrophosphate.

Under the experimental conditions used, the decarboxylation of mevalonate-5-pyrophosphate went to completion and no evidence for reversal was obtained. The enzyme also failed to catalyze an exchange reaction between ADP and ATP, whether substrate was present or not. Since the decarboxylase reaction affords four products, reversibility is exceedingly unlikely on kinetic grounds alone. The addition of avidin to the enzyme system was without any effect on the reaction rate. This finding is in line with other evidence showing that biotin is not a coenzyme in irreversible decarboxylations (Hamilton and Westheimer, 1959).

The "decarboxylase" catalyzes an ATP-dependent decomposition of mevalonate-5-pyrophosphate to stoichiometric amounts of isopentenylpyrophosphate, CO_2, ADP, and P_i, the rates of formation of the four reaction products being identical (de Waard *et al.*, 1959). In discussing our results on squalene synthesis in D_2O, we have pointed out that the conversion of a mevalonic acid derivative to the isopentenyl stage must be concerted, *i.e.*, the elimination of the tertiary hydroxyl group and decarboxylation must be synchronized, and this has been borne out by subsequent enzyme studies (Rilling *et al.*, 1958; Rilling and Bloch, 1959). On the other hand, there is no compelling mechanistic reason why the phosphorylation associated with the decarboxylative elimination must also be part of the synchronized process, *i.e.*, why a phosphorylated derivative of mevalonate-5-pyrophosphate should not be capable of existence. Although attempts to purify the enzyme further with the object of separating a phosphorylation step from the

[1] The acid-catalyzed exchange reaction apparently does not result in racemization, for the mevalonate-5-pyrophosphate was quantitatively converted to isopentenylpyrophosphate. This is an unexpected result which needs to be explored further.

decarboxylation have so far failed, this issue can not yet be regarded as settled.

According to our earlier formulation the decarboxylase reaction is initiated by phosphorylation of the tertiary hydroxyl group of mevalonate-5-pyrophosphate, the elimination of phosphate anion providing the driving force for decarboxylation and formation of the olefin (Bloch *et al.*, 1959b). Decarboxylative eliminations by a concerted mechanism are well known in chemical systems. They have been demonstrated with β-hydroxy acids (Vilkas and Abraham, 1960), β-halo acids (Grovenstein and Lee, 1953; Cristol and Norris, 1953) and β-lactones (Liang and Bartlett, 1958). The tertiary hydroxyl group is likely to be eliminated more readily when esterified, although this has not been proved experimentally. The interesting observation has been made that the decarboxylative elimination of certain tertiary β-hydroxy acids occurs at a lower temperature when polyphosphoric acid is added as a catalyst (Vilkas and Abraham, 1960). This dehydrating agent presumably exerts its effect by phosphorylating the alcoholic function (Cherbuliez *et al.*, 1959).

There is growing evidence for enzymatic mechanisms in which phosphorylation facilitates the elimination of oxygen functions. Watanabe *et al.* (1955) have shown *O*-phosphohomoserine to be an intermediate in the transformation of homoserine to threonine. The studies by Flavin and Slaughter (1960) on the mechanism of this isomerization indicate that phosphohomoserine, after forming a Schiff base with pyridoxal phosphate, undergoes phosphate elimination, the C-O cleavage and proton elimination leading to a vinylglycine intermediate. The olefin then adds water at the β-carbon atom to form the threonine structure. The same interpretation of the role of phosphate may apply to the transformation of 3-enoyl-pyruvyl-5-phosphoshikimic acid to prephenic acid which has been observed by Levin and Sprinson (1960). Here also an oxygen function appears to be eliminated by C-O cleavage of a phosphate ester with concomitant formation of an olefin. Similarly, the pyrophosphate ester structure of isopentenylpyrophosphate can be rationalized as facilitating the elimination of the primary hydroxyl group of mevalonic acid. This case differs from the reactions discussed above in that the electron deficiency created by C-O cleavage does not result in olefin formation, but leads to nucleophilic attack by another isopentenyl unit (Lynen *et al.*, 1958; Rilling and Bloch, 1959). Although the events associated with phosphate anion elimination differ in the various cases discussed, the leaving of a phosphate group is a common feature and presumably provides the driving force for all these reactions. As far as energy aspects are concerned, resonance stabilization of the phosphate anion would account for the greater ease of elimination of phosphate groups than of hydroxide. There is as yet no obvious explanation why phosphate is the leaving anion in some cases and pyrophosphate in others.

The decarboxylase reaction utilizes the bond energy of the terminal phosphate group of ATP for an endergonic process with the over-all formation of ADP and inorganic phosphate. It shares this property with the various synthetase or kinase-type reactions leading to the formation of amide, peptide, or thioester bonds (for reviews see Buchanan *et al.* [1959] and Boyer [1960]). It is characteristic of these ATP-dependent syntheses that they involve the reversible interaction of three substrates and afford three products. The decarboxylase system may be placed in the same category of reactions, although it catalyzes an irreversible process and is concerned with the conversion of two substrates into four products. In this reaction the function of ATP is to facilitate the decomposition of a substrate rather than to promote the synthesis of a larger molecule from two smaller ones. What appears at first sight to be a wasteful expenditure of energy is in fact an activation process. The formation of the reactive nucleophilic exomethylene group permits the synthetic reaction, *i.e.*, condensation with a second "isoprenoid unit," to occur without further expenditure of energy, as shown by the fact that ATP is not required for the synthesis of terpene polymer from isopentenylpyrophosphate (Bloch *et al.*, 1959b) or from isopentenylpyrophosphate and dimethylallylpyrophosphate (Lynen *et al.*, 1959). It is of course realized that the formation of the exomethylene group by the ATP-dependent decarboxylase reaction is only part of the activation process concerned with the formation of carbon-carbon bonds. The second bond-forming carbon of mevalonic acid, originally a primary alcohol, is activated by transformation into a pyrophosphate ester in two ATP-requiring steps. Elimination of pyrophosphate affords the electrophilic center for reaction with the active methylene group of the isopentenyl unit. The energy required for the formation of carbon-carbon bonds in terpene synthesis is thus provided by all three of the ATP molecules consumed in the formation of isopentenylpyrophosphate from mevalonic acid.

The results presented here definitely point to an interaction between the terminal phosphate of ATP and the tertiary hydroxyl group of mevalonic acid pyrophosphate and suggest that this interaction facilitates C-O cleavage. The intermediary formation of a covalent phosphate ester, *i.e.*, 3-phospho-5-pyrophosphomevalonic acid,[2] which could assume the favorable six-membered ring structure, is a possibility (Fig. 1, scheme a), but in the absence of direct evidence alternative formulations cannot be ruled out. For example, we have considered the possibility that in the decarboxylase reaction the carboxyl group rather than the tertiary hydroxyl group of mevalonate-5-pyrophosphate is attacked by the terminal phosphate of ATP. The resulting carboxyl phosphate could form a six-membered cyclic phosphoric anhydride derivative and undergo C-O cleavage with O^{18} transfer to phosphorus, as in Figure 1, scheme b.

In order to test for the formation of an intermediary carboxyl phosphate, the effect of hydroxylamine on the decarboxylase reaction was studied. This reagent did not affect the rate of conversion of

[2] The report from our laboratory of a mevalonic acid derivative containing three phosphorus atoms (Bloch *et al.*, 1959a) has not been verified, and these earlier claims must therefore be withdrawn.

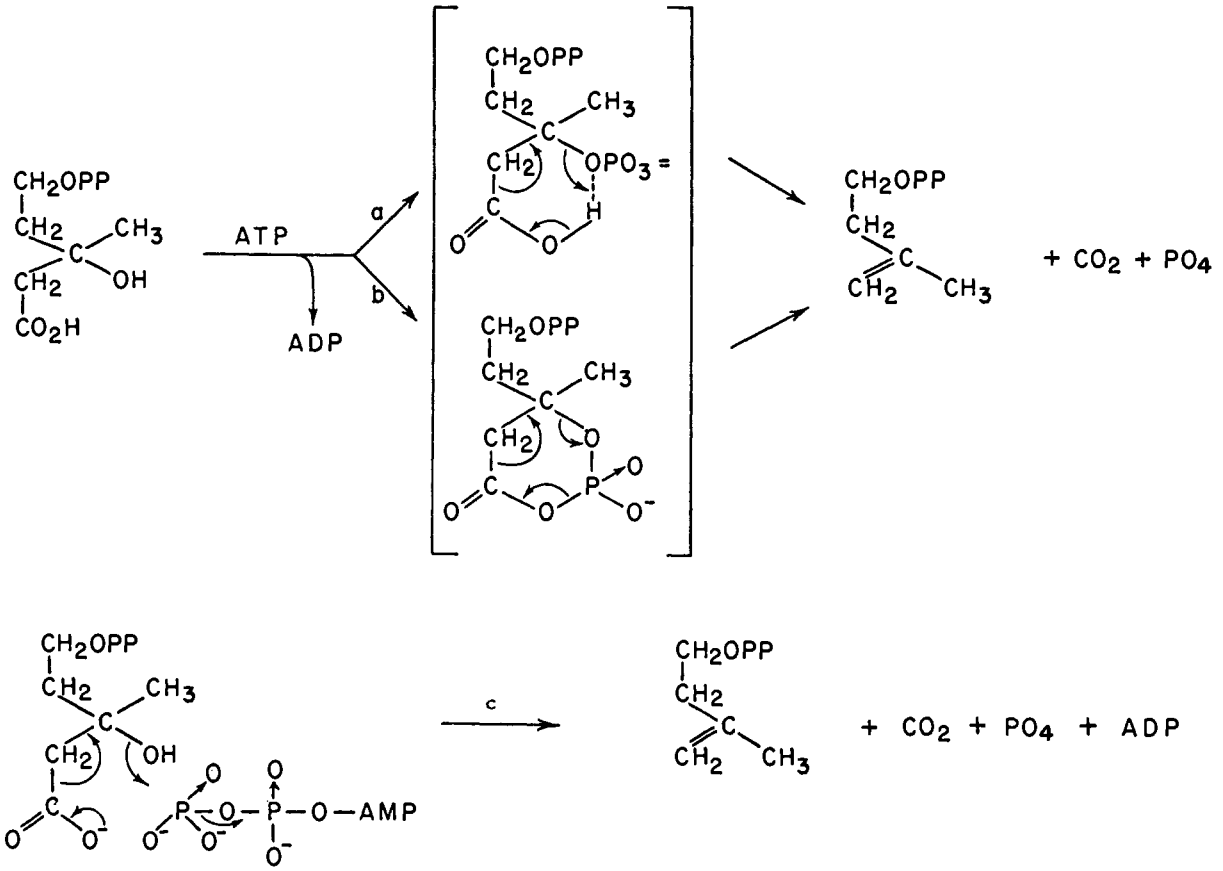

FIG. 1.—Mechanism of decarboxylation of mevalonate-5-pyrophosphate.

mevalonate-5-pyrophosphate to isopentenylpyrophosphate, nor could a hydroxamate derivative be detected. These results make scheme b or any mechanism involving the formation of an anhydride linkage improbable.

The type of transfer of O^{18} from substrate to P_i demonstrated here has been observed in a number of ATP-dependent syntheses—*e.g.*, glutamine (Boyer *et al.*, 1956; Kowalsky *et al.*, 1956), glycinamide-ribotide (Hartman and Buchanan, 1958), γ-glutamylcysteine (Strumeyer and Bloch, 1960), glutathione (Strumeyer, 1959)—yet in no case have the mechanistically probable phosphoric ester or anhydride intermediates been isolated.[3] This may be due to the lack of sufficiently subtle methods for the handling of labile or enzyme-bound intermediates. To explain the apparent nonexistence of classical phosphate intermediates, Buchanan *et al.* (1959) have suggested a concerted mechanism for nucleophilic displacement reactions involving three substrates and three reaction products. The mechanism they propose is applicable to the various syntheses that are coupled to the cleavage of ATP to ADP and P_i and can also be extended to the "decarboxylase" reaction (Fig. 1, scheme c), with the modification that one of the displacements is intra- instead of intermolecular.[4] Although the proposal of a concerted mechanism for reactions of this general type is attractive and rationalizes the majority of the relevant experimental findings, it is perhaps premature to abandon the search for intermediates, which may well be recognizable with the aid of more refined techniques.

Structure of Mevalonic Acid Pyrophosphate.—The structure of mevalonic acid pyrophosphate has not been rigidly proved, but it is well supported by several lines of evidence (Chaykin *et al.*, 1958; Henning *et al.*, 1959; Bloch *et al.*, 1959b). The retention of both phosphorus atoms and the failure of the P of ATP to enter isopentenylpyrophosphate during the decarboxylase reaction, the reversibility of the phosphomevalonic kinase reaction, and the acid stability all are compatible with the proposed pyrophosphate structure. The present experiments with O^{18} provide even stronger evidence for this assignment. The observed transfer of O^{18} from the substrate to inorganic phosphate can take place only if the tertiary hydroxyl group is unesterified, and hence the two phosphorus atoms which the molecule contains must be present as pyrophosphate

[3] The strongest evidence for consecutive events and for the existence of an activated intermediate has been obtained for glutamine synthesis in Meister's laboratory. Even in this case the evidence for an activated intermediate (γ-glutamylphosphate) is circumstantial (Krishnaswamy *et al.*, 1960).

[4] It should be noted that this part of the discussion refers only to the concerted (*versus* consecutive) interaction of the substrate with ATP and the associated cleavage of the nucleotide. The argument for the concerted nature of decarboxylation and hydroxyl group elimination of mevalonate-5-pyrophosphate has a different basis and rests on more direct experimental evidence (Bloch *et al.*, 1959b; deWaard *et al.*, 1959).

attached to the C-5 position. There exists the remote possibility that the product isolated from the phosphomevalonic kinase reaction is the 3,5,-diphosphate ester and that this is isomerized to the 5-pyrophosphate by an internal transphosphorylation (P-O cleavage) prior to decarboxylation. However, in this case the "decarboxylase" would possess still another catalytic function, and in any event the conclusion that mevalonate-5-pyrophosphate is the substrate for decarboxylation would not be invalidated.

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