Confirmation of 5-Pyrophosphomevalonate as an Intermediate in the Enzymatic Synthesis of Bacterial Phytoene from Mevalonate

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(Received June 25, 1963)

It is well known that MVA is a precursor of isoprenoid compounds, such as carotenoids and steroids, which are found universally in the biological world. In the course of their biosynthesis, MVA is believed to react with ATP by specific kinases to form phosphorylated compounds.[1] Though several phosphorylated intermediates have been confirmed in the case of the biosynthesis of steroids,[2-4] 5-P-MVA is the only intermediate which has been reported in carotenogenesis.[5]

The present paper is concerned with the confirmation of 5-PP-MVA as an intermediary product formed during the biosynthesis of phytoene from MVA or 5-P-MVA, and with its enzymatic conversion into phytoene.

As 5-P-MVA and 5-PP-MVA are formed from MVA by means of reactions with ATP catalyzed by enzymes extracted from a phytoene-accumulating mutant of *Staphylococcus*,[6] the pathway of the phytoene biosynthesis by means of this microorganism may be written as follows:

MVA→5-P-MVA→5-PP-MVA

→(Isopentenylpyrophosphate)⇢Phytoene

Experimental and Results

Chemicals and Instruments.[5,8] — The ATP used was equally labeled with ^{32}P at β and γ positions.[7] The prostatic phosphomonoesterase used was the generous gift of Professor Tomoji Suzuki of Kyoto University.

Radioactivity was measured by the instruments and techniques previously described.[5]

Preparation of Enzymes.—For the extraction of the cell-free enzymes from a phytoene-accumulating mutant of *Staphylococcus*, the method described previously was applied.[8] After the adjustment of the protein concentration of the crude extract to 10 mg./ml., a 2% solution of protamine was stirred into the diluted enzyme extract to give a final concentration of 0.1% protamine. The resulting supernatant solution was fractionated with ammonium sulfate. Mevalonic kinase was precipitated at 45~60%, phosphomevalonic kinase at 40~55%, and pyrophosphomevalonic decarboxylase, at 60~75% saturation. For the synthesis of 5-PP-MVA from MVA, the precipitate of the 40~60% ammonium sulfate-saturated fraction (tentatively named enzyme I) was employed, while with that of 5-PP-MVA from 5-P-MVA the 40~55% fraction (tentatively named enzyme II) was employed as the method of enzyme preparation.

A 0.1 M Tris buffer (pH 8.0) solution of the proteins precipitated from the crude extract at 0~90% saturation with ammonium sulfate was used for the synthesis of phytoene from MVA or 5-PP-MVA, as has been reported previously.[8]

Isolation and Identification of 5-Pyrophosphomevalonic Acid. — In the previous paper,[5] it was reported that an undetermined substance Y was found in an earlier stage of the enzymatic conversion of MVA to phytoene but that it disappeared in a later stage. Hence, it is quite reasonable to postulate that compound Y is one of the intermediates in the biosynthesis of phytoene from MVA. The enzymatic formation of compound Y from MVA or 5-P-MVA was established in the following experiment.

A reaction system which contained MVA and enzyme I, or 5-P-MVA and enzyme II, and ATP was incubated as shown in Table I. The incubation was carried out at 30°C for 30 min., and then the enzyme action was stopped by heating the reaction vessel at 100°C for 30 sec. After the denatured proteins had been removed by centrifugation, the

TABLE I. INCUBATION SYSTEMS FOR ENZYMATIC SYNTHESIS OF Y (5-PP-MVA) FROM MVA OR 5-P-MVA

Substrate	DL-MVA	5-P-MVA
	900 mμmol.	500 mμmol.
$MgSO_4$ (or $MnSO_4$)	40 μmol.	40 μmol.
ATP	80 μmol.	80 μmol.
Tris buffer (pH 7.5)	100 μmol.	100 μmol.
Enzyme	Enzyme I 0.5 ml.	Enzyme II 0.3 ml.
Protein concn.	7.5 mg./ml.	6.0 mg./ml.
Total volume	1.2 ml.	1.0 ml.
Incubation	30°C, 30 min.	30°C, 30 min.

* Abbreviations: MVA, Mevalonic acid; 5-P-MVA, 5-Phosphomevalonic acid; 5-PP-MVA, 5-Pyrophosphomevalonic acid.

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TABLE II. R_f VALUES OF COMPOUND Y (5-PP-MVA)

Solvent system	R_f
A	0.03～0.08
B	0.23～0.33
C	0.29～0.36
D	0.06～0.11

Pure compound Y (5-PP-MVA) enzymatically synthesized from 2-^{14}C-MVA was paper chromatographed (ascending) by the following four solvent systems. The component of each system: A, *n*-butanol - formic acid - water (77 : 10 : 13, by volume); B, isobutyric acid - concd. ammonia - water (66 : 3 : 30); C, *t*-butanol - formic acid - water (40 : 10 : 16); D, *t*-amyl alcohol - acetic acid - water (4 : 1 : 2).

supernatants were analyzed by column chromatography or paper chromatography.

By paper chromatographic techniques, using four solvent systems, it was found that the compound Y formed from 2-^{14}C-MVA gave the R_f values shown in Table II. These R_f values are identical with those of 5-PP-MVA previously reported.[3,4,9,10]

When the supernatant (1.1～0.9 ml., incubation scale: Table I) of the enzymatically-synthesized sample is paper chromatographed (paper Toyo-roshi No. 50, size 20 cm.×40 cm.) without prior purification, the observed R_f value of Y is 0.42～0.51 in the methanol - concentrated ammonia - water system. When, however, after the isolation of the material with R_f 0.42～0.51, it was rechromatographed with the same solvent, an R_f value of Y of 0.03～0.08 was observed, corresponding to that of purified 5-PP-MVA. This behavior during the initial chromatography may be attributed to the presence of a large amount of nucleotides.

After purification by paper chromatography using

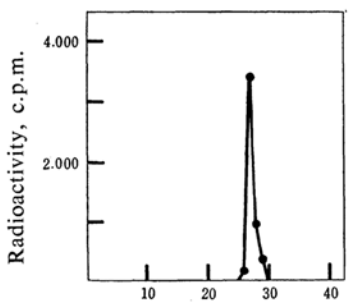

Fig. 1. Chromatographic analysis of compound Y on Dowex 1-formate column (1.7 cm.× 10 cm.).
Sample applied: 5000 c. p. m. (4.5 mμmol.).
Solution used for elution: Fractions 1—16, water; 17—26, 4 N formic acid; 27—31, 0.4 M ammonium formate in 4 N formic acid; 32—35, 0.8 M ammonium formate in 4 N formic acid.

two solvent systems (methanol - concentrated ammonia - water 'R_f 0.42～0.51' and isobutyric acid - concentrated ammonia - water 'R_f 0.23～0.33'), compound Y was further identified using column chromatography on a Dowex 1 - formate column (0.7 cm.×10 cm.). By this technique, too (Fig. 1), compound Y was identified with 5-PP-MVA.[11]

High-voltage paper electrophoresis was performed under the following conditions: buffer solution, pyridine - acetate (pH 6.2); 41 V./cm., 65 mamp.; 120 min.; paper: Toyo-roshi, No. 2. Here compound Y, 5-P-MVA and MVA migrated 21.8 cm., 21.6 cm. and 16.8 cm. respectively. The results also indicate that compound Y is 5-PP-MVA.[3]

Further confirmation was undertaken as follows. The ^{14}C, ^{32}P double-labeled compound Y was enzymatically synthesized from DL-2-^{14}C-MVA, AT^{32}P (β,γ position labeled) and the enzyme preparation described in Table I. The compound Y thus formed was purified at least three times by paper chromatography using the methanol - concentrated ammonia - water or isobutyric acid - concentrated ammonia - water solvent systems. The results are shown in Table III. From the ratio of ^{32}P to ^{14}C in the purified compound Y, it consists of MVA and phosphate in the molar ratio of 1 : 2.

The results of the analysis of the incubation products are summarized in Table IV, where it is shown that these incubation systems are suitable for the preparation of 5-PP-MVA.

TABLE III. RATIO OF ^{32}P TO ^{14}C OF DOUBLY LABELED Y

^{14}C c. p. m.	^{32}P c. p. m.	^{14}C mμ atom	^{32}P mμ atom	^{14}C : ^{32}P
3200	4872	32.0	60.9	1.0 : 1.9

AT^{32}P (80 μmol., specific activity: 8.0×10^4 c. p. m./μmol. in terminal P atom), $MgSO_4$ (40 μmol.), enzyme I (0.5 ml.; protein concentration: 7.5 mg./ml.), Tris buffer (pH 7.5, 100 μmol.) and DL-2-^{14}C-MVA (900 mμmol.; specific activity: 1.0×10^5 c. p. m./μmol.) in a volume of 1.2 ml. were incubated at 30°C for 30 min. The product obtained was purified by the method described in the text and radioactivity was measured in a gas-flow counter with and without aluminum window.

TABLE IV. ANALYSIS OF PRODUCTS AFTER INCUBATION (CF. TABLE I)

	Substrate DL-MVA %	5-P-MVA %
MVA	50	—
5-P-MVA	1	1
Compound Y (5-PP-MVA)	49	99

Enzymatic Hydrolysis of Compound Y. — ^{14}C-Labeled compound Y (4000 c. p. m., 3.6 mμmol. equiv.) and 5-P-MVA (4000 c. p. m., 3.6 mμmol. equiv.) were incubated separately for 30 min. at 37°C in a system which consisted of acetate buffer (pH 5.3, 2 μmol.), magnesium sulfate (1 μmol.),

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and purified prostatic phosphomonoesterase (0.01 ml.; protein concentration; 0.45 mg./ml.) in a volume of 0.1 ml. After the reaction had been stopped by dipping the vessel in boiling water for 30 sec., the incubation mixture was spotted on paper and developed with isobutyric acid - concentrated ammonia - water (66 : 3 : 30) and *t*-butanol - formic acid - water (40 : 10 : 16) systems. The results of analysis by actigraphy are summarized in Table V. The regeneration of 5-P-MVA and MVA by enzymatic hydrolysis was confirmed by paper chromatography.[5]

TABLE V. ENZYMATIC HYDROLYSIS OF COMPOUND Y (5-PP-MVA) AND 5-P-MVA

	Substrate			
	5-P-MVA		Y	
Time of incubation	10	30 min.	10	30 min.
Y	—	—	80	71%
5-P-MVA	2	1%	2	7
MVA	98	99	18	22

Radioactive 5-P-MVA and compound Y (4000 c. p. m., 3.6 mμmol., respectively) were hydrolyzed by prostatic phosphomonoesterase (0.01 ml.; protein concentration : 0.45 mg./ml.) with acetate buffer (pH 5.3, 2 μmol.) and $MgSO_4$ (1 μmol.) in a volume of 0.1 ml. Products were separated and analyzed by actigraphic method after paper chromatography with isobutyric aicd - concentrated ammonia - water and *t*-butanol - formic acid - water systems.

Enzymatic Synthesis of Bacterial Phytoene from 2-^{14}C-5-PP-MVA. — 2-^{14}C-5-PP-MVA (4000 c. p. m., 3.6 mμmol.) or DL-2-^{14}C-MVA (13000 c. p. m., 12 mμmol.) was incubated at 37°C for 3 hr. while being gently shaken with NADP (0.5 mg.), 6-phosphogluconate (2 μmol.), nicotinamide (50 μmol.), ATP (10 μmol.), FAD (0.05 mg.), a Tris buffer (pH 8.0, 40 μmol.), manganese sulfate (10 μmol.), magnesium sulfate (5 μmol.), Tween 20 (final concentration : 0.17%), KF (10 μmol.) and a 0.1 M Tris buffer (pH 8.0) solution of enzyme preparation precipitated at 90% saturation with ammonium sulfate (0.3 ml.; protein concentration : 42 mg./ml.) in a volume of 0.6 ml. The enzymatic reactions were stopped by the addition of 2 ml. of a 10% ethanolic sodium hydroxide solution to the mixture. *p*-Hydroquinone (10 mg.) and a petroleum benzine solution (1.00 ml.) of pure bacterial phytoene (absorbancy : 8.42 at 286 mμ) were added to the mixture simultaneously. After saponification, the unsaponifiable fraction was washed with water three times, and the aliquots were dried on aluminum planchets and their radioactivity counted as infinitely thin samples. The separation of the phytoene in the unsaponifiable fraction was carried out by chromatography on an activated-alumina column. The fractions containing purified phytoene were dried on aluminum planchets, and their radioactivities were measured. The results obtained are summarized in Table VI. The conversion of 5-PP-MVA or MVA to phytoene is evident.

TABLE VI. BIOSYNTHESIS OF PHYTOENE FROM MVA AND 5-PP-MVA

Substrate	Unsaponifiable fraction c. p. m.	Phytoene c. p. m.
DL-MVA (13000 c.p.m.; 12 mμmol.)	2550	1109
5-PP-MVA (4000 c.p.m.; 3.6 mμmol.)	3320	2625

Each system was consisted of NADP (0.5 mg.), 6-phosphogluconate (2 μmol.), nicotinamide (50 μmol.), ATP (10 μmol.), FAD (0.05 mg.), Tris buffer (pH 8.0, 40 μmol.), $MnSO_4$ (10 μmol.), $MgSO_4$ (5 μmol.), Tween 20 (final concentration : 0.17%), KF (10 μmol.), enzyme (0.3 ml., protein concentration : 42 mg./ml.) and the substrates indicated, in a volume of 0.6 ml. Incubation, in nitrogen (99.9%) for 3 hr. at 37°C.

Discussion

Compound Y is detected in an early stage of the biosynthesis of phytoene from MVA and 5-P-MVA. The behavior of this compound has now been shown to be identical with that of 5-PP-MVA, judging from paper chromatographic, column chromatographic, and paper electrophoretic criteria. It contains two atoms of phosphorus per molecule of mevalonic acid. One phosphorus atom has been shown to be located at the 5-position of MVA, as 5-P-MVA is regenerated by the hydrolytic action of prostatic phosphomonoesterase. The liberation of the first phosphorus moiety from compound Y is slow with this enzyme, as has been previously shown using 5-PP-MVA and other phosphomonoesterases.[9] The rapid regeneration of MVA is readily explicable, as the liberation of the second phosphorus is quite rapid, as in Table V shows. Thus, it seems reasonable to conclude that compound Y has one pyrophosphate linkage and that, after one phosphorus atom is liberated, the 5-P-MVA thus formed is readily susceptible to phosphomonoesterase action with the generation of MVA. On the basis of those facts compound Y has been concluded to be 5-PP-MVA, a substance which has been previously found in the pathway of squalene and cholesterol biosynthesis.[1-4]

5-PP-MVA was incorporated into phytoene with more than twice the efficiency of the DL-MVA, thus showing that 5-PP-MVA is also in the biosynthetic pathway of phytoene from MVA or 5-P-MVA.

Summary

In phytoene biosynthesis from mevalonic acid and 5-phosphomevalonic acid by the

enzyme preparation from the mutant of *Staphylococcus aureus*, a phosphorus-containing substance has been isolated as an intermediate compound. After purification by paper chromatography, column chromatography and paper electrophoresis, this substance has been shown to contain phosphorus and mevalonic acid in the molar ratio of 2 : 1. From its behavior with regard to prostatic phosphomonoesterase, together with the above-mentioned results, this substance has been concluded to be 5-pyrophosphomevalonic acid.

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