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STUDIES ON ISOPRENOID BIOSYNTHESIS WITH BACTERIAL INTACT CELLS

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SUMMARY: For the study on the regulation of isoprenoid biosynthesis with intact cells, some strains of bacteria capable of growing on mevalonate as a sole carbon source were isolated from soil. Many of them incorporated [^{14}C]-mevalonate, [^{14}C]isopentenyl- and [^{14}C]farnesyl pyrophosphates into the cells. However, radioactivity was found in their degradation products but not in isoprenoids. Addition of [^{14}C]isopentenyl pyrophosphate, farnesyl pyrophosphate and Mg^{2+} ions in combination to the culture of a strain of *Arthrobacter* gave rise to ^{14}C-incorporation into isoprenoids. Radioactivity was found in polyprenol, its pyrophosphate, monophosphate and fatty acid esters. The reactions of isopentenyl- and farnesyl pyrophosphates syntheses seemed to be rate-limiting steps.

The biosynthesis of isoprenoids in bacteria has been studied mainly with the cell-free system of *Lactobacillus plantarum* (1), *Micrococcus luteus* (2), and *Bacillus subtilis* (3). For the study on the regulatory mechanism of *de novo* biosynthesis of isoprenoids such as ubiquinone, menaquinone and sugar-carrier lipid, however, investigation with *in vivo* system using ^{14}C-labeled substrate is necessary. In this regard, it is well known that mevalonate, a practically important substrate for isoprenoid biosynthesis, is not taken up by most bacterial cells (4) with the following few exceptions: with the cells of *Lactobacilli* (5) and *Streptococcus mutans* (6) incapable of synthesizing the prenyl quinones, ^{14}C-incorporation into undecaprenol from [^{14}C]mevalonate was reported.

In the present investigation, various strains of bacteria which can grow on medium containing mevalonate as a sole carbon source were isolated from soil. Experiments with some strains of these bacteria showed that radioactivity was incorporated into polyprenol and its pyrophosphate, monophosphate

Abbreviations: IPP, isopentenyl pyrophosphate; FPP, farnesyl pyrophosphate

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and fatty acid ester when [^{14}C]isopentenyl pyrophosphate, farnesyl pyrophosphate and Mg^{2+} ions were given in combination to the culture.

MATERIALS AND METHODS

[1-^{14}C]Isopentenyl pyrophosphate (IPP) (specific radioactivity, 57 Ci/mol) and DL-[2-^{14}C]mevalonolactone (specific radioactivity, 53 Ci/mol) were purchased from Amersham International Ltd. and the latter was hydrolyzed with 0.5 M KOH for use. [9, 10-^{3}H(N)]Palmitic acid (specific radioactivity, 15.2 Ci/mmol) was obtained from New England Nuclear. [1-^{14}C]Farnesyl pyrophosphate (FPP) was enzymatically prepared from [^{14}C]IPP as described previously (7). Solanesol and ficaprenol-C_{50}, -C_{55} and -C_{60} were gifts from Dr. H. Fukawa in Central Laboratory of Nisshin Flour Milling Co. Phosphorylation of prenols was carried out by Kandutsch's method (8).

The bacteria which were capable of growing on mevalonate as a sole carbon source were isolated from soil by usual enrichment culture technique. The composition of medium for the isolation was 7 g of K_2HPO_4, 3 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $(NH_4)_2SO_4$, 1 g of $(NH_4)2HPO_4$, 14 g of sodium DL-mevalonate (Sigma Chemical Co.) per 1 l (pH 7.2). For the preculture of bacteria, the medium which was supplemented with 0.5% glycerol and 0.05% Yeast Nitrogen Base (Difco Laboratories, amino acid free) instead of mevalonate was used as a cultivation medium. The bacteria were grown on the medium for 48 h at 30°C. For the incorporation of radioactivity from ^{14}C-labeled substrate into the cells, the substrate and additions were added to the above cultivation medium (10 ml) and the preculture grown was added to the medium for inoculation (2%). The reason for the use of such a synthetic medium in the ^{14}C-incorporation experiments was that glucose, yeast extract and casamino acids strongly depressed the ^{14}C-incorporation. The cultivation was carried out for 72 h at 30°C. The cells were harvested, washed twice with 0.1 M potassium phosphate buffer (pH 6.8) and suspended in the same buffer. Radioactivity incorporated into the cells was counted with an aliquot of the suspension.

Two methods were used for the extraction of radioactive reaction products from the cells. One is petroleum ether extraction after saponification (9). The other is Bligh-Dyer's method (10) using chloroform–methanol (1 : 1) with slight modification. Unless otherwise indicated, analysis of the products was carried out using the following two methods: normal-phase TLC on Silica Gel 60 F_{254} plate using ethyl acetate–benzene (3 : 97) and reversed-phase TLC on Kieselguhr G plate impregnated with liquid paraffin using 90% acetone saturated with liquid paraffin. Radioactivity on the plate was detected with a Packard radiochromatogram scanner (model 7201) and authentic samples on the plate were detected by exposure to iodine vapour. Radioactivity was counted with a Packard liquid scintillation spectrometer (model 3375) using toluene scintillator for petroleum ether extract and toluene–Triton X-100 (2 : 1) scintillator for cell suspension and chloroform extract.

RESULTS

In order to find out bacteria which can take up mevalonate into the cells, an attempt was made to isolate bacteria capable of growing on mevalonate as a sole carbon source. Nineteen strains of bacteria with such a property were isolated from soil. All strains of the bacteria took up [^{14}C]mevalonate included in the medium. Some of the strains took up [^{14}C]IPP and [^{14}C]FPP when it was given with 2.4 mM $MgSO_4$. In these experiments, however, radioactivity

was found only in the compounds which seemed to be the degradation products. To confirm it, radioactivity in carbon dioxide which was expected to be produced from the above ^{14}C-labeled substrates was determined with three strains (including KD-2) belonging to *Arthrobacter*. Radioactive carbon dioxide was found to be formed from all of the substrates (data not shown). After several attempts were made, it was found that the addition of [^{14}C]IPP, FPP and Mg^{2+} ions in combination to the culture gave rise to the incorporation into isoprenoids. Figure 1 shows the incorporations of radioactivity into the cells and chloroform fraction from [^{14}C]IPP in strain KD-2 with increasing concentrations of FPP or Mg^{2+}, keeping the concentrations of other components constant. The incorporations increased with increasing concentrations of FPP and of Mg^{2+}. The use of GPP instead of FPP in these experiments gave a similar increase in the incorporations (data not shown). Replacement of [^{14}C]IPP with [^{14}C]mevalonate in this combination nullified the incorporations. Neither stimulation nor inhibition of the cell growth was observed in these experiments, in spite of the increase in the incorporations. Lipids in the chloroform fraction obtained from KD-2 were analyzed by TLC. The analysis of the lipids by normal-phase TLC gave rise to separation into five fractions (Frac-

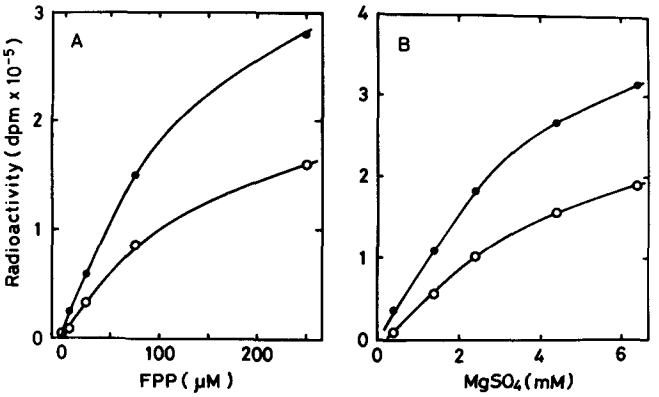

Fig. 1. Increase of ^{14}C-incorporation into the cells and chloroform fraction from [^{14}C]IPP in strain KD-2 with increasing concentrations of FPP(A) and Mg^{2+}(B). The cultivation medium (10 ml) for the ^{14}C-incorporation contained [1-^{14}C]IPP (8.8 x 10^5 dpm), 2.4 mM $MgSO_4$ and various concentrations of FPP in (A) and [1-^{14}C]IPP (5.7 x 10^5 dpm), 80 μM FPP and various concentrations of $MgSO_4$ in (B). The two media were inoculated with the bacterium and incubation was carried out for 72 h at 30°C. Radioactivities incorporated into the cells (●—●) and the chloroform fraction (o—o) were measured. The numbers on the ordinate represent total radioactivities per 10-ml culture.

tions I-V) (Fig. 2-A). The increase in the amount of FPP in the reaction mixture brought about an increase in the total incorporation into the chloroform fraction (Fig. 1) but did not change the relative incorporation into each fraction. Fraction II migrated to nearly the same distance as solanesol (all-*trans* C_{45}-polyprenol) in normal-phase TLC but showed a bigger mobility than solanesol in reversed-phase TLC (Fig. 2-B). This suggests that Fraction II is C_{35}- or C_{40}-polyprenol. Analysis of lipids in KD-2 cells by mass-spectrometry in a separate experiment showed that the cells contained dihydromenaquinone-8 (unpublished data). This fact suggests that Fraction II is all-*trans* C_{40}-polyprenol.

Fraction I was eluted with methanol from the plate (Fig. 2-A) and was separated on Silica Gel 60 F_{254} plate using *i*-propanol—conc. aqueous ammonia—water (6 : 3 : 1). Two radioactive peaks close to the authentic samples of

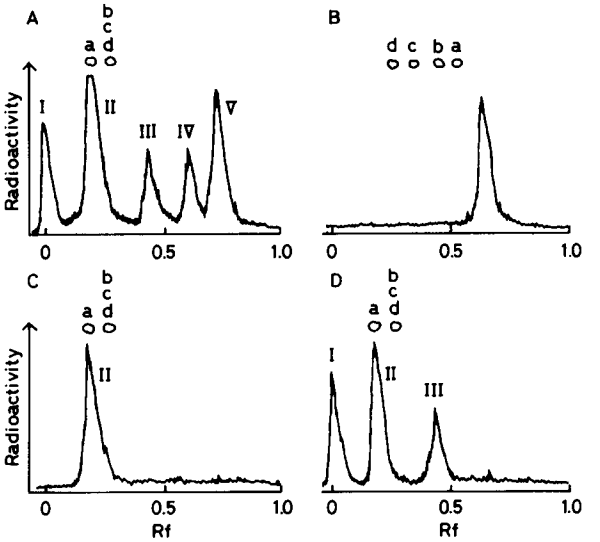

Fig. 2. Analysis of lipids from KD-2 by TLC. KD-2 cells were grown in the cultivation medium containing [1-^{14}C]IPP (8.8 x 10^5 dpm), 2.4 mM $MgSO_4$ and 100 μM FPP for 72 h at 30°C. The harvested cells were divided into two parts. One was subjected to an extraction with chloroform—methanol. The other was saponified as described and lipids were extracted with petroleum ether. The lipids were analyzed by normal- and reversed-phase TLC's. (A) shows normal-phase TLC pattern of lipids extracted with chloroform—methanol from the cells. Five peaks of radioactivity (Fraction I-V) appeared. (B) shows reversed-phase TLC pattern of Fraction II which was obtained by elution with diethyl ether from the plate in (A). (C) shows normal-phase TLC pattern of the substance obtained by extraction with petroleum ether after saponification of Fraction V which was eluted with diethyl ether from the plate in (A). (D) shows normal-phase TLC pattern of lipids extracted from the cells after saponification. The spots on each chromatogram represent the positions of authentic samples: a, solanesol (C_{45}, all-*trans*); b, ficaprenol (*cis*, *trans*-mixed)-C_{50}; c, ficaprenol-C_{55}; d, ficaprenol-C_{60}.

pyrophosphate and monophosphate of solanesol appeared. Treatment of Fraction I with acid phosphatase from potato (Boehringer Mannheim) by the method of Fujii *et al.* (11) gave the same polyprenol as Fraction II. Accordingly, Fraction I was confirmed to contain pyrophosphate and monophosphate of Fraction II.

Figure 2-D shows normal-phase TLC pattern of lipids which were extracted from KD-2 cells with petroleum ether after saponification. Comparison with Fig. 2-A shows that Fractions IV and V disappeared after saponification (Fig. 2-D). Fractions IV and V in Fig. 2-A were eluted from the plate, saponified and were extracted with petroleum ether, respectively. Fraction IV seemed to have been converted to alkali-soluble compound(s), since no radioactivity was detected in the extract. In contrast, the compound obtained by hydrolysis of Fraction V accorded with Fraction II in TLC (Fig. 2-C).

For further characterization of fraction V, KD-2 cells were grown in the cultivation medium containing [^{3}H]palmitate, IPP, FPP and $MgSO_4$. The lipids extracted with chloroform–methanol from the cells were mixed with ^{14}C-labeled Fraction V (Fig. 2-A) and the mixture was subjected to an alumina column chromatography. The fractions corresponding to ^{14}C-labeled Fraction V were col-

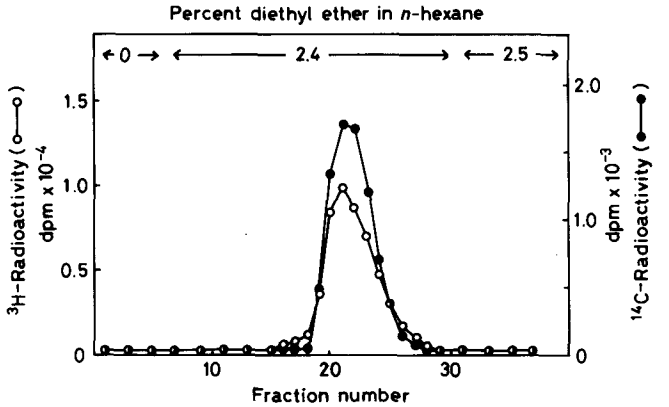

Fig. 3. Rechromatography of the mixture of ^{14}C-labeled Fraction V and the corresponding ^{3}H-labeled fraction on alumina column. KD-2 cells were grown in the cultivation medium (10 ml) containing [9, 10-^{3}H(N)]palmitate (27 μCi), 0.25 mM FPP, 0.54 mM IPP and 4.4 mM $MgSO_4$ for 72 h at 30°C. The lipids (3.5 x 10^6 dpm) extracted with chloroform–methanol from the harvested cells were mixed with Fraction V (3 x 10^4 dpm) in Fig. 2-A and the mixture was separated by chromatography on alumina column. The fractions of ^{14}C-radioactivity were collected and subjected to the similar alumina column (1 x 10 cm) rechromatography. The alumina used was Woelm Neutral Alumina of Brockmann activity grade II. The solvent used for elution was dietyl ether–*n*-hexane. Two-milliliter fractions were collected.

lected and subjected to a rechromatography on alumina column. As seen from Fig. 3, two peaks (^{14}C and ^{3}H) completely coincided with each other. These fractions were collected and saponified. After unsaponifiable fraction was separated by extraction with petroleum ether, the alkali layer was acidified with sulfuric acid and shaken with diethyl ether. From the unsaponifiable fraction, only ^{14}C-labeled polyprenol was detected. The diethyl ether extract gave a single ^{3}H-labeled spot which accorded with authentic sample of palmitic acid on TLC plate.

From these results, Fraction V was found to be fatty acid ester(s) of polyprenol.

Neither Fraction III nor Fraction IV accorded with dihydromenaquinone-8. But both or one of them seem to be related compounds to menaquinone. There was observed a very small mass peak which might correspond to sugar-carrier lipid but no radioactivity was found in it. Further investigation is necessary for the clarification.

DISCUSSION

The present investigation enabled the study of isoprenoid synthesis with intact cells of bacteria to be feasible. FPP and Mg^{2+} were found to be necessary for ^{14}C-incorporation into isoprenoids from [^{14}C]IPP. The stimulatory effect of Mg^{2+} on the incorporation may be explainable in terms of the interpretation that Mg^{2+} negates the negative charges of pyrophosphate group by making complex with the group to make it easy for IPP and FPP to penetrate through the hydrophobic cell membrane. The necessity of FPP in the incorporation was also observed in the polyprenyl transferase reaction in cell-free extracts of *Micrococcus luteus* (2). Sagami *et al.* explained that FPP acted as a primer in the reaction. The effectiveness of FPP for the incorporation *in vivo* system indicates that the intracellular level of FPP is very low. The fact that [^{14}C]IPP plus FPP are effective for the incorporation but not [^{14}C]-mevalonate plus FPP indicates that the intracellular concentration of IPP is very low as well. These phenomena also suggest that the syntheses of IPP and FPP are rate-limiting steps and are regulatory points.

The present investigation revealed the existence of fatty acid esters of polyprenol in bacteria. As such a compound, fatty acid esters of dolichol in pig liver (12) and *Aspergillus niger* (13), of betulaprenols in birch wood (14) and of solanesol in tobacco leaf (15) have been reported. However, no report on the existence of fatty acid ester of polyprenol originating from polyprenyl pyrophosphate for menaquinone synthesis in bacteria seems to have appeared before the present paper. The meaning of ester formation is likely that the excessively produced polyprenyl pyrophosphate ——presumably harmful for cell physiology—— is converted to the inactive fatty acid ester.

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REFERENCES

1. Allen, C.M., Jr., Keenan, K.J. and Sack, J. (1976) Arch. Biochem. Biophys. 175 236-248
2. Sagami, H., Ogura, K. and Seto, S. (1977) Biochemistry 16 4616-4622
3. Takahashi, I., Ogura, K. and Seto, S. (1980) J. Biol. Chem. 255 4539-4543
4. Raman, T.S., Sharma, B.V.S., Jayraman, J. and Ramasarma, T. (1965) Arch. Biochem. Biophys. 110 75-84
5. Thorne, K.J.I. and Kodicek, E. (1966) Biochem. J. 99 123-127
6. Thorne, K.J.I. (1973) Biochem. J. 135 567-568
7. Takatsuji, H., Nishino, T., Izui, K. and Katsuki, H. (1982) J. Biochem. 91 911-921
8. Kandutsch, A.A., Paulus, H., Levin, E. and Bloch, K. (1964) J. Biol. Chem. 239 2507-2515
9. Nagai, J., Katsuki, H., Nishikawa, Y., Nakamura, I., Kamihara, T. and Fukui, S. (1974) Biochem. Biophys. Res. Commun. 60 555-560
10. Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37 911-917
11. Fujii, H., Sagami, H., Koyama, T., Ogura, K., Seto, S., Baba, T. and Allen, C.M. (1980) Biochem. Biophys. Res. Commun. 96 1648-1653
12. Butterworth, P.H.W. and Hemming, F.W. (1968) Arch. Biochem. Biophys. 128 503-508
13. Stone, K.J. and Hemming, F.W. (1968) Biochem. J. 109 877-882
14. Lindgren, B.O. (1965) Acta Chem. Scand. 19 1317-1326
15. Rowland, R.L. and Latimer, P.H. (1959) Tob. Sci. 3 1-3