Formation of Dehydrosqualene in Microsomal Fraction of Rhodotorula Glutinis

AUTHOR(S):

Nishino, Tokuzo; Suzuki, Nobuhiro; Takatsuji, Hiroshi; Katsuki, Hirohiko

CITATION:

Nishino, Tokuzo ...[et al]. Formation of Dehydrosqualene in Microsomal Fraction of Rhodotorula Glutinis. Memoirs of the Faculty of Science, Kyoto University. Series of physics, astrophysics, geophysics and chemistry 1981, 36(1): 67-72

ISSUE DATE:

1981-12

URL:

http://hdl.handle.net/2433/257577

Memoirs of the Faculty of Science, Kyoto University, Series of Physics, Astrophysics, Geophysics and Chemistry, Vol. XXXVI, No. 1, Article 3, 1981

FORMATION OF DEHYDROSQUALENE IN MICROSOMAL FRACTION OF *RHODOTORULA GLUTINIS*

By

Tokuzo NISHINO, Nobuhiro SUZUKI, Hiroshi TAKATSUJI and Hirohiko KATSUKI

Department of Chemistry, Faculty of Science, Kyoto University, Kyoto

(*Received March 6, 1979*)

SUMMARY

When the microsomal fraction obtained from *Rhodotorula glutinis* was incubated with [^{14}C]farnesyl pyrophosphate, ^{14}C-incorporation into dehydrosqualene was demonstrated. The incubation in the presence of NADPH gave squalene instead of dehydrosqualene. When the microsomal fraction was incubated with [^{14}C]geranylgeranyl pyrophosphate, in contrast, radioactivity was incorporated neither into phytoene nor into carotene.

Dehydrosqualene, in the pathway for C_{30}-diapocarotene biosynthesis, is a compound which is believed to occupy a position corresponding to that of phytoene in the pathway for carotene biosynthesis. The fact that dehydrosqualene, not phytoene, was formed in the reaction using microsomal fraction of this yeast suggested that dehydrosqualene formation was catalyzed by the enzyme for squalene synthesis similarly to the case of *Saccharomyces cerevisiae*, but not by that for phytoene synthesis.

INTRODUCTION

We reported that dehydrosqualene was formed when the microsomal fraction obtained from *Saccharomyces cerevisiae* was incubated with farnesyl pyrophosphate. When the reaction was carried out in the presence of NADPH, only squalene was formed instead of dehydrosqualene. These results suggested that dehydrosqualene was formed being catalyzed by the enzyme for squalene synthesis (1).

Dehydrosqualene, in the pathway for C_{30}-diapocarotene synthesis, is a compound which occupies the position corresponding to that of phytoene in the pathway for carotene synthesis. Before the publication of our report (1), the compound had been found only from three species of bacteria, *Staphylococcus aureus* (2), *Halobacterium cutirubrum* (3) and *Streptococcus faecium* (4) which all belong to procaryote and had been reported to contain carotenes (3, 5, 6). With cell-free extracts of *Staph. aureus*, formation of dehydrosqualene from mevalonate was reported (7). After that, the presence of C_{30}-diapocarotene was reported from *Strep. faecium* (4, 8) and *Staph. aureus* (9). Although the possibility that dehydrosqualene functions as a precursor of C_{30}-diapocarotenes were pointed out, no demonstration has been made. As another possibility, if these bacteria do contain C_{40}-carotenes and if phytoene synthetase in the bacteria has a low substrate specificity, dehydrosqualene may be formed from farnesyl pyrophosphate being catalyzed by the enzyme.

As for sterol synthesis of bacteria, in contrast, no sterol has been demonstrated from most of bacteria and squalene also had been believed not to be present until about ten years ago. However, the presence of squalene was not the case and the compound was found in some bacteria including the above three bacteria (3, 4, 10). Especially with *Staph.* aureus, the incorporation of radioactivity from [^{14}C]farnesyl pyrophosphate into squalene was reported (10). This suggests the possibility that dehydrosqualene formation is concerned with squalene synthesis even in the bacteria which produce carotenes.

From these considerations, it is of importance to study the mechanism for dehydrosqualene formation in the organism producing carotenes. For this purpose, the microsomal fraction was prepared from *Rhodotorula glutinis* and it was left to react with [^{14}C]farnesyl pyrophosphate, [^{14}C]presqualene pyrophosphate or [^{14}C]geranylgeranyl pyrophosphate. As a result of experiments, dehydrosqualene was found to be formed from the former two compounds but phytoene was not formed from the latter compound. This strongly suggested that dehydrosqualene formation was concerned with squalene synthesis in *R. glutinis* as well as in *S. cerevisiae* (1).

MATERIALS AND METHODS

[^{14}C]Farnesyl pyrophosphate and [^{14}C]presqualene pyrophosphate (specific radioactivities, 66 and 340 Ci/mol, respectively) were prepared as reported previously (1). [^{14}C]Geranylgeranyl pyrophosphate (specific radioactivity, 114 Ci/mol) was prepared with some modifications according to the method of Ogura *et al.* (11). *Rhodotorula glutinis* IFO 0389 was grown in a medium containing 2% glycerol as a sole carbon source for 20 h at 28°C (12). The harvested cells were disrupted and the microsomal fraction was prepared as described previously (1).

The mixture for the enzyme reaction contained, in a final volume of 1.0 ml, 2 μmol of $MnCl_2$ (or 5 μmol of $MgCl_2$), 0.1 μmol of dithiothreitol, 20 μmol of KF, 30 μmol of potassium phosphate buffer (pH 6.8), the microsomal fraction, and the radioactive substrate described above and additions as indicated. The reaction was carried out in the presence and absence of NADPH (2 μmol) for 1 h at 30°C.

After the reaction, the mixture was heated with 2 N KOH for 1 h at 60°C and nonpolar lipids were extracted with petroleum ether. The radioactive nonpolar lipids were separated by two kinds of TLC as described previously (1) and counted for their radioactivities. Normal-phase TLC was carried out on silica gel using benzene-ethyl acetate (4: 1, V/V) as a solvent system. Reversed-phase TLC was carried out on Kieselguhr G plate impregnated with liquid paraffin using 90% aqueous acetone saturated with liquid paraffin.

RESULTS

Identification of Dehydrosqualene Produced from Farnesyl Pyrophosphate

The microsomal fraction obtained from *R. glutinis* was incubated with [^{14}C]-farnesyl pyrophosphate (155,000 dpm) and Mn^{2+} for 2 h at 30°C. After the reaction, the mixture was treated with alkali and nonpolar lipids were extracted with petroleum ether. Analysis of the lipids (28,000 dpm) by normal-phase TLC showed that about one-fourth the radioactivities was located in the fraction corresponding to squalene. Further analysis of the radioactive lipid eluted from the fraction by reversed-phase

TLC showed that radioactivity was in the fraction corresponding to dehydrosqualene, not to squalene. The radioactive lipid was mixed with the authentic sample of *cis*-dehydrosqualene isolated from *Staph. aureus* (2) and the mixture was subjected to the column chromatography on alumina reported previously (1). The elution pattern of radioactivity accorded completely with that of absorbance at 285 nm where the absorption maximum of *cis*-dehydrosqualene exists. For identification of the structure of radioactive lipid, a part of the mixture, after irradiated under UV light in *n*-hexane containing iodine for *cis-trans* isomerization (2, 13), was subjected to the alumina chromatography similarly as described above. Figure 1 shows coincidence of elution pattern of radioactivity with that of absorbance at 285 nm. Two peaks appeared — one (corresponding to *cis*-dehydrosqualene) eluted with 0.7% ether in *n*-hexane and the other (corresponding to *trans*-dehydrosqualene) eluted with 1.5% ether in *n*-hexane (13, 14). From these results, the radioactive lipid obtained by incubation of the microsomal fraction with [^{14}C]farnesyl pyrophosphate and Mn^{2+} was identified as *cis*-dehydrosqualene.

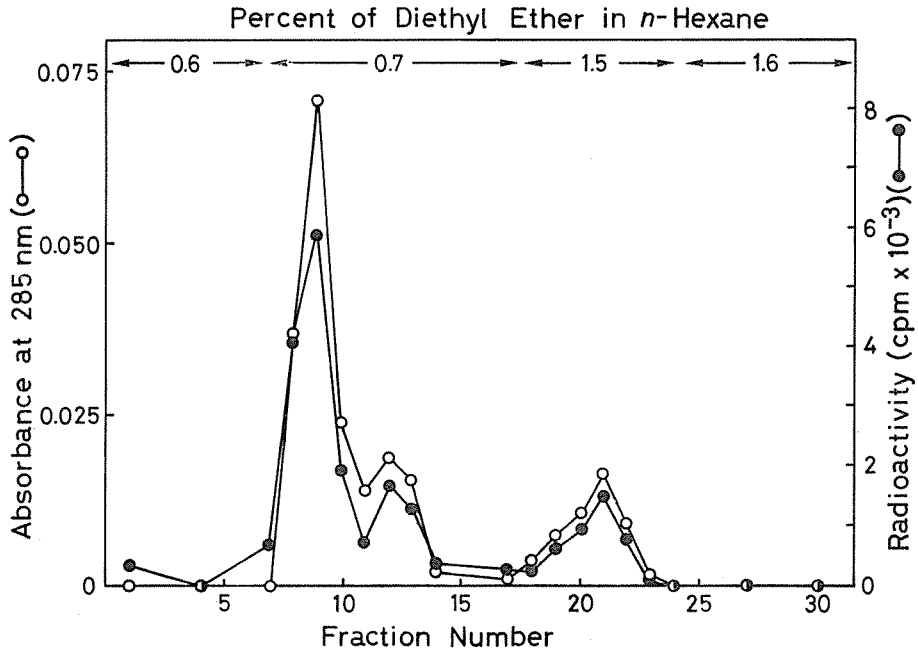

Fig. 1. Coincidence of elution pattern of radioactivity with that of absorbance due to *cis*- and *trans*-dehydrosqualenes in alumina chromatography. The microsomal fraction (1 mg of protein) was incubated with [^{14}C]farnesyl pyrophosphate (1.1 nmol, 155,000 dpm) and 2 mM $MnCl_2$ (final volume, 1 ml) for 2 h at 30°C. Nonpolar lipids which were extracted with petroleum ether from the reaction mixture after alkali treatment were separated by normal-phase TLC. The radioactive lipid eluted from the fraction corresponding to dehydrosqualene was mixed with the authentic sample of *cis*-dehydrosqualene isolated from *Staph. aureus* and the mixture was dissolved in *n*-hexane containing iodine (about 2% of dehydrosqualene in weight) and was irradiated under UV light to allow the *cis-trans* isomerization. The mixture, after treatment with sodium thiosulfate to reduce the iodine, was subjected to the column chromatography on alumina. Elution was made using *n*-hexane containing ether by increasing the concentration of ether stepwisely and radioactivity and absorbance at 285 nm were monitored.

Effects of Mn^{2+} and NADPH on Dehydrosqualene Formation from Farnesyl Pyrophosphate

Table I shows effects of Mn^{2+}, Mg^{2+} and NADPH on ^{14}C-incorporation from [^{14}C]farnesyl pyrophosphate (24,000 dpm) into nonpolar lipids. Total radioactivities incorporated into nonpolar lipids after reaction in the presence of Mn^{2+} were 4,050 dpm. Almost 30% of the radioactivity was found in dehydrosqualene and the remainder in other lipids mostly made up of farnesol and the compound which seemed to be presqualene alcohol. Replacement of Mn^{2+} with Mg^{2+} markedly decreased the incorporation into dehydrosqualene, indicating the effectiveness of Mn^{2+} for dehydrosqualene formation similarly to the case of *S. cerevisiae*. When the reaction was carried out in the presence of NADPH and Mn^{2+}, radioactivity was incorporated only into squalene, not into dehydrosqualene. Separate experiment showed that the formation of squalene was not dependent on the kind of divalent metal ions. It is evident that the formation of dehydrosqualene was an enzymic reaction since replacement of the enzyme with the boiled enzyme in the reaction did not give radioactive products.

Table 1. ^{14}C-Incorporation from [^{14}C]farnesyl phyrophosphate into nonpolar lipids. The reaction mixture (final volume, 1 ml) contained [^{14}C]-farnesyl pyrophosphate (0.17 nmol, 24,000 dpm), 0.1 μmol of dithiothreitol, 20 μmol of KF, 30 μmol of potassium phosphate buffer (pH 6.8) and indicated additions (2 μmol of $MnCl_2$, 5 μmol of $MgCl_2$, 2 μmol of NADPH, the enzyme (0.5 mg of protein or heat-inactivated enzyme). The reaction was carried out for 1 h at 30°C and, after alkali treatment, nonpolar lipids were extracted. They were separated by normal-phase and reversed-phase TLC. The fractions corresponding to dehydrosqualene, farnesol, presqualene alcohol and squalene were scraped from the plates and radioactivity in every fraction was counted.

Additions	Radioactivity of Products (dpm)			
	Total	Dehydro-squalene	Others	Squalene
1. Enzyme, Mn^{2+}	4,050	1,150	2,900	0
2. Enzyme, Mg^{2+}	3,400	220	3,200	0
3. Enzyme, Mn^{2+}, NADPH	7,200	50	200	6,800
4. Boiled enzyme, Mn^{2+}	300	20	250	0

^{14}C-Incorporation from [^{14}C] Presqualene Pyrophosphate into Nonpolar Lipids

When the reactions were carried out using [^{14}C]presqualene pyrophosphate (0.04 nmol, 30,000 dpm) instead of [^{14}C]farnesyl pyrophosphate as a substrate in the presence and absence of NADPH, radioactivities were incorporated into squalene and dehydrosqualene, respectively. Also in this case, Mn^{2+} was much more effective than Mg^{2+} for the dehydrosqualene formation. Differently from the experiment using [^{14}C]farnesyl pyrophosphate, a compound which seemed to be presqualene alcohol was formed, instead of the formation of farnesol, besides dehydrosqualene in the incubation without NADPH.

^{14}C-Incorporation from [^{14}C]Geranylgeranyl Pyrophosphate into Nonpolar Lipids

When similar reaction was carried out using [^{14}C]geranylgeranyl pyrophosphate (0.11 nmol, 28,000 dpm) instead of [^{14}C]farnesyl pyrophosphate, very little radioactivity, almost equal to that in control experiment using boiled enzyme, was incorporated into nonpolar lipids. Separation of the lipids by normal-phase TLC showed that very little activities were found in geranylgeraniol, geranyllinalool and unknown compound which were thought to be contaminants in the substrate. No radioactivity was incorporated into phytoene and lycopersene. Similar pattern was observed in the control experiment. No variation was found in the pattern obtained by the incubation with NADPH. These results indicate that no formation of C_{40}-compound corresponding to squalene in C_{30}-series was found in the microsomal fraction of *R. glutinis*, though squalene or dehydrosqualene was formed depending on the reaction condition. In this regard, Qureshi *et al.* reported that lycopersene was formed by the catalysis of squalene synthetase in *S. cerevisiae* (15). This possibility seemed to be excluded by our present study. Our present studies strongly suggest that dehydrosqualene is not formed by the catalysis of phytoene synthetase owing to its low substrate specificity but the formation is closely related to the squalene synthetase reaction.

DISCUSSION

Some reports appeared on the demonstration of C_{40}-carotenes in *R. glutinis*, such as the finding of β-carotene, γ-carotene, torulene and torularhodin (12, 16, 17), and the demonstration of ^{14}C-incorporation from [^{14}C]mevalonate into torularhodin (18). As for the formation of lycopersene, a compound related to carotene biosynthesis, Qureshi *et al.* reported that squalene synthetase catalyzed a formation of lycopersene from geranylgeranyl pyrophosphate in *S. cerevisiae* (15). However, Ogura *et al.* studied on the substrate specificity of squalene synthetase using the microsomal fraction of pig liver and reported that the enzyme had a high substrate specificity: the product with the longest carbon chain was bis-homo-squalene, and tetra-homo-squalene was not formed (19). Our present studies also showed that the microsomal fraction of *R. glutinis* catalyzed the formation of squalene and dehydrosqualene depending on the reaction condition but not the formation of phytoene or C_{40}-compound from geranylgeranyl pyrophosphate. This suggests that the dehydrosqualene formation was not catalyzed by phytoene synthetase owing to its low substrate specificity and that the C_{40}-compound synthesis was not catalyzed by squalene synthetase. Studies on the intracellular distribution of phytoene synthetase are now in progress.

From the three species of bacteria which produce dehydrosqualene — *Staph. aureus* (2), *H. cutirubrum* (3), and *Strep. faecium* (4) —, squalene was demonstrated (3, 4, 10). These facts suggest that dehydrosqualene is formed in these bacteria in a similar mechanism to that in *S. cerevisiae* and *R. glutinis*. However, the possibility that a distinct enzyme from squalene synthetase is involved in the dehydrosqualene formation and in the succeeding synthesis of C_{30}-diapocarotene can not be excluded, since many kinds of C_{30}-diapocarotenes were found in *Strep. faecium* (4, 8, 20, 21). From consideration of the squalene distribution in some bacteria, its low content and of its unrevealed role in cellular metabolism, it can not be ruled out the possibility

that squalene is formed secondarily being catalyzed by "dehydrosqualene synthetase" in the above described bacteria. The mechanism for dehydrosqualene formation in these bacteria are necessary to be elucidated.

The authors are grateful to Prof. K. Soda of Institute for Chemical Research, Kyoto University, for kind gift of *R. glutinis*. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1) Nishino, T., Takatsuji, H., Hata, S., & Katsuki, H., (1978) *Biochem. Biophys. Res. Commun.* **85**, 867–874.
2) Suzue, G., Tsukada, K., & Tanaka, S., (1968) *Biochim. Biophys. Acta* **164**, 88–93.
3) Kushwaha, S. C., Pugh, E. L., Kramer, J. K. G., & Kates, M., (1972) *Biochim. Biophys. Acta* **260**, 492–506.
4) Taylor, R. F. & Davies, B. H., (1974) *Biochem. J.* **139**, 751–760.
5) Suzue, G. (1959) *J. Biochem.* (Tokyo) **46**, 1497–1504.
6) Cosenza, B. J. & Girard, A. E., (1970) *Bacteriol. Proc.* p. 45.
7) Suzue, G., (1962) *J. Biochem.* (Tokyo) **51**, 246–252.
8) Taylor, R. F. & Davies, B. H., (1974) *Biochem. J.* **139**, 761–769.
9) Shinoi, K., Katayama, K., Hayami, M. & Kanemasa, Y., (1977) *Proc. Jpn. Conf. Biochem. Lipids* (Japan) **19**, 11–13.
10) Suzue, G., Tsukada, K., Nakai, C. & Tanaka, S., (1968) *Arch. Biochem. Biophys.* **123**, 644.
11) Ogura, K., Shinka, T. & Seto, S., (1972) *J. Biochem.* (Tokyo) **72**, 1101–1108.
12) Simpson, K. L. & Chichester, C. O., (1971) *The Yeasts* (Rose, A. H. & Harrison, J. S., ed.) Vol. 2, pp. 493–515, Academic Press, London.
13) Jungalwala, F. B. & Porter, J. W., (1965) *Arch. Biochem. Biophys.* **110**, 291–299.
14) Than, A., Bramley, P. M., Davies, B. H. & Rees, A. F., (1972) *Phytochem.* **11**, 3187–3192.
15) Qureshi, A. A., Barnes, F. J., Semmler, E. J. & Porter, J. W., (1973) *J. Biol. Chem.* **248**, 2755–2767.
16) Kockova-Kratochvilova, A. & Bystricky, S., (1974) *Mycopathologia Mycolog. applicata* **54**, 409–419.
17) Haynma, E. P., Chichester, C. O. & Simpson, K. L., (1974) *Phytochem.* **13**, 1123–1127.
18) Tefft, R. E., Goodwin, T. W. & Simpson, K. L. (1970) *Biochem. J.* **117**, 921–927.
19) Ogura, K., Koyama, T. & Seto, S., (1972) *J. Amer. Chem. Soc.* **94**, 307–309.
20) Davies, B. H., (1975) *Ber. Deutsch. Bot. Ges.* **88**, 7–25.
21) Davies, B. H. & Taylor, R. F., (1976) *Pure Appl. Chem.* **47**, 211–221.