The Journal of Biochemistry, Vol. 61, No. 2, 1967

Detection of Some Isoprenoids and the Influence of Diphenylamine on the Biosynthesis of Isoprenoid by *Sporobolomyces shibatanus*

By Yoshiko Kakutani

(*From the Department of Chemistry, Faculty of Science, Kyoto University, Kyoto*)

(Received for publication, June 20, 1966)

1. Carotenoids, ubiquinone, sterol and an unknown quinone were found to be present in the cells of *Sporobolomyces shibatanus*.

2. The ubiquinone found was identified as CoQ_{10} by its behavior upon paper chromatography.

3. The unknown quinone showed absorption maxima at 265 and 272 mμ in hexane and 274 mμ in ethanol. It also exhibited a spectral change resembling that of ubiquinone on reduction with sodium borohydride.

4. The biosyntheses of isoprenoids in the cells of yeast were shown to vary during the course of cell growth.

5 DPA was observed to inhibit not only the production of carotenoids but also the syntheses of other isoprenoids.

It has been reported that diphenylamine (DPA) specifically inhibits the biosynthesis of carotenoids (*1—4*), and in recent years, this amine was also shown to inhibit electron transport in the mitochondria of plant cells (*5*) and the biosynthesis of ubiquinone in *Rhodospirillum rubrum* (*6*).

As a means of studying the mechanism of carotenogenesis, the author has examined the effect of DPA on carotenogenesis in *Sporobolomyces shibatanus*.

In this paper, it is reported that carotenoids, CoQ_{10}, sterol and an unknown quinone are contained in this yeast, and that the content of these isoprenoids decreases in cells grown in a medium containing DPA.

EXPERIMENTAL

Material—DPA used was of commercial origin and was purified twice by recrystallization from ethanol. Hexane used as solvent was purified by distillation after successive washings with concentrated sulfuric acid, a mixture of concentrated sulfuric acid and concentrated nitric acid (10:1), concentrated sulfuric acid and a 2% aqueous solution of sodium hydroxide. Ethanol used for saponification was purified by distillation after refluxing with zinc and solid potassium hydroxide for 2 hours. Ether used was first treated with *p*-hydroquinone to remove peroxides and then freshly distilled. Other reagents used were from commercial sources.

Culture—Sporobolomyces shibatanus (IFO No. 1103) was provided by the Institute for Fermentation, Osaka. After inoculation, the slant was incubated for 72 hours at 28°C. The growth from one slant was used to inoculate 100 ml. of liquid medium as described in a previous paper (*7*). After a growth period of 48 hours at 28°C, this culture was used as the inoculant for 250 ml. of the same liquid medium contained in a one liter Erlenmyer flask (*7*). The culture was incubated at 28°C with shaking.

Aspergillus niger (IFO No. 4414) and *Neurospora crassa* (IFO No. 6067) was provided by the Institute for Fermentation, Osaka. The cells were grown for 7 days at 28°C without shaking in a medium described by Raman *et al.* (*8*).

E. coli strain W was provided by the Department of Medical Chemistry, Kyoto University, Faculty of Medicine, Kyoto. The cells were grown in a medium containing beef extract, 10 g.; pepton, 10 g. and sodium chloride, 5 g. per liter; pH 7.0 for 24 hours at 28°C with shaking.

Baker's yeast (Oriental Brand, Osaka) was cultured as described by Sugimura and Rudney (*9*).

Isolation of Isoprenoids—Cells were harvested by centrifugation (3,000 r.p.m. for 10 minutes) and washed once with water. After successive freezing and thawing, the cell paste was ground with 2.5 g. of carborundum (600 mesh) per g. of wet cells for 10 minutes under cooling. The ground mass was treated with 15% w/w of potassium hydroxide, 10% w/w of pyrogallol, 150% v/w of ethanol and 10 ml. of hexane. The mixture was heated for 20 minutes at 80°C, and after cooling, it was diluted with water and the unsaponifiable compounds were extracted with hexane (*10*). The extracted unsaponifiable material was washed with distilled water, dried over anhydrous sodium sulfate and then chromatographed according to the following procedure.

Chromatographic Separation—The unsaponifiable material in the extract was fractionated by chromatography on a column (0.7×10 cm.) of methanol deactivated alumina. Deactivated alumina was mixed with 4 volumes of methanol, stirred well for 3 hours, allowed to stand overnight and the suspended particles decanted off. The deactivated alumina was dried at room temperature.

Elution was carried out with hexane containing 6%, 8%, 10% and 15% of ether and finally with ethanol (Fig. 1). The carotenoid fraction eluted with hexane containing 6% ether was rechromatographed on a column (0.7×10 cm.) containing a 5:1 mixture of activated and deactivated alumina. A solvent system which consisted of increasing concentrations of ether (1%, 3%, 5%, 7%, 10% and 20%) in hexane was used to elute carotenoids from the second column (Fig. 2).

Determination and Estimation of Isoprenoids—Since different isoprenoids show characteristic absorption spectra (*10*, *14* and *15*), the absorption spectrum of each fraction eluted from the column was measured at wavelengths between 220 mμ—600 mμ with a Hitachi Recording Spectrophotometer EPS-2. The isoprenoid contents in the fraction eluted from the column as shown in Fig. 1 were determined by measuring the absorption spectrum between 220 mμ—340 mμ and the quantities of individual carotenoid fraction shown in Fig. 2 were determined by measuring the absorbancies in the range between 340 mμ—600 mμ. Isoprenoids were identified by their absorption spectra and quantitatively measured by their absorbancy. Maximal absorbance and extinction value of the compounds are listed in Table I.

Paper Chromatography—Ascending paper chromatography was carried out with Toyō Roshi No. 50 filter paper with 1-propanol-water (4:1) as solvent. Paper had been impregnated beforehand with 5% liquid paraffin in hexane.

TABLE I

Optical Properties of Isoprenoids

Compounds	Absorption		Reference
	Maximum mμ	$E^{1\%}_{1\,cm.}$	
Phytofluene	331 348 367	1,200	*12*
ζ-Carotene	376 396 418	2,000	*11*
Neurosporene	414 437 467	2,900	*11*
γ-Carotene	434 459 490	2,760	*12*
β-Carotene	428 450 480	2,590	*12*
Torulene	458 483 520	1,870[1)]	*13*
Sterol (ergosterol)	260 271 282 293	308	*15*
CoQ_{10}	275	165	*10*

1) $E^{1\%}_{1\,cm.}$ was calculated from moler extinction coefficient.

Ubiquinone was detected with neotetrazolium spray according to the method of Lester and Ramasarma (*16*). Thus, quinone was first reduced by immersing the paper in 0.1% sodium borohydride aqueous solution for 30 seconds. The paper was dried briefly and dipped in 0.1 *N* hydrochloric acid solution for 2 seconds to neutralize any excess borohydride. After drying again, the paper was sprayed with an aqueous solution containing 0.25% w/v neotetrazolium chloride and 0.25 *M* potassium phosphate buffer, pH 7.0, and was heated for 60 seconds at 100°C.

Sterol was chromatographed as described above with methanol, 1-propanol and distilled water (82:15:3) as solvent. Sterol was detected by immersing the paper

in 0.1 *M* aqueous permanganate solution for one second. The excess parmanganate was washed out by dipping the paper in hot water three times.

RESULTS AND DISCUSSION

The presence of several isoprenoids (carotenoids, ubiquinone and sterol) in cell extracts of *Sporobolomyces shibatanus* was demonstrated by chromatography as shown in Fig. 1.

The carotenoids eluted from the column of deactivated alumina (Fig. 1) were resolved into individual carotenes (phytofluene, ζ-carotene, neurosporene, γ-carotene, β-carotene and torulene) by rechromatography with a column of activated and deactivated alumina (5 : 1) as described above (Fig. 2). Each carotene was characterized by its absorption spectrum between 300 mμ—600 mμ and was estimated by measuring the absorbancy at its absorption maximum.

It was found that relatively large amounts of β-carotene and torulene were contained in the cells of yeast grown in medium without DPA. Upon the addition of DPA, the content of the more highly unsaturated carotenes were decreased as compared with cells grown without DPA as shown in Table II.

The ubiquinone fraction eluted from the column (Fig. 1) was identified by measurement of its absorption spectrum (around the 275 mμ region) and by the change in absorption spectrum on reduction with borohydride. On paper chromatography of ubiquinone with the

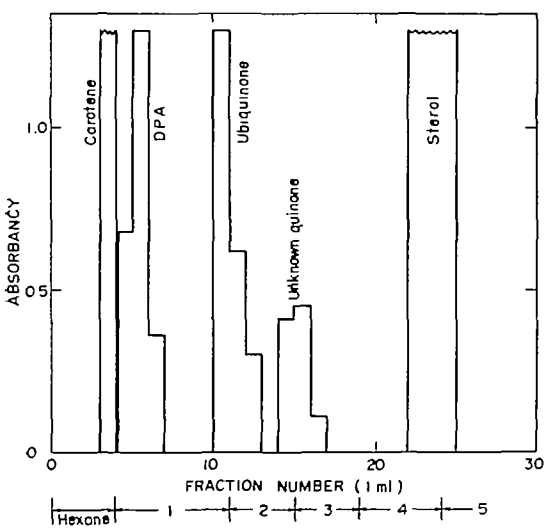

FIG. 1. Separation of isoprenoids by column chromatography on deactivated alumina.

A column of deactivated alumina was eluted successively with hexane containing ether (6% (1), 8% (2), 10% (3) and 15% (4)), and then with ethanol (5). The absorption spectrum of each fraction eluted from the column was measured at wavelengths between 220 mμ—600 mμ. Individual isoprenoids were identified by their characteristic absorption spectra and were measured by their absorbancy at their absorption maxima *i. e.*, ubiquinone at 275 mμ, unknown quinone at 265 mμ and sterol at 282 mμ. DPA showed an absorption maximum at 286 mμ.

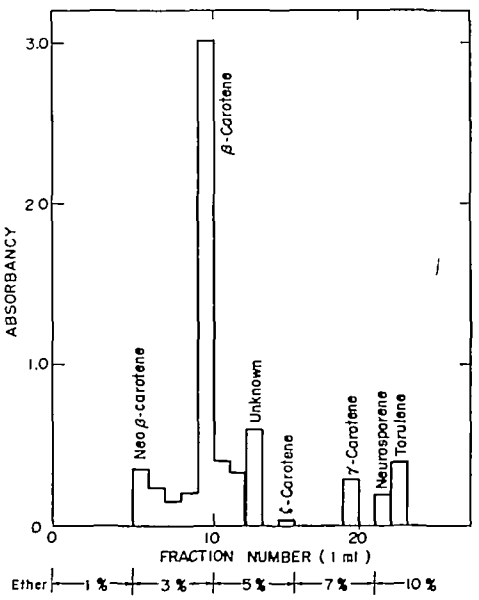

Fig. 2. Separation of carotenoids by column chromatography on alumina.

The carotenoid fraction eluted from the column of deactivated alumina (Fig. 1) was further fractionated by column chromatography on a 5 : 1 mixture of activated and deactivated alumina. Chromatography was carried out with hexane containing ether (1%, 3%, 5%, 10% and 20%) as solvent. The absorption spectrum of each fraction eluted from the column was measured at wavelengths between 340 mμ—600 mμ. Individual carotenes were identified by their absorbancy at their absorption maxima *i. e.*, β-carotene at 450 mμ, ζ-carotene at 396 mμ, γ-carotene at 459 mμ, neurosporene at 437 mμ and torulene at 520 mμ.

solvent system as described above, only a single spot was observed.

The ubiquinone extracted from yeast cells was identified as CoQ_{10} by comparing it with standard samples of ubiquinone (CoQ_6, CoQ_8, CoQ_9 and CoQ_{10}), which were obtained and purified from baker's yeast, *E. coli*, *Aspergillus niger* and *Neurospora crassa*, respectively (Table III).

Sterol extracted from yeast cells was separated by column chromatography on deactivated alumina. This sterol gave a single spot (R_f: 0.64) upon paper chromatogram, showed the same color reaction as ergosterol with the Liebermann-Burchard test, and formed digitonide by the action of digitonine in ethanol solution. The ultraviolet absorption maxima of this sterol were observed to be identical with those of ergosterol at 260, 271, 282 and 293 mμ.

During chromatography on the column (Fig. 1), a substance was eluted between ubiquinone and sterol which had absorption maxima at 265 mμ and 272 mμ in hexane and at 274 mμ in ethanol. The absorption spectrum of this fraction in hexane was different from that of ubiquinone, but this substance was observed to undergo the same change in absorbance at 274 mμ after reduction (Fig. 3). On paper chromatography, this substance showed a behavior different from that of CoQ_{10} although it was recognized to be a quinone by its reaction with neotetrazolium. This quinone had approximately the same R_f

TABLE II

Contents of Colored Carotenoids in Cells Grown in the Presence and Absence of DPA

Carotene	Contents of colored carotenes	
	Control μg./g. (dry weight)	DPA added μg./g. (dry weight)
Phytofluene	—	0.1
ζ-Carotene	0.5	0.1
γ-Carotene	1.8	—
Neurosporene	0.7	—
β-Carotene	7.3	0.6
Torulene	8.0	0.1

Cells precultured for 48 hours at 28°C were transferred to 250 ml. of medium with or without 2 mg. of DPA and were cultured for 77 hours at 28°C with shaking. The content of each carotene was estimated by measuring its absorbancy after separation by column chromatography on alumina.

TABLE III

R_f Values for Paper Chromatography of Ubiquinones Obtained from Different Micro-organisms

Sources	Quinones	R_f
Baker's yeast	CoQ_6	0.82
E. coli	CoQ_8	0.56
Aspergillus niger	CoQ_9	0.37
Neurospora crassa	CoQ_{10}	0.23
Sporobolomyces shibatanus	(CoQ_{10})	0.23
Sporobolomyces shibatanus	Unknown quinone	0.38

Ascending paper chromatography was carried out with the use of 1-propanol-water (4:1) as solvent. Spots were located with neotetrazolium spray after reducing with sodium borohydride.

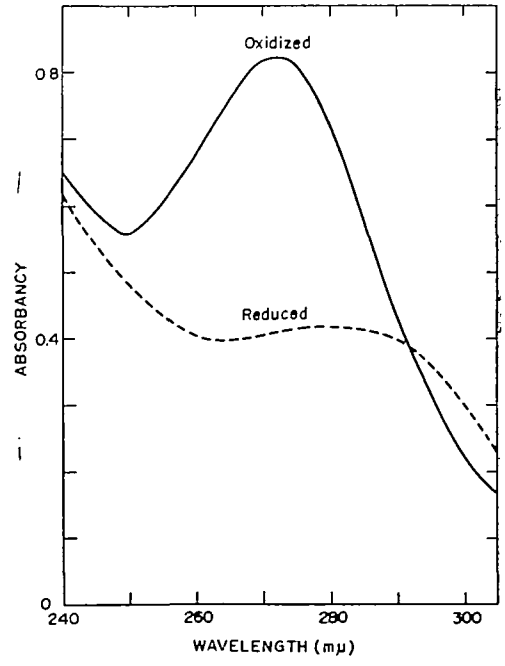

FIG. 3. Change in the absorption spectra of the unknown quinone.

The absorbancy of the unknown quinone was measured before (——) and after (----) reduction with $NaBH_4$.

value as CoQ_9 on the paper chromatogram, but its absorption spectrum in hexane was different from that of CoQ_9. Perhaps this unknown quinone in the yeast cells is a derivative which differs slightly from ubiquinone in structure.

Variations in the isoprenoid content of cells were investigated during proliferation of

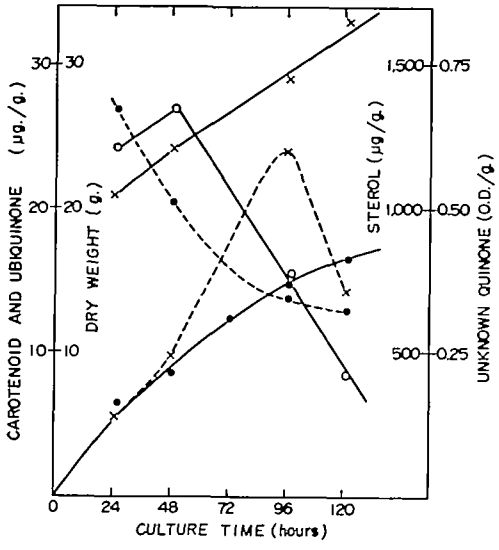

FIG. 4. Isoprenoid content in yeast cultured in the absence of DPA.

Cells grown without DPA were harvested, ground and saponified. The saponifiable matter was separated by column chromatography. The content of individual isoprenoids was measured at various times during culture.

--×--: Total colored carotene
—○—: Unknown quinone
—×—: Ubiquinone --●--: Sterol
—●—: Dry weight

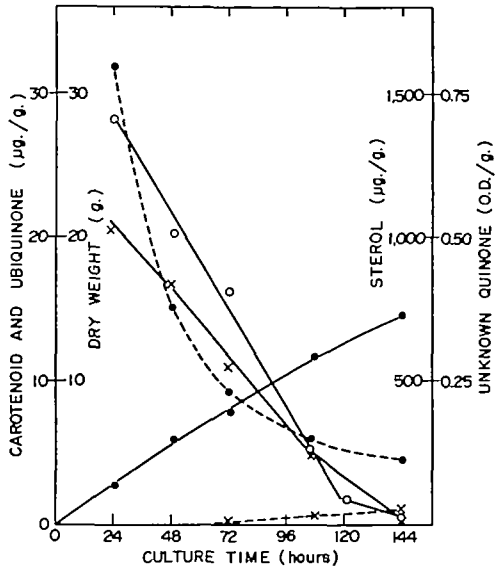

FIG. 5. Isoprenoid content in yeast cultured in the presence of DPA.

Cells grown in the medium containing DPA ($4.8\times10^{-5}M$) were treated as described in Fig. 4.

--×--: Total colored carotene
—○—: Unknown quinone
—×—: Ubiquinone --●--: Sterol
—●—: Dry weight

TABLE IV

Effect of DPA on the Content of Isoprenoids in Sporobolomyces shibatanus

Experiment	DPA added	Culture time hours	Cellular growth dry weight g.	Content of isoprenoids		
				Carotenoid μg./g.	Ubiquinone μg./g.	Sterol μg./g.
I	−	73	4.5	32.0	46.0	1,262
	+	73	5.4	0.5	26.2	698
II	−	77	10.3	15.0	32.0	462
	+	77	9.9	0.8	21.0	320

0.1 ml. of alcohol solution containing 2 mg. of DPA was added to 250 ml. of medium. After culturing the cells at 28°C with shaking, they were ground with carborundum and saponified after freezing and thawing. Extracted isoprenoids were separated by column chromatography on alumina. Individual isoprenoids were estimated by their absorbancy. The total carotenoid content was obtained by adding the values for individual carotenes after rechromatography. In experiments I and II, cultures were performed with mild and violent shaking (30 shaking/minute and 60 shaking/minute), respectively.

the inoculum. In Fig. 4 and Fig. 5 changes in the content of isoprenoids obtained from cells grown in the presence and absence of DPA are shown. It was found that the production of sterol and unknown quinone occurred actively in the initial phase of growth in the medium without DPA.

On the other hand, the contents of carotenoid and ubiquinone were observed to increase remarkably in the matured cells. Upon the addition of DPA to the medium the production of sterol and unknown quinone was observed to decrease strikingly during the logarithmic phase of growth. The content of ubiquinone was observed to decrease reversely with cell growth. Carotenoids were hardly synthesized at all in the cells cultured under these conditions (Fig. 4 and Table IV). DPA was effective in decreasing the biosynthesis of ubiquinone and carotenoids in whole cells.

As shown in this investigation and as previously reported by Goodwin *et al.* (*1*), Rilling (*2*) and Nakagawa and Tatsumi (*3*), DPA inhibits carotenogenesis. Furthermore, the results obtained suggest that DPA affects not only one or more steps in the unsaturation of colorless carotenoids but also other pathways of isoprenoid synthesis.

The author wishes to express thanks to Prof. S. Tanaka for his valuable advice and encouragement throughout this study.

REFERENCES

(*1*) Goodwin, T.W., Jamikorn, M., and Willmer, J.S., *Biochem. J.*, 53, 531 (1953)
(*2*) Rilling, H.C., *Arch. Biochem. Biophys.*, 110, 39 (1965)
(*3*) Nakagawa, M., and Tatsumi, C., *J. Agr. Chem. Soc. Japan* (in Japanese) 34, 199 (1960); *C.A.*, 58, 9429 f (1963)
(*4*) Olson, J.A., and Knizley, H., *Arch. Biochem. Biophys.*, 97, 137 (1962)
(*5*) Baker, J.E., *Arch. Biochem. Biophys.*, 103, 148 (1963)
(*6*) Sugimura, T., and Rudney, H., *Biochim. et Biophys. Acta*, 62, 167 (1962)
(*7*) Kakutani, Y., *J. Biochem.*, 59, 135 (1966)
(*8*) Raman, T.S., Sharma, B.V.S., Jayaraman, J., and Ramasarma, T., *Arch. Biochem. Biophys.*, 110, 75 (1965)
(*9*) Sugimura, T., and Rudney, H., *Biochim. et Biophys. Acta*, 37, 560 (1960)
(*10*) Crane, F.L., and Dilley, R.A., "Methods of Biochemical Analysis", Interscience Publishers, New York, Vol. XI, p. 279 (1963)
(*11*) Eimhjellen, K.E., and Jensen, S.L., *Biochim. et Biophys. Acta*, 82, 21 (1964)
(*12*) Goodwin, T.W., *Biochem. J.*, 50, 550 (1952)
(*13*) Fromageot, C., and Tchang, J.L., *Arch. Mikrogiol.*, 9, 424 (1938)
(*14*) Goodwin, T.W., "Comparative Biochemistry of the Carotenoids", Chapman and Hall Ltd., London, 9-35 (1952)
(*15*) Muber, W., Ewing, G.W., and Kriger, T., *J. Am. Chem. Soc.*, 67, 609 (1945)
(*16*) Lester, R.L., and Ramasarma, T., *J. Biol. Chem.*, 234, 672 (1959)