Attempts to Detect Lycopersene Formation in Yeast

By S. S. SCHARF AND K. L. SIMPSON

Department of Agricultural Chemistry, University of Rhode Island,
Kingston, R.I. 02881, U.S.A.

(Received 26 June 1967)

1. β -Ionone vapour has been shown to cause an increase in the more saturated carotenes and a decrease in the less saturated carotenes of *Rhodotorula glutinis*. Lycopersene (dihydrophytoene) has been proposed as a precursor to phytoene. Attempts were made to isolate lycopersene from β -ionone-treated cultures of *R. glutinis*. 2. Large samples of β -ionone-treated cultures were examined for the presence of lycopersene. Spots were detected on silicic acid plates that could not be differentiated from synthetic lycopersene on the basis of column and thin-layer chromatographic separations and staining techniques. The lycopersene-like substance could be obtained from non-treated pigmented yeast as well as baker's yeast. 3. An extraction of bacterial-grade yeast extract also yielded a lycopersene-like substance. The extracts of *R. glutinis* cells cultured on media not containing yeast extract did not contain the lycopersene-like compound. 4. No significant carbon was incorporated into the lycopersene zone from ^{14}C -labelled mevalonate, acetate and glucose by *R. glutinis* and baker's yeast. 5. These results indicate that compounds may exist with chromatographic properties similar to lycopersene, but that lycopersene could not be detected in either a pigmented or a non-pigmented yeast.

Carotenoid and sterol biosyntheses have been shown to follow similar pathways up to the C_{15} compound, farnesyl pyrophosphate. Squalene is formed by the condensation of two C_{15} units, whereas the first carotenoid is probably formed from two C_{20} pyrophosphates. The formation of squalene from mevalonic acid is a net reductive process, whereas no change in oxidation state would be required for the synthesis of phytoene. Phytoene is generally regarded as the first C_{40} carotenoid formed, although lycopersene (dihydrophytoene) has been proposed as a possible intermediate before phytoene.

Grob, Kirschner & Lynen (1961) reported the synthesis of lycopersene from ^{14}C geranylgeranyl pyrophosphate by a *Neurospora crassa* enzyme system. Grob & Boschetti (1962) reported the isolation of squalene and lycopersene from diphenylamine-inhibited cultures of *N. crassa*. Nusbbaum-Cassuto & Villoutreix (1965) reported the isolation of lycopersene from carrot roots, but were unable to detect the compound in corn oil.

The evidence against the involvement of lycopersene in carotenoid biosynthesis has been mainly indirect. A number of authors detected no lycopersene in carotenoid-forming systems (see Goodwin 1965). Davies, Jones & Goodwin (1963) found no evidence for the presence of lycopersene in diphenylamine-inhibited cultures of *N. crassa*, nor did they

detect any radioactivity associated with the lycopersene zone from the mould cultured on $[2-^{14}\text{C}]$ mevalonic acid. Anderson & Porter (1962) found phytoene and not lycopersene in the extracts of a tomato-plastid system. Rilling (1962) found only phytoene in a *Mycobacterium* sp. that was incubated under conditions where dehydrogenations were inhibited. He concluded that lycopersene was not involved in formation of phytoene, since such a transformation would require oxidation and reduction.

Simpson, Nakayama & Chichester (1964a) found that β -ionone inhibited the formation of the cyclic and more unsaturated carotenoids in *Rhodotorula glutinis* cultures. A large increase in the concentrations of phytoene, phytofluene, ζ -carotene and neurosporene was reported in these cultures.

The present paper reports an attempt to detect lycopersene in β -ionone-inhibited cultures of *R. glutinis*. If lycopersene is formed as an intermediate before phytoene, one might expect to find it under conditions of a large accumulation of phytoene.

EXPERIMENTAL

Organism. *Rhodotorula glutinis* (Fres.) Harrison 48-23T was kindly provided by Dr H. J. Phaff of the University of California, Davis, Calif., U.S.A. Some of the characteristics of this strain have been reported by Nakayama, Mackinney & Phaff (1954) and Simpson *et al.* (1964a). Baker's yeast

(*Saccharomyces* spp.) was obtained from Fleischmann Division of Standard Brands Inc. New York, N.Y., U.S.A.

Cultural conditions. The yeast was grown on a medium containing 0.5% Difco yeast extract and 5.0% glucose. Difco Bacto-agar (2.5%) was added to the broth for solid surface growth. On occasion the yeast was cultured on a defined medium suggested by Peterson, Evans, Lecce, Bell & Etchells (1958). The cultures were incubated in flat whisky-type bottles or in 250ml. Erlenmeyer flasks, as described previously (Simpson *et al.* 1964a,b).

The β -ionone gave the best balance between growth and inhibition of pigment development when it was added within 3 hr. for the solid cultures and 24 hr. for the shake cultures.

β -Ionone (0.1 ml.) was added to non-absorbent cotton plugs to saturate as large an area as possible inside the flask. The flat whisky-type bottles and the 250 ml. flasks had neck internal diameters of 2.0 cm. and 3.0 cm. respectively. More uniform pigment inhibition was obtained when the flat bottles were incubated in a vertical position after 3 or 4 days in a horizontal plane.

Solvents. Spectral-grade pentane was prepared by treating 95% practical-grade pentane (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) with conc. H_2SO_4 . The pentane was washed free of the acid, distilled and percolated through a column of silica gel that had been activated before use for 6 hr. at 400°. Only those fractions that gave negligible absorption ($E_{0.025}$) down to 225 m μ were used as spectral-grade pentane. Anhydrous diethyl ether, A.R. grade, was distilled and before use was passed through neutral grade Al_2O_3 (Woelm) to remove peroxides. Acetone (A.R.) was distilled before use.

Chromatographic adsorbents. Al_2O_3 (Woelm, neutral) was obtained from Alupharm Chemicals, New Orleans, La., U.S.A. Silical gel G (Merck) was obtained from Brinkman Instruments, Westbury, L.I., N.Y., U.S.A. MgO (Sea-Sorb 43) and Hyflo Super-Cel were obtained from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.

Radioactive chemicals. All radioactive carbon compounds were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Other chemicals. Squalene (98%) was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. β -Ionone (technical grade) was obtained from Matheson, Coleman and Bell. Synthetic lycopersene was a gift from Dr O. Isler, F. Hoffmann-La Roche, Basle, Switzerland.

Disruption of the cells, extraction and chromatographic procedures. For volumes greater than 500 ml. a colloid mill (model QV6; Gifford Wood Co., Hudson, N.Y., U.S.A.) was used (Simpson *et al.* 1964b). More consistent results were obtained when the disruption of the cells was performed under N_2 . For volumes of cells less than 100 ml., a French press (American Instrument Co., Silver Springs, Md., U.S.A.) was used (Simpson, Wilson, Burton, Nakayama, & Chichester, 1963).

The yeast cells were extracted, saponified and chromatographed on MgO-Super-Cel, as described by Simpson *et al.* (1964a,b).

The crude phytoene from the MgO-Super-Cel column was rechromatographed on a 25 g. column of neutral Al_2O_3 (1.3 cm. internal diameter). Spectral-grade pentane (100 ml.) was passed through the column before the addition of the crude phytoene. Six 10 ml. fractions were collected before

diethyl ether-pentane (1:99, v/v) was added to develop the column. The fractions from the Al_2O_3 column were concentrated and chromatographed on thin-layer plates. Squalene, lycopersene and phytoene generally ran with R_F values 0.4, 0.3 and 0.2 respectively. There were slight variations in the R_F values. Identification was based on R_F values and authentic samples of phytoene, lycopersene and squalene chromatographed on the same plates. I_2 vapour was used to develop the spots, although on occasion SbCl_3 was also used (see Mercer, Davies & Goodwin, 1963; Davies *et al.* 1963). Squalene, synthetic lycopersene and phytoene were usually eluted from the Al_2O_3 column in the thirteenth, sixteenth and nineteenth 10 ml. fraction respectively.

When ^{14}C -labelled substrates were used, the area on the thin-layer plates corresponding to the spots was scraped from the plate and added directly to scintillation-counting vials.

The I_2 was allowed to resublime before the addition of phosphors, although the I_2 did not quench the samples. The phosphor-counting solution consisted of 0.05% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.6% 2,5-diphenyloxazole and 4% (w/v) Cab-O-Sil (thixotropic gel powder) in toluene. Nuclear-Chicago solubilizer was added to solubilize radioactive substrates not soluble in toluene.

Radioactivity measurements were made on a Nuclear-Chicago model 725 scintillation counter. Counting efficiency was determined by the channels-ratio method (Baillie, 1960). Samples that contained thin-layer scrapings had to be held in the dark before being counted to avoid errors due to phosphorescence.

Spectroscopic determinations. Absorption spectra of the polyenes were made with a Cary model 15 recording spectrophotometer. Samples from the Al_2O_3 column were taken to dryness in a stream of N_2 and were redissolved in pentane before analysis.

RESULTS

Cultural methods. In previous experiments (K. L. Simpson & T. W. Goodwin, unpublished work) a lycopersene-like substance was detected on thin-layer plates from the extracts of β -ionone-treated *R. glutinis* cells. Shake cultures had been used in the previous study, but larger volumes of cells were necessary if conclusive results were to be obtained on the existence of lycopersene in yeast. Larger volumes of β -ionone-treated cells were obtained from the yeast grown on agar surfaces. The addition of 0.1 ml. of β -ionone to each cotton plug gave a reasonable balance between inhibition of the red-pigment formation and cell growth. The larger volumes of cells were disrupted in the colloid mill.

Purity of solvents and reagents. Light petroleum, diethyl ether and acetone contained a number of impurities that have R_F values on silica-gel plates similar to that of synthetic lycopersene. Anhydrous sodium sulphate and neutral alumina (grade I) also contained iodine-staining substances. These contaminants had been observed by Mercer *et al.* (1963) and Davies *et al.* (1963), and the precautions that these authors suggested were followed in the present study. Spectral-grade pentane was used in place

Table 1. *Changes in the concentration of the carotenes as a function of the growth period in β -ionone-inhibited *R. glutinis* cultures*

Six 50 ml. broth cultures in 250 ml. flasks were inoculated, and β -ionone was added 1 day later (Simpson *et al.* 1964a).

Carotene	Growth period	Concn. of carotene (μ g./g. dry wt.)		
		3 days	6 days	12 days
Phytoene		14.2	13.1	26.5
Phytofluene		—	Trace	4.9
ζ -Carotene		—	0.7	1.0
Neurosporene		0.5	0.8	0.9
β -Zeaxanthene		1.2	1.4	3.1
γ -Carotene		2.2	3.6	5.4
β -Carotene		5.7	7.4	16.8
Torulene		3.5	3.7	9.1
Total carotenes		27.3	30.7	67.7

Table 2. *Isolation of a lycopersene-like compound from β -ionone-treated cultures of *R. glutinis**

Expt. no.	Cell material (g. dry wt.)	β -Ionone added	Amount of phytoene (μ g./g. dry wt.)	Lycopersene- like substance isolated
1	50.0	+	13.6	+
2	53.4	+	23.0	—
3	23.5	—	7.7	—
4	43.2	+	21	+
5	8.3	—	—	+

of distilled light petroleum. The impurity in the sodium sulphate was removed by washing the compound with pentane. It could not be removed by heating to 400° for 2 hr.

The complete disruption, extraction and chromatographic procedure was run without cell material as a further check on the system. No lycopersene-like material was observed in the blank system, in which β -carotene and squalene were added as markers.

Attempts to isomerize squalene, phytoene and lycopersene. Jungalwala & Porter (1965) showed that natural phytoene exists as the 15,15'-*cis* isomer. These authors isomerized natural phytoene with iodine and u.v. light and isolated different isomers on alumina columns.

An isomer of natural phytoene or squalene might co-chromatograph with synthetic lycopersene. The isomerization method of Jungalwala & Porter (1965) was attempted on natural phytoene, squalene and synthetic lycopersene. The compounds could still clearly be separated from each other on thin-layer plates. Thus, in conjunction with the staining

techniques described by Davies *et al.* (1963), the compounds could be resolved.

*β -Ionone inhibition of carotene formation in *R. glutinis*.* The effect of β -ionone on the formation of the carotenoids of *R. glutinis* was reported by Simpson *et al.* (1964a). In this study no attempt was made to isolate lycopersene or to follow the effect of the inhibitor on the carotene formation over a period of time.

In the present study, the accumulation of the carotenes of the yeast was determined 3, 6 and 12 days after the addition of β -ionone (Table 1). Torularhodin was removed in the saponification step and its concentration was not determined. The 12-day growth period gave the greatest accumulation of phytoene, phytofluene and carotenes. Similar results were obtained for solid cultures, and the 12–15-day growth period was used for large volumes of yeast cells cultured in the flat bottles.

Table 2 summarizes the attempts that were made to isolate lycopersene on thin-layer plates. The isolation of the lycopersene-like spot was not always possible. It was isolated from untreated cultures of *R. glutinis* as well as from β -ionone-treated cultures.

Attempts to incorporate ^{14}C into lycopersene. *R. glutinis* was grown in the presence of β -ionone vapour on the shaker for 12 days with ^{14}C -labelled substrates. The French press was used for the disruption of the small volume of cells used in the radioactivity studies. Synthetic lycopersene (100 μ g.) was added to the *R. glutinis* yeast extract as carrier. Phytoene, squalene and lycopersene spots were removed from the plates and the amount of radioactivity was determined by liquid-scintillation counting. The β -carotene was crystallized from pentane before its addition to the vials and was bleached (Yokoyama, Nakayama & Chichester, 1962).

Total activities in disintegrations/min. for the polyenes of *R. glutinis* are given in Table 3 for 12-day cultures. Lycopersene was also not detected in 3- or 6-day cultures grown on either [2- ^{14}C]-acetate or [2- ^{14}C]-mevalonate.

Attempts to detect lycopersene in baker's yeast. Dried as well as pressed cakes of baker's yeast (*Fleischmann; Saccharomyces* spp.) were extracted and chromatographed in the same manner as the pigmented yeast. An iodine-staining compound was isolated at the same R_F as synthetic lycopersene and gave the same colour reactions with several reagents. A large amount of squalene was detected, but no phytoene was isolated.

Table 4 gives the results of culturing the baker's yeast on ^{14}C -labelled substrates.

Yeast extract was used in the media for growing the *R. glutinis* cultures. All the solvents and adsorbents (except the culture media) had been

Table 3. *Incorporation of ^{14}C into squalene and the carotenes of *R. glutinis* grown for 12 days on ^{14}C -labelled substrates in an atmosphere of β -ionone*

Eighteen 250 ml. flasks each containing 50 ml. of yeast extract-glucose broth were inoculated with *R. glutinis* cells from 6-day-old agar slopes. The cultures were incubated at 25° on a shaker. The radioactive substrates were added to the broth of each of six flasks, and β -ionone (0.1 ml.) was added to the cotton plugs 24 hr. after inoculation.

Substrate	$10^{-6} \times$ Total radioactivity added (disintegrations/min.)	Total radioactivity isolated (disintegrations/min.)			
		Squalene	Lycopersene	Phytoene	β -Carotene
DL-[2- ^{14}C]Mevalonic acid	6.0	1298	0	667	184
[2- ^{14}C]Acetic acid	5.4	100	3	353	64
D-[U- ^{14}C]Glucose	37.5	209	1	229	25

Table 4. *Incorporation of ^{14}C into squalene and lycopersene by dried commercial baker's yeast*

Two 11. flasks each containing 500 ml. of yeast extract-glucose broth were inoculated with dried baker's yeast. The cultures were incubated at 25° without shaking, the radioactive substrates were added to their respective flasks, and the yeast was harvested 12 days after inoculation.

Substrate	$10^{-6} \times$ Total radioactivity added (disintegrations/min.)	Total radioactivity isolated (disintegrations/min.)	
		Squalene	Lycopersene
DL-[2- ^{14}C]Mevalonic acid	6.3	1720	0
[2- ^{14}C]Acetic acid	19.5	142 000	31

checked for lycopersene-like impurities. When the yeast extract was extracted, a lycopersene-like substance was found on thin-layer plates.

R. glutinis was cultured on a defined medium (Peterson *et al.* 1958) and 50.0 g. of β -ionone-treated cells was obtained. No lycopersene-like substances were detected under these conditions.

DISCUSSION

The present work represents another attempt to isolate lycopersene under conditions that might be conducive to its formation. A lycopersene-like substance was isolated from cultures treated with β -ionone and from untreated cultures. The source of the lycopersene-like substance was found to be the yeast-extract component of the growth media. The fact that the substance was not a biosynthetic product was confirmed by the lack of any incorporation of ^{14}C from radioactive acetate, mevalonate and glucose by a carotene-forming yeast into the lycopersene zone on thin-layer plates.

The substance was isolated from dried as well as pressed cakes of Fleischmann baker's yeast, but could not be isolated from another brand of baker's yeast that was tested. The lack of incorporation of ^{14}C from mevalonate and the negligible incorporation from acetate casts doubt on the biosynthetic role of this substance in baker's yeast. The 31 dis-

integrations/min. in the lycopersene zone, against 142 000 in squalene can be explained by a contamination of radioactivity from squalene into the lycopersene zone, as Mercer *et al.* (1963) reported.

No evidence was found in this study for the existence of lycopersene in the carotene-forming yeast, *R. glutinis*.

In a non-carotene-forming yeast a compound was found that co-chromatographed with synthetic lycopersene. The lack of any significant incorporation of ^{14}C into the lycopersene zone casts doubt on the substance's being a metabolic product of yeast.

The evidence for the natural existence of lycopersene is found in two reports on its isolation from *N. crassa* and one report on its isolation from carrots. Davies *et al.* (1963) could not isolate lycopersene from the same culture as that used by Grob & Boschetti (1962). Nusbaum-Cassuto & Villoutreix (1965) reported the isolation of lycopersene from carrot root, but Anderson & Porter (1962) found no evidence of the presence of the compound in carrots. Carrot roots were extracted according to the method described by Nusbaum-Cassuto & Villoutreix (1965) during the present investigation on yeast pigments, and there was no evidence for the presence of lycopersene.

The present results do not clear up the apparent discrepancy that has occurred in the literature. They suggest, however, that there are substances having properties similar to those of synthetic

lycopersene that are confused with lycopersene. These results agree with the findings of other workers that lycopersene is not involved in carotene biosynthesis.

We thank Dr H. J. Phaff of the University of California for the cultures used in this work, and Dr O. Isler of F. Hoffman-La Roche, Basle, Switzerland, for providing the synthetic lycopersene. The work was supported in part by National Science Foundation Grant GB3582. This work was initiated during the tenure (by K.L.S.) of a National Science Post-doctoral Fellowship in the Department of Biochemistry, University College of Wales, Aberystwyth. This paper is contribution no. 1234 of the Rhode Island Experiment Station.

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