BIOSYNTHESIS OF CAROTENOIDS BY FUNGI

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The occurrence of carotenoids in species of *Penicillia* has never been observed with the only one exception. Luteraan and Choay (1) mentioned an unspecified *Penicillium* as a carotene producer but full details of this investigation are not yet available.

The author conducted a survey of carotenogenic activity of a number of fungus strains, and found *Penicillium sclerotiorum* to produce a considerable amount of carotenoid pigments. In addition to this, *Pen. multicolor*, *Pen. lapidosum* and *Rhizoctonia zeae* have also been found to be carotene producers. The present report describes the occurrence and identification of the major carotenoid pigments in the above four strains, some culture conditions affecting the carotene formation of *Pen. sclerotiorum*, and discussion on the biosynthetic pathway of the carotenoid.

EXPERIMENTAL

1. Culture of Fungi and Determination of Carotene Content

Pen. sclerotiorum, *Pen. multicolor*, *Pen. lapidosum*, and *Rhiz. zeae* were grown at 25° in a liquid medium (designated Medium No. 1) which contained the following ingredients: Difco malt extract, 15 g; maltose, 12.5 g; soluble starch, 2.5 g; glycerol, 2.5 g; dibasic potassium phosphate, 1 g; ammonium chloride, 1 g; Difco peptone, 1 g; distilled water to make 1000 ml. The pH of the medium was adjusted to approximately 5 with 10 N sulfuric acid and then sterilized at 120° for 15 min. Cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of the medium. After a suitable period of growth the mycelial mats were harvested on a Buchner funnel, washed with distilled water, and extracted in a Waring blender with acetone and hexane (100:75, v/v). The mycelial mat was filtered and extracted twice more with 200-ml portions of acetone. The filtrates were combined in a separatory funnel and treated with 250 ml of water. The aqueous hypophase was discarded, and the hexane epiphase was washed three times with the mixture containing 300-ml portions of water and one 50-ml portion of 90% aqueous methanol. The methanol extract contained a colored substance of undetermined nature.

The hexane epiphase solution was extracted with 50 ml of methanolic potassium hydroxide solution (methanol-water-potassium hydroxide, 90:10:20, v/v/w), and then with another 50 ml of 90% methanol. This final extract

was colorless. The epiphase was washed four times with equal volumes of water, and finally filtered through a layer of anhydrous sodium sulfate. The methanolic extracts and washings were discarded. The hexane extract was made to 100 ml in a volumetric flask.

The absorbance of the hexane solution was determined at 450 m μ with a Beckman DU spectrophotometer. Total carotene, calculated as β -carotene, was determined from the following formula:

$$\mu\text{g carotene} = \frac{\text{absorbance}}{258} \times 1000 \times 100$$

To determine the weight of the dry mycelia, a duplicate mycelial mat of each culture was heated at 100° for 15 min, filtered on a Buchner funnel, washed three times with distilled water, dried at 105–110° for 4.5 hr and weighed.

2. Identification of the Pigment

The strains were cultured for 15 days at 25° in thirty 500-ml Erlenmeyer flasks containing 100 ml of the medium No. 1. The mycelial mats were collected, and the carotenes were extracted in dry hexane solution by the procedure shown elsewhere (2). Preliminary separations were made on magnesia-supercel (1:1) and complete separation on lime. All major zones were removed from the developed column by sectioning, eluted with ethyl ether, concentrated to dryness, and redissolved in hexane or isooctane. Complete absorption spectra were obtained in the range 520 to 275 m μ . These pigments were identified from the chromatographic behaviors and absorption spectra.

3. Environmental Effects on Carotenogenesis

Pen. sclerotiorum was grown under various environmental conditions on the medium No. 1 or chemically defined media (designated No. 2), with some modification occasionally, which contained the following basal ingredients: Glucose, 40 g; citric acid, 10 g; (NH₄)₂SO₄, 1.5 g; KH₂PO₄, 0.5 g; MgCl₂·6 H₂O, 0.5 g; B (as Na₂B₄O₇·10 H₂O), 10 μ g; Mo (as (NH₄)₆Mo₇O₂₇·4 H₂O), 20 μ g; Zn (as ZnSO₄·7 H₂O), 200 μ g; Cu (as CuCl₂·2 H₂O), 100 μ g; Mn (as MnCl₂·4 H₂O), 20 μ g; Fe (as FeSO₄·7 H₂O), 250 μ g; Co (as CoCl₂·6 H₂O), 5 μ g; distilled water to make 1000 ml.

Effects of various culture conditions on its growth and carotene formation were investigated in some detail. Extraction of pigments and determination of total carotene were conducted as shown previously.

RESULTS AND DISCUSSION

1. Survey

A survey of carotenogenic activity of 72 fungus strains were conducted. In addition to the strains known as carotene producers, *Pen. sclerotiorum*, *Pen. multicolor*, *Pen. lapidosum*, and *Rhizoctonia zeae* were found to produce carotene when grown on Difco wort agar media. Table I shows the growth and total carotene content of the new carotene producers, and the carotenoids have been shown to be absent from the fungi listed in Table II.

Among the tested strains, *Pen. sclerotiorum* NRRL 2074 was found to be

TABLE I
Growth and Total Carotene Content on Wort Agar (at 25°)

Strain	NRRL No.	Duration of incubation	Dry weight per 100 ml medium	Total carotene
		days	mg	µg per g dry
<i>Pen. sclerotiorum</i>	2074	14	773	696
"	2076	14	792	9
<i>Pen. multicolor</i>	764	15	947	13
"	2058	15	848	12
"	2059	15	790	25
"	2060	15	750	8
"	A-2324	15	1107	9
<i>Pen. lapidosum</i>	718	21	897	32
"	2084	16	968	14
<i>Rhizoctonia zeae</i>	—	12	556	88

TABLE II
Fungi in Which Carotenoids Have Not Been Shown to Be Present

<i>Penicillium asperum</i>	<i>Penicillium parvum</i>	<i>Aspergillus chevalieri</i>
" <i>avellaneum</i>	" <i>purpurogenum</i>	" <i>citrosporus</i>
" <i>baarnense</i>	" <i>pusillum</i>	" <i>flavus</i>
" <i>chrysogenum</i>	" <i>roqueforti</i>	" <i>ochraceus</i>
" <i>duclauxi</i>	" <i>rubrum</i>	" <i>oryzae</i>
" <i>funiculosum</i>	" <i>stipitatum</i>	" <i>tamarii</i>
" <i>gladioli</i>	" <i>striatum</i>	" <i>ustus</i>
" <i>implicatum</i>	" <i>substeritium</i>	<i>Gibberella zeae</i>
" <i>janthinellum</i>	" <i>thomii</i>	<i>Monascus purpurea</i>
" <i>javanicum</i>	" <i>turbatum</i>	<i>Rhizopus delemar</i>
" <i>luteum</i>	" <i>wortmanii</i>	<i>Trichoderma liquerum</i>
" <i>notatum</i>		

the most promising carotene producer. This species, belonging to the *Penicillium thomii* series, was first isolated in Java in 1935 (3) and has been maintained in the NRRL collection as strain NRRL 2074. This species has been represented also by *Pen. sclerotiorum* NRRL 2076 which was isolated from soil collected in Brazil (4).

Pen. sclerotiorum has been of special interest because of the brilliant orange-red color of the mycelia which characteristically surround the sclerotia of this species when cultured on Czapek-Dox medium. In 1940 Curtin and Reilly (5) isolated an acidic pigment from *Pen. sclerotiorum* in a yield comprising 2% of the dry mycelial mat. This acidic pigment, which was isolated also from *Pen. multicolor* (6, 7), was named sclerotiorine. It is extractable from hexane with alkali. Hence it is easily distinguished from carotene by this property. Characteristically media such as Czapek-Dox agar, which favor the production of copious quantities of sclerotiorine, inhibited the formation of carotenoids. The inverse effect of different media on the formation of both sclerotiorine and carotene suggests that possibly sclerotiorine is a precursor for carotene or a metabolic product of carotenogenesis in culture 2074.

From the analysis of sclerotiorine and its derivatives, the most probable

empirical formula was shown to be $C_{21}H_{23}O_5Cl$ (6, 7). When sclerotiorine was warmed with alkali, it was hydrolyzed to a crystalline monobasic acid containing conjugated double bonds, 4,6-dimethyl-octa-2,4-dienoic acid. The isoprenoic nature of sclerotiorine and of the carotenes lends support to the possibility that they have a metabolic interrelationship.

Culture 2074 produced no extracellular carotene in the agar or liquid portion of the media, but the color of the mycelial mats was greater between the mats and the culture media.

2. Identification of Pigments

A preliminary separation of extracted total carotene was made on magnesia-Supercel, and a complete separation was made on lime. Each major zone was removed from the chromatograms and their properties are summarized in

TABLE III
Wavelength of Maximum Absorption of Various Carotenoids Produced
by *Pen. sclerotiorum* (in Hexane)

Pigment no.	Carotene indicated	Maxima								Per cent of total pigment
		1		2		3		Cis peak		
		m μ	A ^a	m μ	A	m μ	A	m μ	A	
1	<i>α</i> -Carotene	475	0.860	445	1.000 ^b	422	0.718	331	0.210	0.2
2	<i>Neo</i> - β -carotene-1	470	0.801	447	1.000			336	0.561	1.5
3	<i>Neo</i> - β -carotene-2	472	0.832	445	1.000			338	0.385	2.9
4	β -Carotene	478	0.902	450	1.000			335	0.050	64.6
5	<i>Neo</i> - β -carotene-3	474	0.875	445	1.000			325	0.135	1.2
6	Zeacarotene-1	452	0.841	426	1.000	406	0.715			0.3
7	Zeacarotene-2	454	0.854	427	1.000	404	0.712	286	1.190	0.4
8	<i>Neo</i> - γ -carotene-1	486	0.781	456	1.000	434	0.764	347	0.329	1.1
9	<i>Neo</i> - γ -carotene-2	486	0.801	456	1.000	432	0.734	348	0.271	1.7
10	γ -Carotene	492	0.862	461	1.000	436	0.710	350	0.063	25.8
11	Unknown	517	0.716	483	1.000	456	0.905			0.3

^a A = $\log I_0/I$.

^b The highest absorption maximum of each carotenoid was arbitrarily given a value of 1.000.

The same are applicable in the following Tables.

TABLE IV
Wavelength of Maximum Absorption of Various Carotenoids Produced
by *Pen. multicolor* (in Hexane)

Pigment no.	Carotene indicated	Maxima						Per cent of total pigment
		1		2		3		
		m μ	A	m μ	A	m μ	A	
1	β -Carotene	478	0.902	450	1.000			17.9
2	Zeacarotene	452	0.841	426	1.000	406	0.715	1.2
3	γ -Carotene	492	0.862	461	1.000	436	0.710	33.0
4	Neurosporene	468.5	0.975	439.5	1.000	416	0.689	3.4

Tables III—VI.

TABLE V
Wavelength of Maximum Absorption of Various Carotenoids Produced
by *Pen. lapidosum* (in Hexane)

Pigment no.	Carotene indicated	Maxima								Per cent of total pigment
		1		2		3		4		
		m μ	A	m μ	A	m μ	A	m μ	A	
1	Ergosterol	293.5	0.573	282	1.000	271	0.945	262	0.671	—
2	γ -Carotene	492	0.843	461	1.000	436	0.714			17.2
3	Pigment L	504.5	0.799	474.5	1.000	360	0.145			43.4

TABLE VI
Wavelength of Maximum Absorption of Various Carotenoids
Produced by *Rhizoctonia zeae*

Pigment no.	Carotene indicated	Solvent	Maxima						Per cent of total pigment
			1		2		3		
			m μ	A	m μ	A	m μ	A	
1	Phytofluene	Isooctane	367	0.568	348	1.000	332	0.920	2.1
2	β -Carotene	Hexane	478	0.898	450	1.000	335	0.065	33.4
3	γ -Carotene	Hexane	491.5	0.856	460.5	1.000	436	0.721	5.8

Pen. sclerotiorum NRRL 2074 produced about 1 mg of total carotene per g of mycelium dry weight, of which approximately 65% was β -carotene and approximately 25% was γ -carotene. In addition, as minor pigments, α -carotene, 3-cis-isomers of β -carotene, 2-cis-isomers of γ -carotene, and zeacarotene were identified.

Pen. multicolor NRRL 2059 produced about 50 μ g of total carotene per gram of mycelium dry weight, of which ca. 18% was β -carotene, ca. 33% γ -carotene, ca. 3% neurosporene, and ca. 1% zeacarotene.

Pen. lapidosum NRRL 718 produced about 70 μ g of total carotene per g of mycelium dry weight, of which ca. 43% was the pigment L (unidentified), and ca. 17% γ -carotene. In addition, ergosterol was identified.

Rhizoctonia zeae produced about 90 μ g of total carotene per g of mycelium dry weight, of which ca. 33% was β -carotene, ca. 6% γ -carotene, and ca. 2% phytofluene.

3. Environmental Effects on Carotenogenesis

pH of Medium—*Pen. sclerotiorum* NRRL 2074 was cultured on several media. As shown in Fig. 1 and Tables VII—IX for instance, in all cases showed the minimum value in 6 to 9 days after inoculation and thereafter increased gradually. Its carotene production seemed to be markedly affected

FIG. 1 Time Course Data during Incubation of *Penicillium sclerotiorum*

- × Mycelium dry weight, g/100 ml medium
- Total carotene, mg/g dry mycelium
- Δ pH of medium
- Residual sugar (initial content = 1.0)
- ▲ Residual nitrogen (initial content = 1.0)

by the pH value of the medium after it attained to the minimum value. The optimum pH for its carotenogenesis was found to be 2.00 ± 0.25 .

The total carotene produced on an acidic medium (initial pH: 2.31) was about twice as much as that on neutral media (initial pH: 4.59 and 6.98). On the neutral media xanthophylls and acidic

TABLE VII
Effect of pH (1) (25°, 15 days)

pH of medium		Yield of dry matter	Total carotene
Initial	Final		
		mg/100 ml	μg/g dry
1.03	1.00	0	—
1.58	1.39	541	43
2.10	1.59	549	268
2.61	1.71	643	436
3.11	1.80	690	536
4.11	1.82	640	734
5.00	1.88	710	689
5.80	1.90	651	784
6.58	2.00	658	801
7.08	2.19	685	935
7.90	2.80	706	734
8.15	4.60	747	234

TABLE VIII
Effect of pH (2) (25°, 15 days)

pH of medium		Yield of dry matter	Total carotene
Initial	Final		
		mg/100 ml	μg/g dry
1.70	1.36	622	201
1.93	1.57	664	318
2.19	1.70	734	462
2.43	1.86	722	725
2.69	2.09	784	1227
3.15	6.31	806	765
4.15	8.02	884	298
5.10	7.89	870	336

pigments were produced, but not on the acidic medium. Although the amounts of β -carotene and zeacarotene produced on those three media did not much vary, the amount of γ -carotene produced on the acidic medium was much greater than the amounts on the neutral media. Production of γ -carotene appeared to be accelerated on the acidic medium.

General Conditions — Growth of *Pen. sclerotiorum* was observed at temperatures between 12 and 32°, but maximum carotene production was attained at 24°. The data are shown in Fig. 2. The optimum depth of the medium for its carotenogenesis seemed to be approximately 1 cm. The smaller the medium concentration, the greater was the total carotene per g dry mat.

TABLE IX
Effect of pH (3) (25°, 15 days)

pH of medium		Yield of dry matter	Total carotene
Initial	Final		
		mg/100 ml	μg/g dry
1.35	1.20	0	—
1.75	1.39	344	247
1.95	1.59	372	394
2.30	1.75	454	505
2.60	1.95	524	331
3.40	2.90	678	86
4.48	4.05	714	54
6.20	4.85	736	77
7.10	4.92	525	77

Even after maximum growth was attained and nitrogen assimilation was ceased, consumption of sugar was further continued, which seemed to be used for carotene formation.

The time course data during incubation was shown in Fig. 1.

The optimum light intensity was about 1 foot candle for 15 day cultivation. Stronger light seemed to inhibit carotene formation or to stimulate

FIG. 2 Effect of Temperature on the Carotenogenesis of *Pen. sclerotiorum*

× Mycelium dry weight, g/100 ml medium
○ Total carotene, mg/g dry mycelium

TABLE X
Effect of Carbon Sources (25°)

Carbon source	15 days			20 days		
	Final pH	Yield of dry matter	Total carotene	Final pH	Yield of dry matter	Total carotene
		mg/100 ml	μg/g dry		mg/100 ml	μg/g dry
Acetic acid	2.30	0	0	2.30	0	0
L(+)-Arabinose	1.75	548	1242	1.76	529	1440
D(+)-Cellobiose	1.80	594	950	1.82	581	1370
Citric acid	2.25	631	756	3.80	547	850
Dextrin	1.74	543	568	1.75	546	320
Diglycerol	1.82	247	548	1.80	236	284
Ethyl alcohol	2.30	0	0	2.30	0	0
D(-)-Fructose	1.72	672	440	1.70	673	595
D(+)-Galactose	1.80	730	630	1.83	751	690
D-Glucose	1.77	633	959	1.81	620	886
Glycerol	1.70	577	981	1.69	605	990
Lactose	2.02	508	386	2.04	424	716
D(+)-Maltose	1.82	567	441	1.81	629	586
Mannitol	1.70	526	671	1.73	521	524
Pectin	3.00	620	431	3.25	608	174
D(+)-Raffinose	1.82	635	598	1.90	640	804
D-Ribose	1.79	634	1193	1.84	629	1096
Sorbitol	1.70	492	736	1.65	525	707
Soluble starch	1.75	555	673	1.70	567	655
Succinic acid	1.81	515	476	1.75	654	897
Saccharose	1.74	625	726	1.80	617	738
D(+)-Xylose	1.80	656	1173	1.80	678	1318

carotene destruction, while weaker light appeared to stimulate the formation of xanthophylls and acidic pigments. Direct contact with the air seemed to be necessary for the carotene formation.

The suitable inoculum size was found to be approximately 1600 spores per ml medium.

Carbon and Nitrogen Sources—It is conceivable that if nitrogen is in excess the most part of carbon is used up in protein synthesis, leaving scarcely

TABLE XI
Effect of Nitrogen Sources (25°)

Nitrogen source	12 days			20 days		
	Final pH	Yield of dry matter	Total carotene	Final pH	Yield of dry matter	Total carotene
		mg/100 ml	μg/g dry		mg/100 ml	μg/g dry
NH ₄ Cl	1.76	760	237	1.78	785	181
NH ₄ NO ₃	3.82	827	335	3.80	888	153
(NH ₄) ₂ HPO ₄	1.95	693	530	1.98	750	445
(NH ₄) ₂ SO ₄	1.72	573	708	1.80	687	772
DL-α-Alanine	2.22	650	423	2.30	703	721
L-Arginine	2.10	391	220	2.20	414	404
L-Asparagine	2.27	662	754	2.50	667	975
L-Aspartic acid	2.29	688	503	2.50	751	715
β-Alanine	1.81	777	676	1.70	762	906
Betaine	2.00	660	1370	2.00	782	1850
Casein	2.25	619	324	2.24	656	1071
Choline·Cl	1.71	592	1280	1.70	701	1295
L-Cysteine·HCl	1.55	500	900	1.60	595	1022
L-Cystine	1.80	257	983	1.62	685	750
Ethanolamine	1.80	454	782	1.88	687	829
γ-Aminobutyric acid	1.81	807	606	1.75	751	878
L-Glutamic acid	2.28	686	475	2.38	772	712
Glycine	2.29	654	338	2.48	732	726
L-Histidine	2.02	614	376	2.20	676	801
DL-Isoleucine	2.22	527	696	2.40	840	851
L-Leucine	2.15	652	690	2.41	884	914
L-Lysine	2.15	125	384	2.00	506	596
DL-Methionine	2.00	472	142	1.88	690	537
Peptone	2.18	539	475	2.19	541	1291
KNO ₃	5.60	898	101	5.75	1188	120
KNO ₂	2.30	0	0	2.30	0	0
L-Phenylalanine	2.20	601	276	2.37	286	748
L-Proline	2.30	682	318	2.50	766	602
DL-Serine	2.26	677	207	2.54	859	485
Thiourea	2.30	0	0	2.30	0	0
DL-Threonine	2.28	586	701	2.58	820	686
L-Tryptophan	2.28	748	350	2.75	986	717
DL-Tyrosine	2.20	631	369	2.41	548	670
Urea	2.38	112	250	2.35	116	302
Uric acid	2.30	0	0	2.30	0	0
DL-Valine	2.22	537	714	2.49	670	890

any carbon available for carotene synthesis, and that, on the other hand, if carbon is in excess, there remain plenty to spare for carotene production. In the carotenogenesis, the controlling factor seems to be the amount of assimilable carbon, the C:N ratio having only limited significance.

Effects of carbon and nitrogen sources were investigated, and the results obtained were shown in Tables X and XI. Arabinose, cellobiose, xylose, ribose, and glycerol were good carbon sources for the carotene formation; generally, pentoses were better than hexoses. Alanine and leucine were able to be carbon sources for the carotenogenesis. Leucine, isoleucine, valine, betaine, choline, cysteine, asparagine, and β -alanine were found to be good nitrogen sources. It is very interesting that methyl donors, such as betaine and choline, were best nitrogen sources for the carotene formation by *Pen. sclerotiorum*.

Mineral Elements—Tables XII and XIII show the effects of mineral

TABLE XII
Effect of Mineral Elements (I) (25°)

Element	g/l	12 days			20 days		
		Final pH	Yield of dry matter	Total carotene	Final pH	Yield of dry matter	Total carotene
			mg/100 ml	$\mu\text{g/g dry}$		mg/100 ml	$\mu\text{g/g dry}$
K	0	2.30	39	0	2.25	52	0
	0.033	1.72	531	755	1.78	587	980
	0.065	1.75	509	748	1.80	621	1021
	0.13	1.75	495	579	1.82	598	995
	0.26	1.80	487	620	1.83	605	1058
	0.52	1.82	512	570	1.85	602	1006
Mg	0	2.30	0	0	2.32	0	0
	0.03	1.70	613	615	1.75	715	930
	0.06	1.70	566	748	1.77	777	987
	0.12	1.70	613	633	1.76	680	1130
	0.24	1.70	570	690	1.75	669	958
Cl	0	1.78	584	595	1.80	624	1044
	0.044	1.78	645	526	1.80	658	976
	0.087	1.80	635	569	1.80	682	954
	0.175	1.78	666	564	1.82	694	1092
	0.35	1.80	655	601	1.83	677	981
	0.70	1.79	596	597	1.80	638	990
PO ₄	0	2.28	0	0	2.31	0	0
	0.088	1.70	572	690	1.86	600	999
	0.175	1.70	527	524	1.82	580	976
	0.35	1.70	550	572	1.80	596	940
	0.70	1.71	562	655	1.82	628	1062
	1.40	1.80	567	738	1.84	613	1038
SO ₄	0	2.57	51	0	2.55	60	0
	0.273	1.78	656	418	1.78	663	880
	0.545	1.79	593	479	1.82	569	684
	1.09	1.82	572	609	1.88	780	1018
	2.18	1.82	618	536	1.84	760	972
	4.36	1.88	696	515	1.89	890	1046

elements upon the growth and carotenogenesis of *Pen. sclerotiorum*. It was found that C, H, O, P, K, N, S, and Mg were essential elements; Fe, Zn, Mn, Ni, and Li were stimulatory ones; and Al, B, Ba, Ca, Cl, Co, Cu, F, I, Mo,

TABLE XIII
Effect of Mineral Elements (2) (25°)

	15 days			20 days		
	Final	Yield of	Total	Final	Yield of	Total
	pH	dry matter	carotene	pH	dry matter	carotene
		mg/100 ml	μg/g dry		mg/100 ml	μg/g dry
— B	1.75	506	1360	1.76	566	1520
— Mo	1.75	500	1305	—	—	—
— Zn	1.75	441	431	1.75	617	700
— Cu	1.75	464	1425	—	—	—
— Mn	1.75	507	601	1.74	583	1110
— Fe	1.75	468	1150	1.75	643	974
— Co	1.75	484	1321	—	—	—
— all	1.85	363	408	1.78	478	500
+ all	1.75	494	1352	1.78	574	1482
+ 1/10 all	1.77	452	725	1.77	509	807
+ 1/10 Zn	—	—	—	—	635	865
+ 1/10 Mn	—	—	—	—	594	1438
+ 1/10 Fe	—	—	—	—	659	972

— Not determined

Na, and Sn were unnecessary.

Vitamins — Table XIV shows the additional effects of various vitamins, and Tables XV—XIX show the effects of niacin, *p*-aminobenzoic acid, folic acid, calcium pantothenate, and menadione, respectively. Carotene production of *Pen. sclerotiorum* was stimulated by *p*-amino benzoic acid, folic acid, niacin, and menadione. Calcium pantothenate stimulated growth but inhibited caro-

TABLE XIV
Effect of Vitamins (25°)

Vitamin	Concentration	15 days		20 days	
		Yield of	Total	Yield of	Total
		dry matter	carotene	dry matter	carotene
	mg/l	mg/100 ml	μg/g dry	mg/100 ml	μg/g dry
None	—	519	682	534	873
Thiamine	100	524	638	532	889
Riboflavin	100	530	664	555	862
Niacin	1000	691	755	864	1096
PABA	100	352	915	423	1250
Pyridoxine	100	569	700	588	824
Ca-pantothenate	100	608	612	715	636
Inositol	1000	548	618	581	771
Biotin	1	538	583	554	814
Folic acid	100	546	976	543	1195
Choline	1000	838	532	943	859
B ₁₂	1	525	674	561	895
Ascorbic acid	100	566	669	576	867
α-Lipoic acid ^a	10	540	802	548	988
α-Tocopherol ^a	100	528	786	556	1022
Menadione ^a	100	0	—	0	—
Ethanol	5 cc	535	701	549	1044

^a Added as ethanol solutions, and the media contained 0.5% ethanol when inoculated.

TABLE XV
Effect of Niacin (25°, 15 days)

Concentration of niacin	Yield of dry matter	Total carotene
mg/l	mg/100 ml	μg/g dry
0	493	707
10	487	812
100	538	769
1,000	671	895
10,000	694	1076

TABLE XVII
Effect of Folic Acid (25°, 15 days)

Folic acid	Yield, dry matter	Total carotene
mg/l	mg/100 ml	μg/g dry
0	525	668
1	531	673
10	529	789
100	536	970
1,000	548	882

TABLE XVI
Effect of p-Aminobenzoic Acid (25°, 15 days)

Concentration of PABA	Yield of dry matter	Total carotene
mg/l	mg/100 ml	μg/g dry
0	526	712
1	473	763
10	451	906
100	417	974
1,000	389	500

TABLE XVIII
Effect of Calcium Pantothenate (25°, 15 days)

Concentration of Ca pantothenate	Yield of dry matter	Total carotene
mg/l	mg/100 ml	μg/g dry
0	496	663
0.1	514	637
1	509	673
10	547	628
100	575	604
1,000	693	313

tene production.

Organic Acids—Table XX shows the effects of organic acids used as a sole carbon source, and Table XXI the effects of organic acids added to sugar-containing media. Succinic and fumaric acids were good carbon sources for the carotenogenesis of *Pen. sclerotiorum*. Mevalonic, dimethylacrylic, levulinic, kojic, and fumaric acids stimulated its carotene

production. Formic, acetic, propionic, and butyric acids inhibited its growth.

Other Compounds—Table XXII shows the effects of several possible precursors on the carotenogenesis. α -, β -, and ψ -ionones, phytol, isophytol, methylheptenone, squalene, and vitamin A stimulated the carotene production to some extent; while citral, citronellal, geraniol, or isoprene failed to show such an effect.

4. Spectral and Stereochemical Properties of Carotenes

In order to study the biosynthesis of the carotenoid belonging to a conjugated polyene, it seems to be one of the most important problems to investigate

TABLE XIX
Effect of Menadione (25°, 15 days)

Concentration of menadione	Yield of dry matter	Total carotene
mg/l	mg/100 ml	μg/g dry
0	535	768
1	524	1136
10	187	975
100	0	—

TABLE XX
*Effect of Organic Acids Used as Sole
Carbon Source (25°, 15 days)*

Organic acid or sugar	Final pH	Yield of	Total
		dry matter	carotene
		mg/100 ml	μg/g dry
Acetic	2.30	0	—
Aconitic	2.22	66	334
Ascorbic	2.31	37	0
Citric	2.70	547	950
Crotonic	2.31	0	—
Diglycolic	2.30	0	—
Fumaric	1.90	407	949
Glutamic	5.85	551	138
Glycolic	2.32	0	—
Lactic	2.20	180	23
Levulinic	2.40	trace	—
Maleic	2.30	0	—
Malic	4.25	656	315
Malonic	2.30	0	—
Oxalic	2.88	138	0
Pyruvic	2.34	0	—
Succinic	1.94	654	897
Tartaric	6.60	328	485
Arabinose	1.72	545	1317
Glucose	1.62	610	826
Glycerol	1.97	596	964
Xylose	1.75	628	1250

TABLE XXI
*Effect of Organic Acids Added to Basal
Medium (25°, 15 days)*

Organic acid (1%)	Final pH	Yield of	Total
		dry matter	carotene
		mg/100 ml	μg/g dry
None	1.71	582	808
Acetic	2.30	0	—
Adipic	1.90	610	666
Butyric	2.30	0	—
Citric	2.62	716	660
Formic	2.30	0	—
Fumaric	1.90	429	1170
Glutaric	1.87	688	712
α-Ketoglutaric	1.92	712	524
Kojic	1.92	698	895
Lactic	4.03	325	540
Levulinic	1.70	122	230
Malic	3.18	343	713
Dimethylacry- lic	2.30	0	—
Mevalonic	2.30	0	—
Oxalic	5.49	516	270
Propionic	2.30	0	—
Pyruvic	2.30	0	—
Succinic	1.90	674	684
Tartaric	3.42	321	360

their spectral characteristics and stereochemical properties.

Different solvents vary both the wavelength of maximum absorption and the absorbance of β -carotene. The former shows a correlation with the refractive index squared, and the latter a logarithmic correlation with wavelength. The solvent effects may be explained as dipolar interactions between β -carotene and the solvent.

Lycopene and β -carotene contain the same chromophore group, a series of 11 conjugated double bonds, but there are three distinguishing differences in their comparative spectral properties: (1) as already noted, the absorption for β -carotene are much less than for lycopene, (2) the wavelength of maximum absorption for β -carotene is displaced 21 m μ hypochromically, and (3) the spectral fine structure characteristic of acyclic polyenes is reduced in the spectrum of β -carotene. These distinguishing properties can be explained on the basis of the unique properties of the β -ionone rings which terminate the conjugated double bonds in β -carotene. The spectral properties of several polyenes are listed in Table XXIII and the wavelengths of their maximum absorption in hexane are plotted against the number of conjugated double bonds in Fig. 3.

The effect one β -ionone ring in carotenes, *e.g.*, α - and γ -carotenes, is to impart properties intermediate between β -carotene and the acyclic analogues. It is apparent that an α -ionone ring does not affect the chromophore of carotenoids because the double bond of α -ionone is not conjugated. A comparison of the spectral properties of known polyene related to the carotenoids shows that the wavelength of maximum absorptions is a function of the logarithm

TABLE XXII
Effect of Several Organic Compounds (25°, 15 days)

Compound	Concen- tration	Yield of dry matter	Total carotene	Compound	Concen- tration	Yield of dry matter	Total carotene
	mg/l	mg/100 ml	μg/g dry		mg/l	mg/100 ml	μg/g dry
Citral	0	564	1052	Isophytol	0	564	1054
	0.1	550	1129		0.1	565	1429
	1	575	1096		1	569	1061
	10	553	1132		0	569	958
	100	576	798		100	579	1039
Citronellal	0	552	1039	Isoprene	0	555	1098
	0.1	562	1020		1	560	1066
	1	552	1050		10	554	1034
	10	560	1029		100	559	1088
	100	554	893		1000	556	1105
Geraniol	0	545	1075	Methyl heptenone	0	548	1077
	0.1	578	1021		1	562	1060
	1	576	955		10	565	1071
	10	564	971		100	521	1265
	100	504	982		1000	0	—
α-Ionone	0	574	1037	Phytol	0	562	1029
	0.1	582	1067		0.1	572	1204
	1	566	1120		1	581	1085
	10	569	1312		10	579	966
	100	498	954		100	575	1041
β-Ionone	0	568	1034	Squalene	0	559	1070
	0.1	564	1234		0.1	533	1053
	1	557	1255		1	562	959
	10	552	1450		10	529	1209
	100	489	894		100	538	1061
ψ-Ionone	0	568	1088	Vitamin A	0	548	1058
	1	579	1134		1	620	1044
	10	580	1306		10	631	1009
	100	475	904		100	502	1257
	1000	0	—		1000	165	1302

TABLE XXIII
Absorption of Carotenes and Polyenes in Hexane

Polyene	λ_{max}	<i>A</i>	$\epsilon \times 10^3$	<i>N</i>	<i>N_e</i>	Ref.
	<i>mμ</i>					
Lycopene	472	348	186	11	11	(8)
γ-Carotene	462	272	146	11	10.3	(8)
δ-Carotene	456	320	172	10	10	(9)
β-Carotene	451	256	137	11	9.6	
α-Carotene	446	272	146	10	9.3	(10)
Neurosporene	439	299	161	9	9	(11)
ζ-Carotene	400	220	119	7	7	(12)
Phytofluene	348	152	82	5	5	(13)
Phytoene	286	85	46	3	3.3	(14)
α-Eleostearic acid	272	170	47	3	3	(15)

A, absorbance (mg/ml)

ϵ , molar absorbance (moles/liter)

N, number of conjugated double bonds

N_e, apparent number of conjugated double bonds

FIG. 3 Relationship between Maximum Absorption of Polyenes and Number of Conjugated Double Bonds

of number of conjugated double bonds. Extrapolation of the wavelengths of maximum absorption of those carotenes containing β -ionone rings suggests that each β -ionone ring contributes in effect only 0.3 of a double bond to the conjugated chromophore. It could be explained that the β -carotene molecule is capable of undergoing transition only when the conjugated double bonds are coplanar. There is a steric hindrance to coplanarity between the 2 methyl groups at C_1 and the hydrogen at C_8 in β -carotene molecule.

This steric overlap cannot be relieved by bending C-C bond at C_6 because of the proximity of the hydrogen at C_7 to the methyl group at C_5 . Since the steric overlap cannot be relieved by bending the bond angles, the plane of the β -ionone ring takes a position angular to the plane

of the conjugated double bonds. In so far as the two planes are transangular, maximum resonance or maximum orbital overlap cannot be achieved, and the electronic transition is forbidden.

The concept of steric hindrance to a coplanar arrangement with its consequent effects on molecular spectra is well established (16). The fact that allene and ketene do not show a K-band above 200 mμ (17) because the π -electron orbitals are at right angles to each other, lends support to the limited contribution of the β -ionone double bond to the conjugated system in β -carotene.

The transangular nature of β -ionone ring may possibly offer an explanation for its unique ability to undergo a kind of selective reduction in carotenes (18).

5. Biosynthetic Pathway of Carotenoids

Succinic acid-1- C^{14} was added to the culture medium of *Pen. sclerotiorum* containing succinic acid as the sole carbon source, and the incubation was continued. Pigment separation and measurement of the radioactivity were made 15 days after inoculation. The activity of β -carotene was pretty low in 15 days, but much increased in 20 days. The activity of γ -carotene was also increased in 20 days. The pathway of carotenoid formation was discussed as follows:

Conceptual possibilities and pathways for the phytosynthesis of carotenoids were stimulated by the Porter-Lincoln theory of carotenogenesis in the ripening tomato (19). Theories of carotene formation have been reviewed with a detailed consideration given to the Porter-Lincoln concept of a series of quadri-dehydrogenation steps (20). If the dehydrogenation pathway is taken into

consideration as a provisional hypothesis, the relevant evidence and observations should possibly be composed into a unified rationale which may be tested by investigators in related disciplines. The implications inherent in a coordinated rationale of more recent evidence bearing on carotene biosynthesis merits candid consideration.

Chemical studies on the acyclic polyene (Porter-Lincoln) series have indicated that all are isoprenic and bear an isologous relationship to lycopene. Chemical dehydrogenation of the polyenes, phytoene and phytofluene, into the more unsaturated members of the acyclic polyene series (21), and the well-known oxygen requirement for carotenoid formation in plants and microorganisms lend some support to the phyto-dehydrogenation mechanism as a general possibility. As a rationalization β -carotene may be deemed the objective of carotene phytosynthesis; however, β -carotene is not necessarily constrained to a static state. It apparently exists in a dynamic state in green leaves (22) and may be catabolized via its epoxide and furanoid (23).

Phytoene bears a structural similarity to squalene which is usually associated with animal metabolism. All evidence, including infrared evidence, points to a definite quasi-cyclic structure for squalene with the double bonds in a *trans* configuration (24, 25). In this configuration, squalene is cyclized by a concerted, cationic, non-stop reaction (26) to lanosterol (27). Phytoene has strong infrared absorption bands at 6.1 and 12.9 μ (14) which indicates that the central double bond of the triene conjugation in phytoene is *cis* (28). Otherwise the infrared absorption spectrum is identical to that of squalene bearing out its structural relationship to squalene. If phytoene does not function as a carotene precursor, it is interesting to consider its possible role as a precursor to plant steroids and triterpenes or as a reservoir of metabolic "isoprene."

η -Carotene, which is possibly a cyclized analog of ζ -carotene (29, 30) has been found among plant carotenoids and probably exists in tomatoes and carrots. It is proposed for purposes of this discussion that cyclization occurs with ζ -carotene to form η -carotene.

There are a number of reasons militating against considering the cyclization of lycopene to form β -carotene (20). Among them are the greater thermodynamic stability of lycopene and the overt steric hindrance to direct cyclizations indicated previously. These same arguments apply in part to a consideration of neurosporene as an immediate precursor to β -carotene. If cyclization were to occur at the phytoene level, a steroid-like molecule could very well result from a concerted, non-stop reaction.

In order to unify the known polyenes and carotenes into a rational synthetic relationship, phytoene is considered to be the initial member arising from fundamental processes of "isoprene" synthesis. According to the proposed concept, bis-dehydrogenation first produces phytofluene and then ζ -carotene which is deemed the pivotal polyene. On the one hand, cyclization of ζ -carotene may lead to η -carotene, quadri-dehydrogenation of η -carotene to ε -carotene, and isomerization of ε -carotene to α -carotene and then to β -carotene. On the other hand, bis-dehydrogenation of ζ -carotene leads to neurosporene which in

turn may be dehydrogenated to lycopene, or which by sequential cyclization, dehydrogenation, and isomerization may lead to δ - and then to γ -carotene.

The remarkable observations of Claes on mutants of *Chlorella vulgaris* seem to substantiate this dehydrogenation concept and confirm its application to chlorophyllous tissue (31). One mutant (5/871) accumulated phytoene and another accumulated phytoene, phytofluene, and ζ -carotene. This observation can be explained by a post-phytoene dehydrogenation block and post- ζ -carotene cyclization block, respectively. There were no mutants which accumulated phytofluene exclusively or both phytoene and phytofluene possibly because the same reaction (perhaps the same enzyme) dehydrogenating phytoene to phytofluene also forms ζ -carotene. Zeaxanthin and lutein (xanthophyll) were blocked in the mutants which produced phytoene, phytofluene, and ζ -carotene; however, one mutant (9a) produced xanthophyll, but no α - or β -carotene. This observation suggests that the phytosynthesis of carotenes and xanthophylls diverge at some level subsequent to the synthesis of ζ -carotene. A logical explanation would be that oxygen is introduced in position 3 of the carotene at the η - or ϵ -carotene level. From this point the syntheses of carotenes and xanthophylls could follow parallel pathways.

SUMMARY

1. In addition to the strains known as carotene producers, *Pen. sclerotiorum*, *Pen. multicolor*, *Pen. lapidosum*, and *Rhizoctonia zeae* were found to be new carotene producers, and carotenoid pigments produced by these fungus strains were identified and determined.

2. *Pen. sclerotiorum* NRRL 2074, the best carotene producer, was investigated on its culture condition in some detail, and its carotene content was enhanced up to 1.85 mg total carotene per gram dry mat. Particularly, low pH value of culture medium ($\text{pH} = 2 \pm 0.25$), and addition of nitrogen-containing methyl donor, *e.g.*, betaine and choline, into the medium, were found to stimulate its carotene production remarkably.

3. The stereochemical and spectral properties of the carotenoid and its biosynthetic pathway were discussed.

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