Carotenoid Formation by *Staphylococcus aureus*

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The carotenoid pigments of *Staphylococcus aureus* U-71 were identified as phytoene; ζ-carotene; δ-carotene; phytofluenol; a phytofluenol-like carotenoid, rubixanthin; and three rubixanthin-like carotenoids after extraction, saponification, chromatographic separation, and determination of their absorption spectra. There was no evidence of carotenoid esters or glycoside ethers in the extract before saponification. During the aerobic growth cycle the total carotenoids increased from 45 to 1,000 nmoles per g (dry weight), with the greatest increases in the polar, hydroxylated carotenoids. During the anaerobic growth cycle, the total carotenoids increased from 20 nmoles per g (dry weight) to 80 nmoles per g (dry weight), and only traces of the polar carotenoids were formed. Light had no effect on carotenoid synthesis. About 0.14% of the mevalonate-*2-^{14}C* added to the culture was incorporated into the carotenoids during each bacterial doubling. The total carotenoids did not lose radioactivity when grown in the absence of ^{14}C for 2.5 bacterial doublings. The total carotenoids did not lose radioactivity when grown in the absence of ^{14}C for 2.5 bacterial doublings. The incorporation and turnover of ^{14}C indicated the carotenes were sequentially desaturated and hydroxylated to form the polar carotenoids.

Carotenoids are isoprenoid lipids containing 40 carbon atoms (eight isoprene units) and are detectable in the cell membranes of many microorganisms (for a review see reference 4). The degree of unsaturation and conjugation of the double bonds ranges between colorless saturated polyenes to conjugated, hydroxylated carotenoids which are intensely colored.

Microbial carotenogenesis has been reviewed by Jensen (9) and more recently by Ciegler (4). Colonies of *Staphylococcus aureus* become deeply colored when allowed to grow to stationary phase. This color was attributed to the presence of carotenoids by Zopf (18) in 1889, but no identification of the carotenoids of *S. aureus* was published until 1933 when Chargaff (3) reported that zeaxanthin was the only carotenoid detectable in lipid extracts of *S. aureus*. However, later studies (14, 15) indicated that phytoene, ζ-carotene, δ-carotene and rubixanthin were formed by most strains of *S. aureus*. Other investigators (5) have indicated the presence of sarcinaxanthin and sarcinene in some strains of *S. aureus*. Since Chargaff's study (3), only one other report of zeaxanthin in *S. aureus* has been published (1).

Suzue (15, 16) has studied the synthesis of carotenoids in *S. aureus*. He finds that the initial formation of a C_{40} compound and sequential desaturation to form other carotenoids is similar to that reported for tomato carotenoids by Porter and Lincoln (11). In this study, the carotenoid pigments of strain U-71 were characterized and the general features of their metabolism are described.

MATERIALS AND METHODS

Materials. Commercially available, analytical grade organic solvents were used without further purification. Dibenzylethylenediamine-DL-mevalonate-*2-^{14}C* was purchased from New England Nuclear Corp., Boston, Mass.

Growth of the bacteria. The strain and harvesting conditions of the bacteria have been described (7). A semi-synthetic growth medium used throughout this study consisted of 0.3% (w/v) trypticase, 0.2% (w/v) yeast extract, 50 mM sodium gluconate (15 mM glucose for anaerobic cultures), 25 mM phosphate, 0.4 mM $MgCl_2$, 15 μM $Fe(NH_4)_2(SO_4)_2$, 35 mM NaCl, 4 nM biotin, 0.7 μM thiamine, 8 μM nicotinic acid, and 0.1 mM uracil, adenine, and xanthine. For the identification of the pigments, 8 liters of medium was inoculated with 200 ml of exponentially growing *S. aureus*, and the culture was incubated at room temperature (24 to 28 C) with vigorous stirring and aeration for 18 hr. The cells were harvested in the stationary phase and the lipids were extracted. For measurement of carotenoids during the growth cycle, 4 liters of medium was inoculated with 100 ml of an exponentially growing culture and incubated at 24 to 28 C. Periodically, 400-ml portions were withdrawn, cells were harvested, and the lipids were extracted. Bacteria were grown anaerobically at 37 C in medium sparged with nitrogen

from just after autoclaving through the end of the growth cycle. The nitrogen was purified by passage over hot copper chips (7). Incorporation of ^{14}C was measured during growth in 1,500 ml of medium for 2.5 doublings in the presence of 50 μc of mevalonate-2-^{14}C. To measure turnover, bacteria were grown at 37 C in 250 ml of medium for three doublings in the presence of 25 μc of mevalonate-2-^{14}C. The culture was centrifuged at 37 C; the cells were washed by gently adding medium over the pellet, and were suspended in 1,800 ml of nonradioactive medium for continued incubation. A 1,500-ml culture was grown for 14 hr in the presence of 25 μc of mevalonate-2-^{14}C to provide radioactive carotenoids for autoradiography.

Extraction of the lipids and isolation of the carotenoids. The lipids were extracted by a modified Bligh and Dyer procedure (2). The modification was as follows. Methanol and chloroform were added to the bacteria suspended in 50 mM phosphate buffer, *p*H 7.6 (10 ml of buffer per 30 mg, dry weight of cells), in a separatory funnel such that the proportions were 0.8:2.0:1.0 (v/v), buffer-methanol-chloroform; this mixture was shaken vigorously. The one-phase mixture was allowed to stand for a least 2 hr; then the proportions were made to 0.9:1.0:1.0 (v/v), buffer-methanol-chloroform by adding water and chloroform. The mixture was shaken vigorously and the layers were allowed to separate. The lower layer (chloroform) was filtered through anhydrous sodium sulfate (about 1 g per 10 ml of chloroform) and evaporated to dryness in vacuo. The residue was dissolved in methanol containing 10% (v/v) water (approximately 1 ml per 3 mg, dry weight of cells extracted). Potassium hydroxide was added to the solution to a final concentration of 5% by weight. The lipids were saponified by stirring at room temperature for 2 hr. Between 0.1 and 0.2 volumes of saturated, aqueous sodium chloride was added, and the mixture was extracted three times with a volume of benzene-petroleum ether (1:1, v/v) equal to the volume of the saponification mixture. The combined benzene-petroleum ether extracts were washed once with an equal volume of water. The organic phase was evaporated to dryness in vacuo. The nonsaponifiable lipid residue was dissolved in hexane (1 ml per 3 mg of cells extracted) and stored under nitrogen at 0 C. All these steps were carried out in a minimum of illumination and under nitrogen where possible.

Paper chromatography. Two cycles of paper chromatography were used to separate the carotenoids. The carotenoid mixture was spotted as a series of adjacent spots along the base of a sheet of alumina-impregnated paper (SS-288, Carl Schleicher and Schuell, Co., Keene, N.H.) with less than 15 nmoles per spot. Before chromatography, the paper was activated in a 102 C oven for 2 hr and stored in a dessicating cabinet for not longer than 3 days. Descending chromatography in a solvent of hexane (25 cm in approximately 1 hr) separated the carotenoids as follows: R_F 0.1 to 0.3, the polar carotenoids; R_F 0.40 to 0.45, a pale yellow band; R_F 0.65 to 0.70, a deep yellow band; and R_F 0.85 to 0.90, a colorless band, detected as a pale-green band with a longwave mineral light (Ultra Violet Products, Pasadena, Calif.). As soon as the paper dried, each band was cut out and eluted by soaking in 2% (v/v) acetone in hexane (5 ml per μmole of carotenoid). Ten per cent acetone in hexane was used to elute the polar carotenoids.

The polar mixture (R_F 0.1 to 0.3 in first chromatography) was separated by descending chromatography on alumina-impregnated paper as before with a solvent of 2% acetone in hexane (v/v) into six components as follows: R_F 0.10 to 0.15, a yellow band; R_F value 0.20 to 0.25, a red band; R_F 0.40 to 0.45, an orange band; R_F 0.50 to 0.55, a yellow band; R_F 0.60 to 0.65, a colorless band detected as a dark-blue band with ultraviolet (UV) light; and R_F 0.80 to 0.85, a colorless band detected as a pale-blue band with UV light. Each of these carotenoids was extracted from the paper with 10% acetone in hexane (v/v). The radioautogram was prepared after ascending chromatography on alumina impregnated paper with a solvent of 2% (v/v) acetone in hexane.

Absorption spectra—The purified carotenoids were dissolved in hexane and the absorption spectra measured with the Cary Model 15 spectrophotometer. Carotenoid concentrations were calculated using molar extinction coefficients ($cm^{-1}M^{-1}$) of 6.65×10^4 for phytoene, 1.23×10^5 for ζ-carotene, 1.50×10^5 for δ-carotene, 1.25×10^5 for the rubixanthins, and 1.20×10^5 for the phytofluenols (8).

Assay of radioactivity. Radioactivity was measured in a Packard Scintillation Spectrometer (model 2311) in a scintillation fluid of 9.28 mM 2,5-bis-[2(5-tert-butyl-benzoxazoyl)]-thiophene (BBOT) in toluene. Radioactive samples were dried in the scintillation vials, and a drop of Br_2 was added to each vial. The vials were then placed in a vacuum oven at 45 C for 3 hr, after which 5 ml of scintillation fluid was added. Samples containing ^{14}C were counted under conditions such that the efficiency of counting was 82%. Autoradiograms were prepared by placing chromatograms on Kodak No-Screen X-ray film (17). After 12 days, the film was developed.

RESULTS

The extraction of the carotenoids in this study involved a total lipid extraction with chloroform-methanol rather than the usual methanol extraction. The methanol extraction method as described by Suzue (15) yielded less than 10% of the carotenoids recovered in the total lipid extract. Reextraction of the aqueous phase with chloroform yielded no detectable carotenoids, indicating that the modified Bligh and Dyer (2) extraction removed all chloroform-extractable carotenoids. The cell debris was white after extraction, indicating that no appreciable amount of polar carotenoids remained in the residue. Figure 1 illustrates the extraction and purification procedure used to detect nine carotenoid fractions. In the crude lipid preparation, the content of rubixanthins plus δ-carotene, the two phytofluenols plus ζ-carotene, and the phytoene could be estimated from the absorption spectrum. Comparison of the

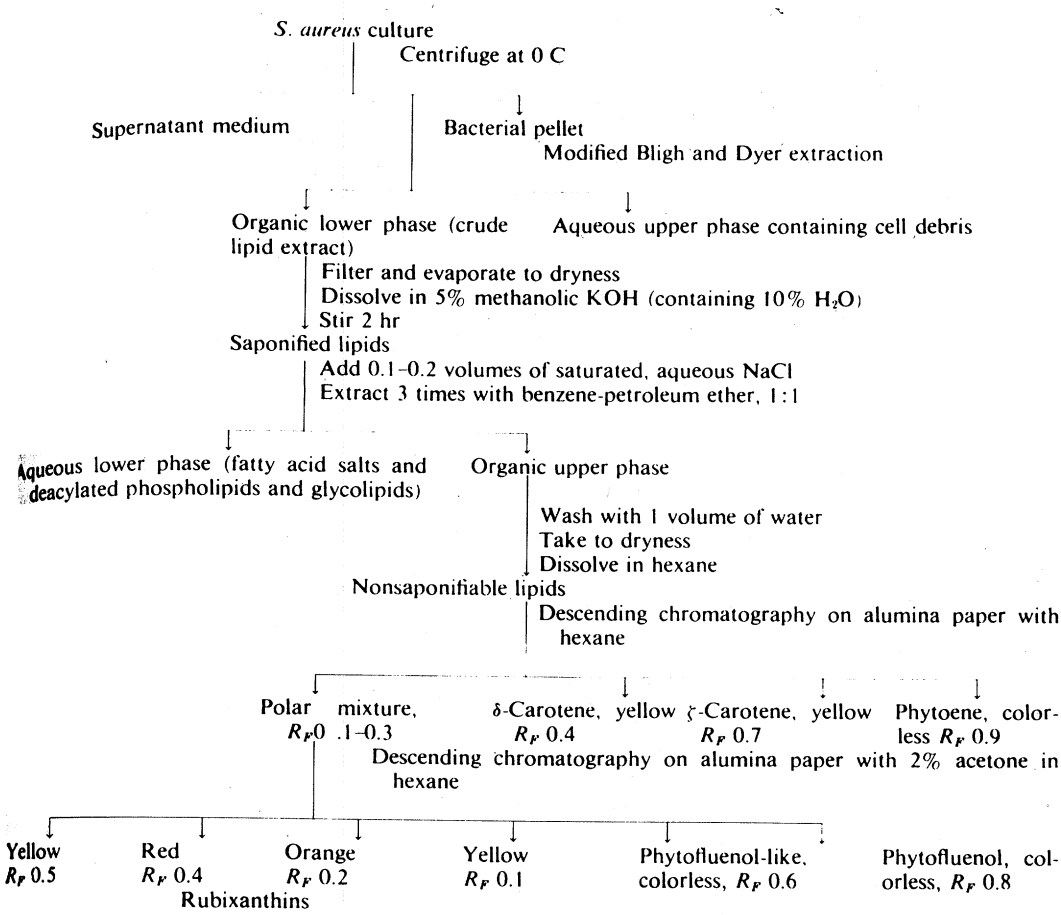

FIG. 1. *Flow chart for isolation and purification of the carotenoids of S. aureus U-71.*

estimates from the spectra of the lipid extract to the yields after saponification indicated that about 6% of the carotenoid mixture was lost. As far as could be determined, there was no differential loss of any carotenoid. Comparison of the amounts of carotenoids from the spectrum of the non-saponifiable lipid mixture with the individual carotenoids after chromatographic separation indicated that recovery after chromatography was quantitative.

When the saponification step was omitted, the recovery of carotenoids was quantitative. If the extract was not saponified, carotenoid separation required removal of the phospholipids by an acetone precipitation at room temperature (25 C). The crude lipid extract was dissolved in a minimal volume of hexane (0.5 ml per 1.0 nmole of carotenoid), and acetone was added dropwise until a precipitate appeared. The mixture was filtered through Whatman no. 4 paper, and the residue was washed with acetone until colorless. The filtrate was then used for chromatography. The carotenoids were detected with the same mobilities and in the same proportions as in preparations that had been saponified. This indicated that neither carotenoid esters nor carotenoid glycosides were present in this strain as had been found in other bacteria (13).

Absorption spectra of the carotenoids. The characteristic absorption spectrum of carotenoids could be demonstrated for each of the purified components of the non-saponifiable lipid extract (Fig. 2). The components were identified as follows.

Phytoene. The R_F 0.85 to 0.90 nonpolar component had the chromatographic mobility and absorption spectrum (maxima at 277, 286, and 300 nm) of phytoene (8).

ζ-Carotene. The R_F 0.65 to 0.70 nonpolar component had the chromatographic mobility and absorption spectrum (maxima at 378, 398, and 425 nm) of ζ-carotene (8).

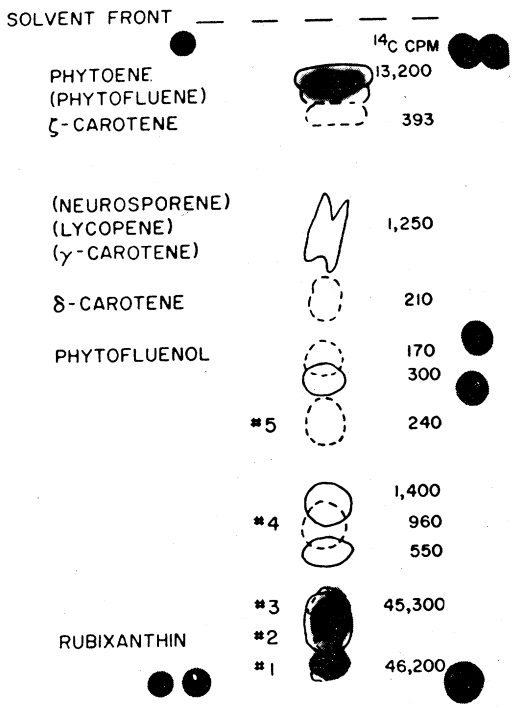

FIG. 2. *Autoradiogram after separation of the carotenoids from S. aureus U-71. Cells were grown with mevalonate-2-^{14}C for 14 hr, and the carotenoids were extracted, saponified, and separated by ascending chromatography on alumina-impregnated paper with a solvent of 2% (v/v) acetone in hexane. Carotenoids were identified by their color in visible light or fluorescence in ultraviolet light as indicated by the dotted lines. Solid lines indicate the major radioactive components; the ^{14}C (counts per minute) for each spot is indicated at the right. Approximately 100 nmoles of carotenoids containing 115,000 counts/min of ^{14}C was applied to the origin.*

δ-Carotene. The R_F 0.40 to 0.45 nonpolar component had the chromatographic mobility and absorption spectrum (maxima at 430, 456, and 488 nm) of δ-carotene (8).

Phytofluenol. The R_F 0.80 to 0.85 polar component had the chromatographic mobility and absorption spectrum (maxima at 333, 348, and 368 nm) of phytofluenol (8). The R_F 0.65 to 0.70 polar component had a chromatographic mobility and absorption spectrum (maxima at 349, 373, and 395 nm) similar to phytofluenol. This component will be referred to as carotenoid 5.

Rubixanthin. The R_F 0.20 to 0.25 polar component had the chromatographic mobility and absorption spectrum (maxima at 437, 461, and 494 nm) similar to rubixanthin (8). This component was red. Three other polar components had chromatographic mobilities and absorption spectra very similar to rubixanthin: R_F 0.10 to 0.15 (maxima at 438, 466, and 499 nm); R_F 0.40 to 0.45 (maxima at 432, 458, and 482 nm); and R_F 0.50 to 0.55 (maxima at 428, 456, and 478 nm). Each of these components, when rechromatographed in the same system, migrated as a single spot. The rubixanthin will be referred to as carotenoid 2, the slower xanthophyll as 1, and the other two xanthophylls as 3 and 4.

Autoradioautography. Once the nine principal carotenoids had been identified by chromatographic mobility and absorption spectra, trace components were examined by autoradioautography. Cells were grown with 25 μc of mevalonate-2-^{14}C in 1,500 ml for 14 hr at 37 C, and the carotenoids were extracted, saponified, and separated on alumina-impregnated paper (Fig. 3). Examination of the chromatogram with UV light showed the characteristic pale-green fluorescence of phytoene at R_F value 0.90; fluorescence extended below the spot on the autoradiogram. The pale-yellow ζ-carotene (R_F 0.84) was clearly separated from the phytoene complex in this chromatogram. In most cases, preliminary chromatography with hexane was necessary to separate phytoene and ζ-carotene. δ-Carotene (R_F 0.58) was yellow. Phytofluenol fluoresced pale blue at R_F 0.49 and carotenoid 5 fluoresced deep

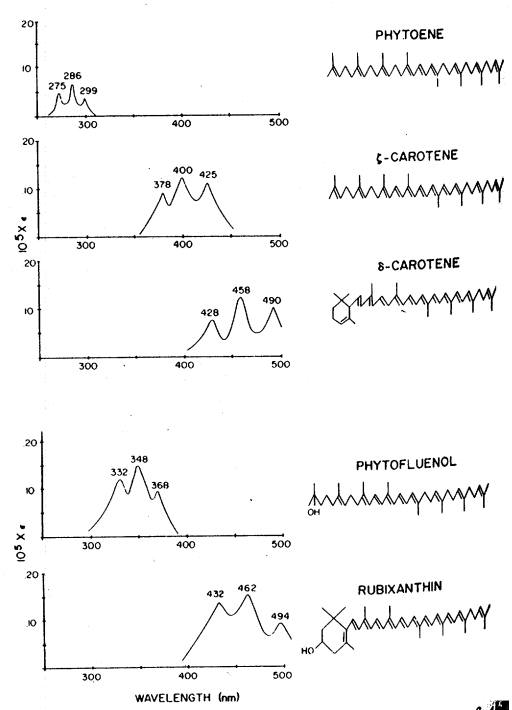

FIG. 3. *Absorption spectra and structures of the major carotenoids of S. aureus U-71. Absorption spectra were measured in hexane.*

blue at R_F 0.38 in UV light. The xanthophyll 4 (R_F 0.23) was yellow; 3 (R_F 0.12) was orange; 2, the rubixanthin (R_F 0.05) was red; and 1, just above the origin was yellow. Some components were detected only on the autoradiogram. At R_F 0.67, a complex spot with the mobility expected for neurosporene, lycopene, and γ-carotene was detected. There were apparently three components migrating with the phytofluenols: phytofluenol (R_F 0.49), an unknown radioactive component (R_F 0.46), and component 5 (R_F 0.38). Two components were detected on the autoradiogram with the xanthophyll component 4, one at R_F 0.19 and one at R_F 0.26. Rubixanthin and the xanthophyll 3 were so heavily labeled that their separation did not show on the autoradiogram. These components account for 96% of ^{14}C applied to the chromatogram. The nine components that have been identified by absorption spectra account for 97% of the ^{14}C recovered from the chromatogram.

Carotenoid formation during aerobic and anaerobic growth. The carotenoid concentration in aerobically growing *S. aureus* is illustrated in Fig. 4. Early in growth, the nonpolar carotenoids phytoene, ζ-carotene, and δ-carotene and the polar phytofluenols predominate. However, later in growth, the rubixanthins became predominant. Growth of cells in a lighted laboratory had no effect on the carotenoid levels when compared to cells grown in total darkness.

The carotenoid concentrations in anaerobically growing *S. aureus* are illustrated in Fig. 5. The nonpolar carotenoids remained predominant throughout the anaerobic growth cycle and the rubixanthins reached approximately 1% of the level found after aerobic growth.

Incorporation of mevalonate-2-^{14}C. The incorporation of mevalonate-*2-^{14}C* into each carotenoid during aerobic growth is illustrated in Fig. 6. The nonpolar carotenoids phytoene, ζ-carotene, and δ-carotene and the polar phytofluenols rapidly became labeled. The specific activities of the nonpolar carotenoids and phytofluenols reached a maximum and then began to decrease at about the time ^{14}C appeared in the rubixanthins.

Turnover of carotenoids labeled with mevalonate-2-^{14}C. Bacteria were grown with mevalonate-*2-^{14}C* for three bacterial doublings. The total radioactivity in each carotenoid during continued growth in the absence of ^{14}C is illustrated in Fig. 7. Phytoene, ζ-carotene, and phytofluenol lost radioactivity rapidly, whereas the total radioac-

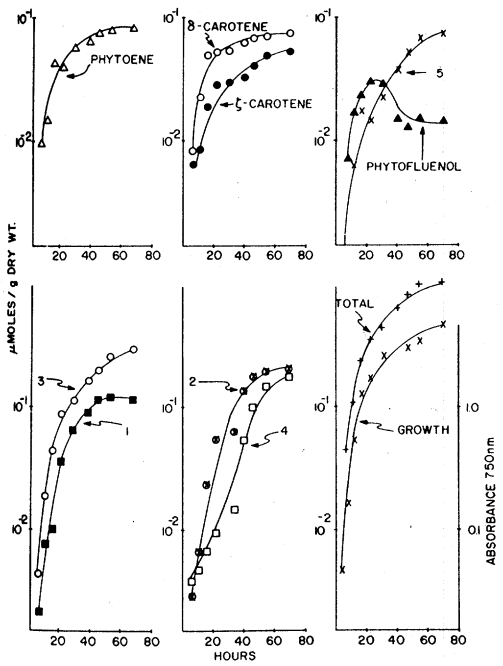

FIG. 4. *Carotenoid formation by S. aureus during aerobic growth. Samples of 400 ml were withdrawn from aerobically growing S. aureus, the lipids were extracted, and the carotenoids were purified and assayed spectrophotometrically. The rubixanthins are designated as 1, 2, 3, and 4 and represent the polar carotenoids with R_F values of 0.5, 0.4, 0.2, and 0.1 in Fig. 1. The phytofluenol-like carotenoid is designated as 5 (R_F 0.6).*

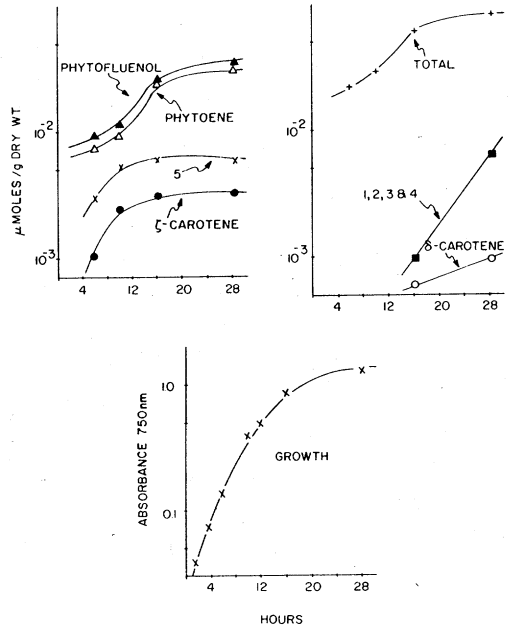

FIG. 5. *Carotenoid formation by S. aureus during anaerobic growth. The carotenoids isolated from an anaerobic culture were treated as in Fig. 4.*

tivity of the rubixanthins increased. The increase in radioactivity in the rubixanthins equaled the decrease in radioactivity in the precursors. During growth in nonradioactive medium, the radioactivity of the total carotenoid did not change.

DISCUSSION

In this study, *S. aureus* U-71 has been shown to contain three nonpolar carotenoids and six xanthophylls as the primary pigments. Possibly seven other unidentified components were present; these minor components accounted for less than 3% of the ^{14}C in the carotenoid fraction. Phytoene, ζ-carotene, and δ-carotene and rubixanthin have been reported previously in *S. aureus* (14, 15). However, the presence of three other rubixanthin-like carotenoids and two phytofluenol-like carotenoids in *S. aureus* has not previously been reported.

The carotenoid concentrations during aerobic growth of *S. aureus* changed over wide limits. Aerobically, there was a 22-fold increase in the concentration of carotenoids per bacterium between the exponential and stationary growth phases (Fig. 4). At the maximal level of carotenoid, the rubixanthins represented 75% of the total compared to 35% in the exponential phase. In the anaerobic growth cycle the total carotenoids increased fourfold (Fig. 5). The rubixanthins represented 8% of the total in the stationary phase and were undetectable ($<10^{-5}$ μmoles per g, dry weight) in the exponential phase. In the anaerobic stationary phase, the rubixanthins

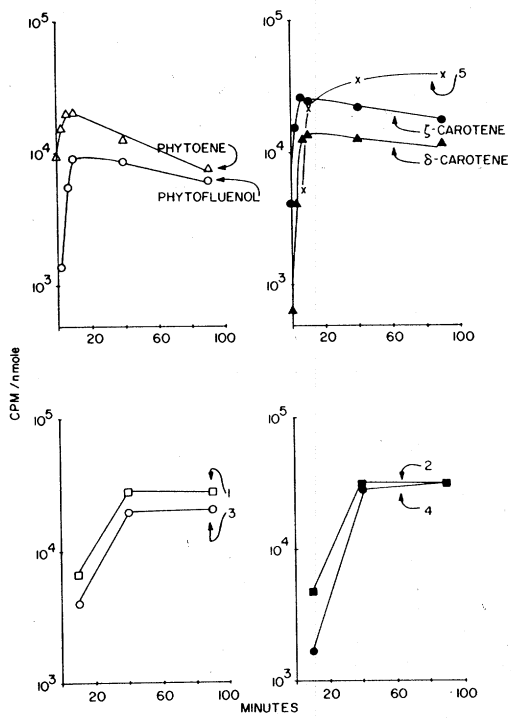

FIG. 6. *Incorporation of mevalonate-2-^{14}C into carotenoids by S. aureus. Samples of 250 ml were withdrawn from a culture of S. aureus growing in the presence of 50 μc of mevalonate-2-^{14}C per 1,500 ml, the lipids were extracted, and the carotenoids were purified and assayed for radioactivity. Carotenoids are indicated as in Fig. 4.*

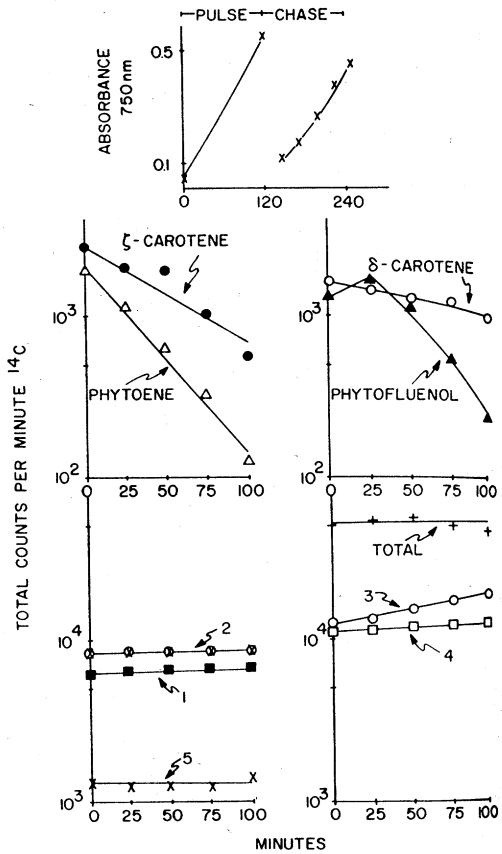

FIG. 7. *Turnover of carotenoids labeled with mevalonate-2-^{14}C in S. aureus. S. aureus, grown for three doublings in the presence of 25 μc of mevalonate-2-^{14}C per 250 ml of culture, were centrifuged at 35 C, the pellet was resuspended in 1,700 ml of nonradioactive medium, and samples of 300 ml were withdrawn. The lipids were extracted, and the carotenoids were purified and assayed for radioactivity as in Fig. 6. The upper graph illustrates the bacterial density during both periods of growth. The carotenoids are designated as in Fig. 4.*

represented 0.8% of the level in the aerobic stationary phase.

The considerably higher concentrations of rubixanthins in the aerobic cells may reflect the oxygen requirement for the hydroxylation of the carotenoids which apparently is common in bacteria (6, 10). An alternate pathway to the rubixanthins has been detected during anaerobic growth (Fig. 5). Possibly the anaerobic pathway involves the hydration of a terminal double bond of phytoene to form phytofluenol. The reaction sequence postulated for carotenoid biosynthesis in *S. aureus* U-71 is illustrated in Fig. 8. Components in parentheses in Fig. 8 were not characterized, although these components could be detected by autoradioautography (Fig. 2). The incorporation of mevalonate-*2-*^{14}*C* into the carotenoids was consistent with the dehydrogenation of the nonpolar, relatively saturated phytoene to the more unsaturated ζ-carotene. The ζ-carotene was then dehydrogenated and cyclized to δ-carotene. The δ-carotene was then hydroxylated to form the rubixanthins.

The turnover of carotenoids labeled with ^{14}C (Fig. 7) was also consistent with the pathways illustrated in Fig. 8. The loss of ^{14}C from phytoene, ζ-carotene, and δ-carotene equaled 80% of the increase in radioactivity in the rubixanthins. Presumably this represented the activity of the aerobic hydroxylation pathway. The loss of ^{14}C from the phytofluenols accounted for the other 20% of the increase in ^{14}C in the rubixanthins. Presumably this represented rubixanthin synthesis by hydration to phytofluenol and subsequent cyclization. The hydration pathway appeared to function both anaerobically and aerobically.

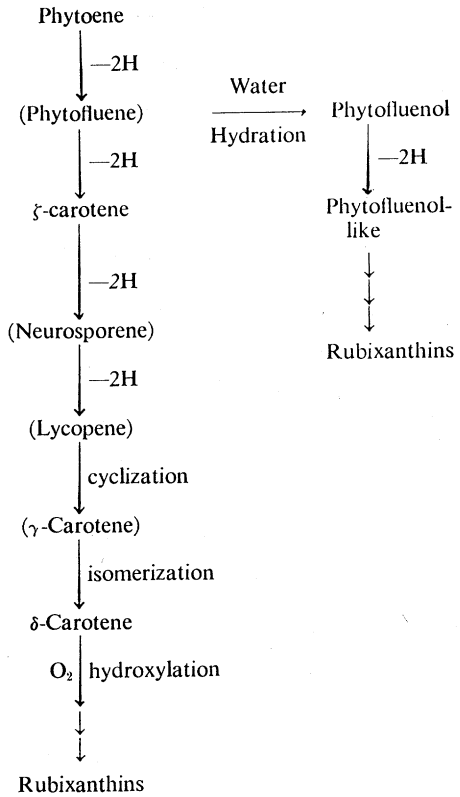

FIG. 8. *Proposed scheme for the terminal reactions of carotenoid biosynthesis in S. aureus. The carotenoids in parentheses have not been characterized from this organism, although evidence for their presence has been obtained (Fig. 2).*

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