JOURNAL OF BACTERIOLOGY, Sept. 1970, p. 607-610
Vol. 103, No. 3

Printed in U.S.A.

Inhibition of Carotenoid Hydroxylation in *Staphylococcus aureus* by Mixed-Function Oxidase Inhibitors

RAY K. HAMMOND AND DAVID C. WHITE

Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40506

Compounds known to be inhibitors of mixed-function oxidase systems inhibited the aerobic synthesis of hydroxylated carotenoids in *Staphylococcus aureus* U-71. Growth of the cells in the presence of 2-diethylaminoethyl-2,2-diphenyl valerate, 2,4-dichloro-6-phenylphenoxyethylamine, 2,4-dichloro-6-phenylphenoxyethyldiethylamine, and piperonyl butoxide reduced the levels of the rubixanthins found in stationary-phase cells by 75 to 97%. In cells grown with mevalonate-*2-*^{14}C, the turnover rate of phytoene was reduced and the turnover rate of phytofluenol was increased in the presence of these inhibitors. The ζ- and δ-carotenes, which turn over in the absence of the inhibitors, accumulated ^{14}C in the presence of the inhibitors. This suggested that a mixed-function oxidase was responsible for the aerobic hydroxylation of δ-carotene in *S. aureus* U-71.

The hydroxylation of cyclic hydrocarbon carotenoids to form xanthophylls in many bacterial species requires molecular oxygen (9). Xanthophylls can also be formed by the anaerobic hydration of noncyclic hydrocarbon carotenoids as in the photosynthetic bacteria (15). Other oxygen-requiring enzymatic reactions involve the introduction of one atom of molecular oxygen into a wide variety of substrates and are termed mixed-function oxidase systems (11). The mixed-function hydroxylases responsible for steroid hydroxylation in adrenal microsomes (3) and for drug and carcinogen hydroxylations in liver microsomes involve a heme protein, cytochrome P_{450} (4, 14). The cytochrome P_{450} of the mixed-function oxidase system can be inhibited specifically by 2-diethylaminoethyl-2,2-diphenyl valerate (SKF 525-A), 2,4-dichloro-6-phenylphenoxyethylamine (DPEA), 2,4-dichloro-6-phenylphenoxyethyldiethylamine (DPDA), and piperonyl butoxide (PB; references 2, 5, 8, 10).

The marked stimulation of rubixanthin synthesis by oxygen in *Staphylococcus aureus* (6, 7) suggested that molecular oxygen could be involved. This study shows that the specific inhibitors of mixed-function oxidases inhibit the oxygen-requiring hydroxylation of δ-carotene to form the rubixanthins.

MATERIALS AND METHODS

Materials. DPEA and DPDA were the gifts of the Lilly Research Laboratory, Indianapolis, Ind. SKF 525-A was supplied by Smith, Kline and French Laboratories, Philadelphia, Pa.; 2,4-diamino-5,6,7,8-tetrahydro quinazoline was the gift of N. A. Nugent and R. C. Fuller. PB was supplied by K & K Laboratories, Inc., Plainview, N.Y. Other materials were as described (6, 7, 16).

Growth of S. aureus. The strain, medium, growth conditions and harvesting procedures have been described (6). The effect of inhibitors was studied by dissolving them in dimethyl sulfoxide. The inhibitors were added to 250-ml Erlenmeyer flasks containing 25 ml of medium such that the final concentration of dimethyl sulfoxide was 0.4% (v/v). Cultures were shaken rapidly for 18 hr at 37 C. Bacterial density was measured at 750 nm in 13-mm round test tubes (16). For the turnover experiments, cultures were grown for two to three divisions in 25 μc of mevalonate-*2-*^{14}C in 250 ml of medium (6). The cultures were then centrifuged at 37 C, suspended in 1,700 ml of nonradioactive medium containing either 50 μg of DPDA or 100 μg of PB per ml of medium, and sampled as described (6).

Extraction and assay of the carotenoids. Lipids were extracted by the Bligh and Dyer procedure (1), the extract was saponified, and the carotenoids were separated chromatographically and assayed spectrophotometrically as described (6). The small samples used to determine the effects of the inhibitors were assayed differently. The crude extract was dissolved in hexane and the absorption spectrum was recorded. The absorbance at 460 nm (rubixanthins plus δ-carotene, $\epsilon=125$), at 400 nm (ζ-carotene, $\epsilon=120$), at 375 nm (the phytofluenols, $\epsilon=120$), and at 286 nm (phytoene, $\epsilon=67$) was used to assay the carotenoids. A portion of the carotenoid mixture was chromatographed on alumina-impregnated paper no. 288 (Schleicher and Schuell, Keene, N.H.), with a solvent of 2% (v/v) acetone in hexane, which moves the nonpolar carotenoids (phytoene and the carotenes

R_F value 0.5 to 0.8) from the polar carotenoids and phospholipids, which remain at the origin. The origin was eluted (16), and the absorption spectrum was compared with that of the total carotenoid extract to determine the proportion of the absorption at 460 nm corresponding to the δ-carotene.

Radioactivity. Radioactivity was measured with a scintillation spectrometer as described (6).

RESULTS

Effects of the inhibitors on growth. The cell yield after 18 hr of growth with the inhibitors is illustrated in Table 1. The amines DPDA and DPEA inhibited growth substantially above 50 μg/ml. At 400 μg of PB per ml, there was substantial growth. After centrifugation the bacterial pellets showed a progressive decrease in color with increasing inhibitor concentration.

Effects on carotenoid formation. Analysis of the carotenoid levels at the end of the growth cycle in the cultures used in Table 1 revealed that, as the concentration of PB was increased, phytoene accumulated and δ-carotene decreased. The most striking effect was the substantial decrease in the rubixanthin concentration (Fig. 1). Cells growing in the presence of 50 μg of PB per ml formed only 10% as much of the rubixanthins as the control culture. As the concentrations of DPDA and DPEA were increased, similar effects were detected. In the presence of 25 μg of DPEA or DPDA, 13 to 25% as much rubixanthin was formed as in the control culture (Fig. 2). The phytoene concentration decreased, and δ-carotene increased as the concentration of the amines was raised (Fig. 2). As the concentration of SKF 525-A increased, δ-carotene accumulated and the rubixanthins again decreased substan-

TABLE 1. *Effects of the mixed-function oxidase inhibitors on growth of S. aureus*

Inhibitor[a]	Turbidity[b] (μg of inhibitor/ml of medium)							
	0	6.25	12.5	25	50	100	200	400
PB.....	4.2	5.0	5.1	4.3	4.6	4.1	3.4	2.4
DPEA.....	4.2	3.9	3.9	3.1	2.0	1.0		
DPDA.....	4.2	4.0	3.9	4.5	2.7	0.09		
SKF 525-A..	4.1	4.5	4.2	3.8	3.7	3.2		

[a] Inhibitors were added in DMSO to 250-ml Erlenmeyer flasks containing 25 ml of medium so that the final concentration of DMSO was 0.4% (v/v) in all flasks.

[b] Absorbancy at 750 nm was measured in 13-mm round test tubes after 18 hr at 37 C. Cultures were aerated by vigorous shaking. Cultures with absorbancies greater than 0.6 were diluted with water before reading.

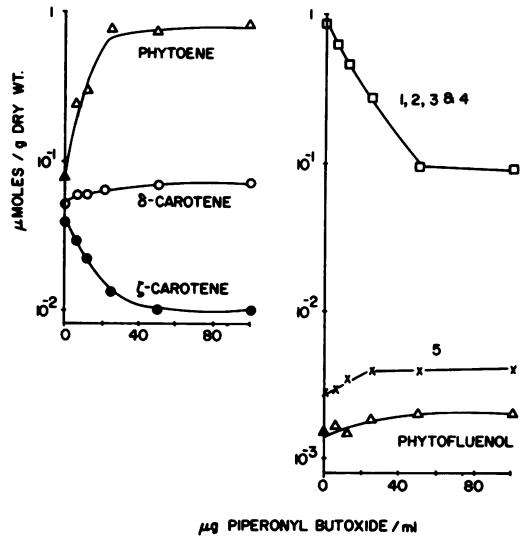

FIG. 1. *Carotenoid levels in S. aureus U-71 after growth in PB. Carotenoids were isolated from the cells and assayed spectrophotometrically as described in Materials and Methods. Cells were harvested after 18 hr of aerobic growth. The numerals 1, 2, 3, and 4 refer to rubixanthin and the three rubixanthin-like carotenoids; 5 refers to a phytofluenol-like carotenoid.*

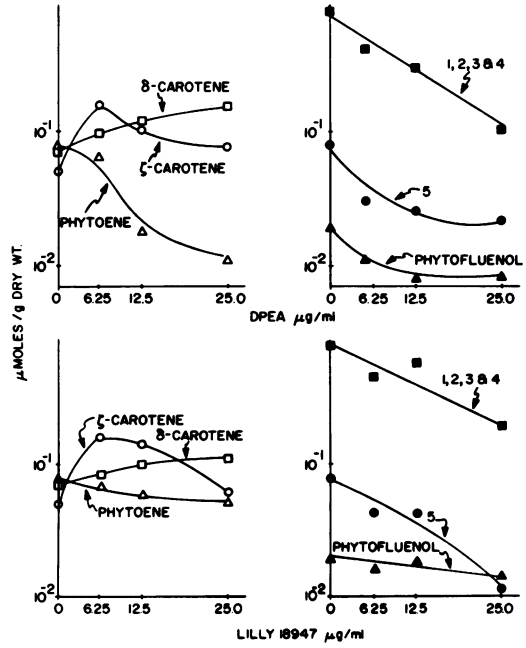

FIG. 2. *Carotenoid levels in S. aureus U-71 after growth with DPEA and DPDA (Lilly 18947). Carotenoids were assayed as in Fig. 1.*

tially (Fig. 3). Cells growing in the presence of 50 μg of SKF 525-A per ml formed only 3% as much of the rubixanthins as the control cultures. None of these inhibitors had any effect on the vitamin K_2 or phospholipid levels of the cells.

The pteridine inhibitor 2,4-diamino-5,6,7,8-tetrahydroquinazoline (12) had no effect on aerobic growth or carotenoid formation in *S. aureus* U-71 at concentrations up to 100 μg/ml.

Turnover of carotenoids in the presence of inhibitors. The ^{14}C in the carotenoids of cells grown with mevalonate-2-^{14}C for two divisions, was measured after transfer of the cells to nonradioactive medium containing 100 μg of PB per ml (Fig. 4). Phytoene lost 52% of its radioactivity in 50 min. The ^{14}C accumulated in the carotenes in this 50-min period of growth in nonradioactive medium. In control cultures, phytoene lost 72% of its radioactivity, ζ-carotene lost 48% of its radioactivity, and δ-carotene lost 20% of its radioactivity during the same period (6). In the presence of PB, the phytofluenols lost 53% of their radioactivity in contrast to 34% in the control cultures (6). There was no turnover of ^{14}C in the total carotenoid in the presence or absence of PB. At 100 μg of PB per ml, there was no effect on the growth rate.

In a similar experiment, cells grown with mevalonate-2-^{14}C were suspended in nonradioactive medium containing 50 μg of DPDA per ml, and the ^{14}C in the carotenoids was determined (Fig. 5). Phytoene lost 51% of its radioactivity,

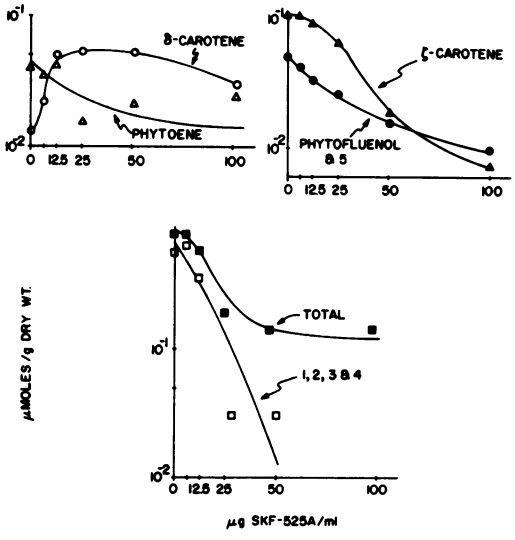

FIG. 3. *Carotenoid levels in S. aureus U-71 after growth with SKF 525-A. Carotenoids were isolated as in Fig. 1.*

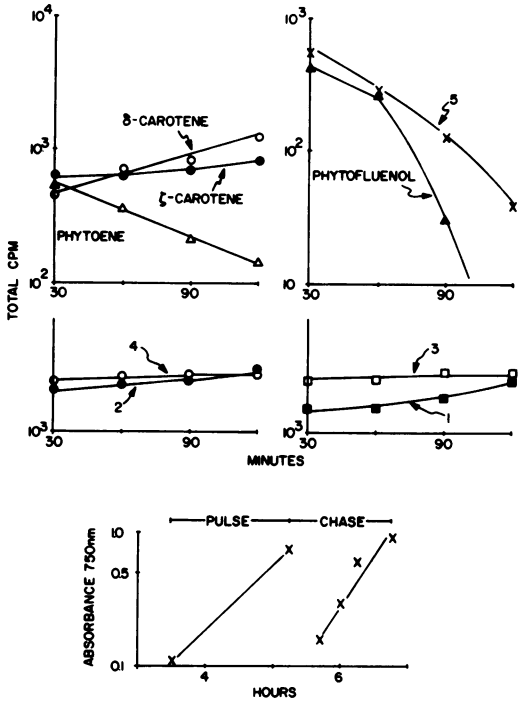

FIG. 4. *Turnover of carotenoids during growth in the presence of PB. Cultures were incubated in the presence of mevalonate-2-^{14}C for two divisions, washed, and diluted into nonradioactive medium containing 100 μg of PB per ml as illustrated in the lower curve. After 30 min of growth in the nonradioactive medium, the cultures were sampled periodically, the lipids were extracted, and the carotenoids were separated and assayed for ^{14}C as indicated in the upper curves. Carotenoids are indicated as in Fig. 1.*

the carotenes accumulated ^{14}C, and the phytofluenols lost 32% of their radioactivity in 50 min after the transfer to nonradioactive medium. Again there was no turnover of the total carotenoid ^{14}C. DPDA at 50 μg/ml had no effect on the growth rate.

DISCUSSION

The mixed-function oxidase inhibitors DPEA, DPDA, SKF 525-A, and PB inhibited the aerobic synthesis of the rubixanthins by 75 to 97% at concentrations which did not affect the growth rate. Total carotenoid levels were not affected by PB. The increase in phytoene and the carotenes was matched by the decrease in the rubixanthins (Fig. 1). DPDA, DPEA, and SKF 525-A substantially decreased both the total carotenoid level and the rubixanthins (Fig. 2, 3). The rate of loss of radioactivity from phytoene was de-

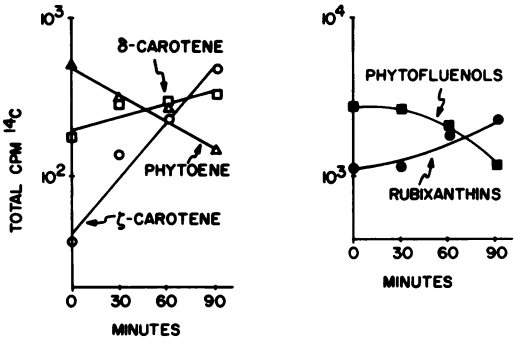

FIG. 5. *Turnover of carotenoids during growth in the presence of DPDA. The experimental conditions were the same as in Fig. 4 except that 50 μg of DPDA per ml was added after the transfer to nonradioactive medium.*

creased by 20% in the presence of PB and DPDA (Fig. 4, 5). The carotenes, which lose radioactivity in a pulse-chase experiment, accumulated ^{14}C in the presence of PB and DPDA.

These inhibitors are relatively specific for the P_{450} mixed-function oxidase activities of the mammalian microsomes which hydroxylate a variety of substrates with molecular oxygen (8, 10, 14). The facts that aeration greatly stimulates rubixanthin formation, that incorporation and turnover data are compatible with a pathway from phytoene via ζ-carotene to δ-carotene to the rubixanthins (6), and that these specific inhibitors block rubixanthin formation and cause accumulation of δ-carotene suggest very strongly that a mixed-function oxidase hydroxylates δ-carotene to form the rubixanthins in aerated *S. aureus*. In a difference spectrum of *S. aureus* which compares cells with respiratory pigments reduced in the presence of L-lactate with cells with pigments reduced and saturated with carbon monoxide, a maximum between 448 and 460 nm appears that is similar to the P_{450} pigment in liver (13). The *S. aureus* carbon monoxide-binding pigment was induced when anaerobic cultures were aerated (Joyce and White, *unpublished data*).

A second pathway involving the hydration of the noncyclic carotenes to phytofluenol with subsequent cyclization to the rubixanthins accounted for the slow anaerobic formation of rubixanthins in *S. aureus* U-71 (6, 7). Aerobic growth in the presence of PB may stimulate this secondary pathway, as there was an increased loss of radioactivity from the phytofluenols (Fig. 4). Growth with DPDA did not increase the rate of loss of radioactivity from the phytofluenols above the control (Fig. 5).

ACKNOWLEDGMENTS

This investigation was supported by contract 12-14-100-9517 (73) with the Agricultural Research Service, U.S. Department of Agriculture, administered by the Eastern Utilization and Development Division, Philadelphia, Pa., and by Public Health Service grant GM-10285 from the National Institute of General Medical Sciences.

R.K.H. was supported by grant 5-T01-GM-01026 from the National Institute of General Medical Sciences to the Department of Biochemistry, University of Kentucky Medical Center.

Generous gifts of DPEA and DPDA from R. E. McMahon, Lilly Research Laboratories, Indianapolis, Ind., SKF 525-A from G. E. Ulloy, Smith, Kline and French Laboratories, Philadelphia, Pa., and DTHQ from N. A. Nugent and R. C. Fuller, Biology Division, Oak Ridge National Laboratories, Oak Ridge, Tenn., made this study possible.

LITERATURE CITED

1. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.
2. Brodie, B. B., J. Axelrod, J. R. Cooper, L. E. Gaudette, B. N. LaDu, C. Mitoma, and S. Undefriend. 1955. Detoxication of drugs and other foreign compounds by liver microsomes. Science 121:603–604.
3. Cooper, D. Y., R. W. Estabrook, and O. Rosenthal. 1963. The stoichiometry of C_{21} hydroxylation of steroids by adrenocortical microsomes. J. Biol. Chem. 238:1320–1323.
4. Cooper, D. Y., S. Levine, S. Narasimhulu, O. Rosenthal, and R. W. Estabrook. 1965. Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. Science 147:400–402.
5. Fauts, J. R., and B. B. Brodie. 1955. Inhibition of drug metabolic pathways by the potentiating agent 2,4-dichloro-6-phenyl-phenoxyethyl diethylamine. J. Pharmacol. Exp. Ther. 115:68–73.
6. Hammond, R. K., and D. C. White. 1970. Carotenoid formation by *Staphylococcus aureus*. *J. Bacteriol.* 103:191–198.
7. Hammond, R. K., and D. C. White. 1970. Inhibition of vitamin K_2 and carotenoid synthesis in *Staphylococcus aureus* by diphenylamine. *J. Bacteriol.* 103:611–615.
8. Jaffe, H., K. Fujii, H. Guerin, M. Sengupta, and S. S. Epstein. 1969. Bimodal effect of piperonyl butoxide on the o- and p-hydroxylations of biphenyl by mouse liver microsomes. Biochem. Pharmacol. 18:1045–1051.
9. Liaaen-Jensen, S. 1965. Biosynthesis and function of carotenoid pigments in microorganisms. Annu. Rev. Microbiol. 19:163–182.
10. McMahon, R. E., J. Mills, H. W. Culp, W. R. Gibson, W. M. Miller, and F. J. Marshall. 1969. Demethylation studies. VI. The inhibition of hepatic microsomal oxygenation by 2,4-dichloro (6-phenylphenoxy)ethylamine and related compounds. J. Med. Chem. 12:207–211.
11. Mason, H. S. 1957. Mechanisms of oxygen metabolism. Advan. Enzymol. 19:79–233.
12. Nugent, N. A., and R. C. Fuller. 1967. Carotenoid biosynthesis in *Rhodospirillum rubrum:* Effect of pteridine inhibitor. Science 158:922–924.
13. Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239:2370–2378.
14. Omura, T., R. Sato, D. Y. Cooper, O. Rosenthal, and R. W. Estabrook. 1965. Function of cytochrome P_{450} of microsomes. Fed. Proc. 24:1181–1189.
15. Rilling, H. 1962. Anaerobic carotenoid synthesis by an aerobic microorganism. Biochim. Biophys. Acta 65:156–158.
16. White, D. C., and F. E. Frerman. 1967. Extraction, characterization, and cellular localization of the lipids of *Staphylococcus aureus*. J. Bacteriol. 94:1854–1867.