JOURNAL OF BACTERIOLOGY, Sept. 1970, p. 611-615
Vol. 103, No. 3
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Inhibition of Vitamin K_2 and Carotenoid Synthesis in *Staphylococcus aureus* by Diphenylamine

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Received for publication 26 January 1970

Diphenylamine at concentrations which did not effect the growth rate inhibited the synthesis of vitamin K_2 in both anaerobic and aerobic cultures by about 50%. At this concentration, diphenylamine inhibited the synthesis of the cyclic carotenoids δ-carotene and the rubixanthins 25 to 35% anaerobically and 60 to 90% aerobically. The inhibition of synthesis of cyclic carotenoids and vitamin K_2 by diphenylamine had no detectable effect on the formation of the membrane-bound electron transport system.

Diphenylamine (DPA) inhibits the synthesis of carotenoid pigments in several microorganisms at concentrations which do not affect the growth rate (12, 17). Depending on the concentration and the organism, DPA inhibits (12) or does not inhibit (13) steroid synthesis or affects (4) or does not affect (13) fatty acid synthesis. DPA does not affect the synthesis of triglyceride in yeast (17). There is a marked inhibition of benzoquinone formation (12, 18) and a somewhat less severe inhibition of naphthoquinone formation in a number of microorganisms (16). Even though synthesis of various lipid components of the membrane can be inhibited, the total mass of the membrane formed by cells grown in DPA appears to be normal (15).

In view of the possible involvement of membrane lipids in the formation of the electron transport system of *Staphylococcus aureus* (5), the effect of DPA on the synthesis of lipids and the formation of the membrane-bound electron transport system were examined.

MATERIALS AND METHODS

Materials. DPA (Fisher Chemical Co., St. Louis, Mo.) was used without further purification. Other materials were as described (7, 9, 22).

Growth of S. aureus U-71. The media, culture conditions, and harvesting procedures have been described (9). For anaerobic growth, the medium was sparged with deoxygenated nitrogen beginning just after autoclaving and continuing throughout the experiment (5, 22). The formation of the electron transport system was induced by aerating anaerobic cultures growing exponentially as described (5). The bacterial density was determined by the absorbancy at 750 nm (22). DPA was dissolved in dimethyl sulfoxide and added to the culture, so that the final concentration of DPA was 74 μM and dimethyl sulfoxide was 0.4% (v/v).

Extraction of the lipids. The lipids were extracted by the Bligh and Dyer procedure (3) as modified (9). A portion of the lipid extract was saponified, and the carotenoids were separated chromatographically and assayed spectrophotometrically (9). A second portion of the lipid extract was purified by thin-layer chromatography, and the vitamin K_2 was assayed spectrophotometrically (7, 8). In lipids from bacteria grown with DPA, the vitamin K_2 fraction isolated from the thin-layer plates was applied to a second Silica Gel G thin-layer plate and chromatographed with isooctane-chloroform (2:1, v/v). The vitamin K_2 band (R_F value 0.3 to 0.4) was recovered and had no spectral evidence of DPA contamination. None of the purified carotenoid fractions showed spectral evidence of DPA contamination. Phospholipids were analyzed as described previously (22).

Assay of the electron transport system. Cytochromes were measured by difference spectroscopy, oxygen utilization was measured with the polarograph in the presence of L-lactate, and protoheme was measured by the reduced pyridine hemochrome as described previously (22).

RESULTS

Isoprenoid lipids of S. aureus. *S. aureus* U-71 has been shown to contain the carotenoids phytoene, ζ-carotene, δ-carotene, phytofluenol, a phytofluenol-like carotenoid, rubixanthin, and three rubixanthin-like carotenoids as the major components (9). Vitamin K_2 isoprenologues with side chains containing zero to nine isoprene units have been identified in this strain of *S. aureus* (7, 8). In cells grown for six to eight divisions in the presence of 5 μc of mevalonate-2-^{14}C per 50 ml, 50% of the lipid ^{14}C was recovered in the vitamin K_2 and the nine carotenoids.

Inhibition of growth by DPA. DPA inhibits growth above 148 μM (Table 1). At a DPA concentration of 74 μM, the final yield of cells was re-

TABLE 1. *Growth yield of S. aureus U-71 grown with DPA*

DPA concn[a]	Growth yield[b]	Color of cells[c]
μM		
0	4.1	Orange
37	4.2	Orange
74	3.8	White
148	2.8	White
296	0.05	—[d]
592	0.01	—

[a] DPA was added in dimethyl sulfoxide with the final dimethyl sulfoxide concentration 0.4% (v/v). Cultures were grown in 250-ml Erlenmeyer flasks for 18 hr at 37 C (10).

[b] Bacterial density was measured as absorbancy at 750 nm in 13-mm diameter round test tubes (22). When the absorbancy was greater than 0.6, the cultures were diluted 1 to 10 with water before measurement.

[c] Color of the bacterial pellet after centrifugation at 4,000 × *g* for 10 min.

[d] No color detected.

duced by less than 10%, and the growth rate was not affected. In cells grown with 74 μM DPA, approximately 2% of the DPA added to the culture was recovered in the lipid extract. The remainder was in the medium after the cells were harvested by centrifugation.

Inhibition of carotenoid synthesis by DPA. In the anaerobic growth cycle, DPA had no effect on the growth rate or the synthesis of phytoene, phytofluenol, the phytofluenol-like pigment or ζ-carotene (Fig. 1). There was a threefold inhibition of the level of the trace cyclic carotenoids (δ-carotene and the rubixanthins) early in stationary phase. By late stationary phase, the cyclic carotenoid concentration was depressed by 24% in cells grown with DPA. The cyclic carotenoids represent such a small portion of the total carotenoids in anaerobic cells that the total carotenoid was depressed by less than 1% by the end of the growth cycle.

Oxygen markedly stimulates the synthesis of carotenoids in *S. aureus* (9). When an anaerobic, exponentially growing culture was aerated, a rapid synthesis of the cyclic carotenoids, δ-carotene, and the rubixanthins occurred (Fig. 2). At the onset of aeration, there was a sharp decrease in the content of phytoene and the phytofluenols that was followed by a marked increase in the phytofluenols (Fig. 2).

When DPA was added at the onset of aeration, there was an initial decrease in phytoene as in the control, but the phytoene concentration never recovered (Fig. 3). The synthesis of the other carotenoids was markedly inhibited. After 4 hr of aerobic growth, the control culture contained 156 nmoles of carotenoid per g (dry weight), of which the phytoene represented 35 nmoles, the δ-carotene 22 nmoles, and the rubixanthins 72 nmoles. In the presence of DPA after 4 hr of aerobic growth, the cells contained 60 nmoles of carotenoid per g (dry weight), of which phytoene represented 22 nmoles, δ-carotene 1.1 nmoles, and the rubixanthins 1.5 nmoles (Fig. 2, 3).

Inhibition of vitamin K_2 formation. During the anaerobic growth cycle, the presence of 74 μM DPA reduced the vitamin K_2 level by about half without affecting the growth rate (Fig. 4). When air was added to logarithmically growing cells, there was a stimulation of growth and vitamin K_2 formation. Addition of air and DPA showed no effect on growth, but the final level of vitamin K_2 achieved (1.1 μmoles per g, dry weight) was again about half that found in logarithmically growing aerobic cells (2.0 μmoles per g dry weight). In this strain of *S. aureus*, both anaerobic and aerobic cultures achieved the same level of vitamin K_2 in the stationary phase (2.0 to 2.4 μmoles per g dry weight).

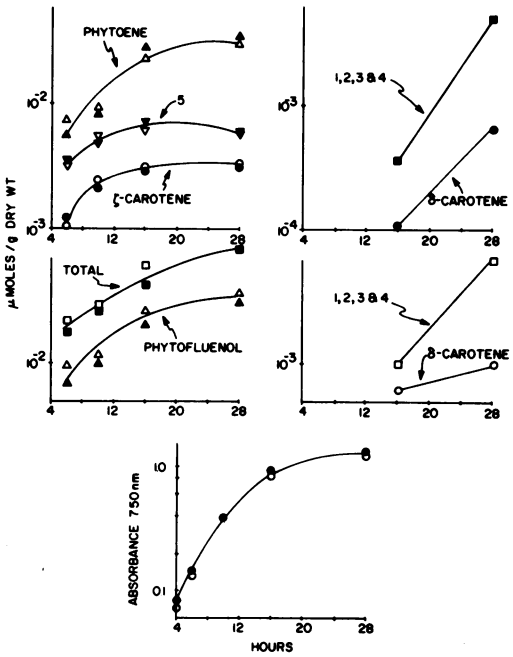

FIG. 1. *Carotenoid levels in S. aureus U-71 during anaerobic growth in the presence of 74 μM DPA. Bacteria were grown anaerobically (5, 9) as illustrated in the lower graph. Solid symbols represent growth with DPA and open symbols without DPA. The cells were harvested, the lipid was extracted and saponified, the carotenoids were separated chromatographically, and the pigments were assayed spectrophotometrically (9). The numerals 1, 2, 3, and 4 refer to rubixanthin and the three rubixanthin-like carotenoids (9); 5 refers to a phytofluenol-like carotenoid (9).*

Phospholipid concentration. Anaerobically growing *S. aureus* in the exponential phase contained about 34 μmoles of phospholipid per g (dry weight). The phospholipids increase to 50 μmoles per g (dry weight) in the anaerobic stationary phase. The addition of 74 μM DPA to the culture had no effect on the total phospholipid content of anaerobically growing cells. When anaerobically growing cells in the exponential growth phase were aerated, the total phospholipid increased rapidly to about 50 μmoles per g (dry weight) and was maintained at this level until the organism entered the stationary growth phase. When DPA was added at the time of aeration, the phospholipid increased to 50 μmoles per g (dry weight) and was maintained at that level. DPA at 74 μM had little effect on the phospholipid concentration of *S. aureus*.

Formation of the electron transport system. The switch from anaerobic to aerobic growth caused the induction of the membrane-bound electron transport system (5). When 74 μM DPA was added at the onset of aerobic growth, there was no

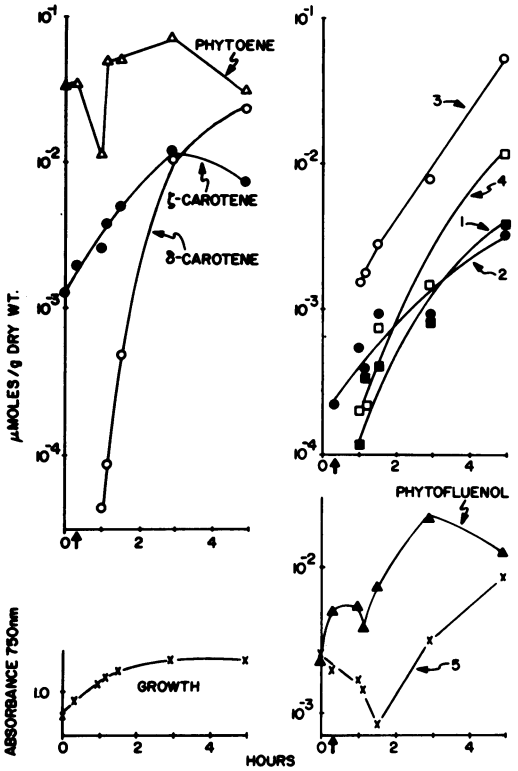

FIG. 2. *Carotenoid levels during the switch from anaerobic to aerobic growth. Exponentially growing cells incubated anaerobically were aerated at the time indicated (↑) with 6 liters of air per min per 8 liters of medium as described (5). Samples were withdrawn and analyzed as in Fig. 1.*

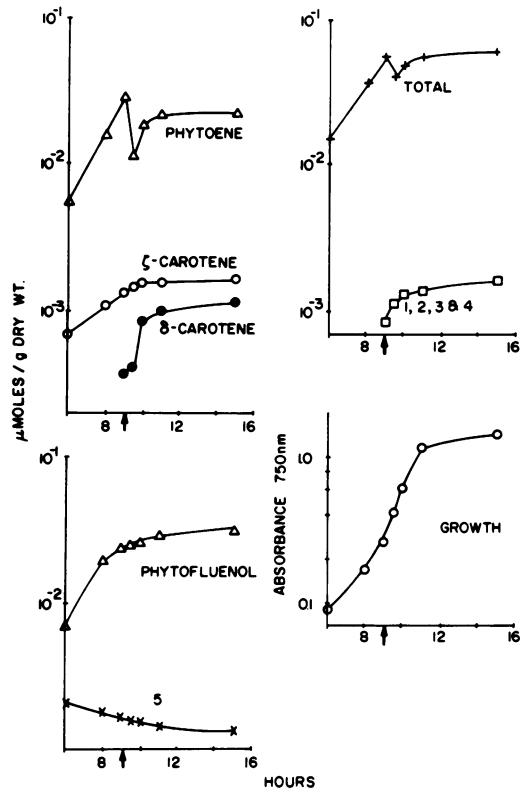

FIG. 3. *Carotenoid levels during the switch from anaerobic to aerobic growth in the presence of DPA. In an experiment like that illustrated in Fig. 2, DPA (74 μM) was added at the time of aeration (↑), and the carotenoids were assayed as in Fig. 1.*

detectable change in the rate of growth, the synthesis of protoheme, or the appearance of enzymatically reducible cytochromes *b*, *o*, and *a* (Fig. 5). The final levels of these pigments were similar to those in the control experiments. There was no detectable difference in the rate of oxygen utilization in the presence of L-lactate or in the critical oxygen concentration between control and cells grown with DPA. The critical oxygen concentration is a sensitive measure of the function of the electron transport system (5, 20).

DISCUSSION

DPA had multiple effects on the two major isoprenoid lipids in *S. aureus* U-71 at 74 μM, a concentration which had no effect on bacterial growth. Vitamin K_2 biosynthesis was depressed by about 50% both anaerobically and aerobically (Fig. 4). This strain of *S. aureus* was unusual in that it formed as much vitamin K_2 in the anaerobic stationary phase as it did when growing aerobically. Usually anaerobically growing *S. aureus* forms very little vitamin K_2 (2).

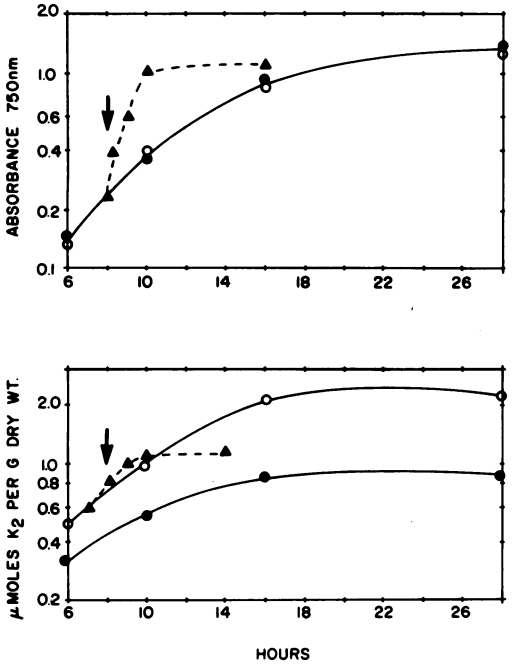

FIG. 4. *Vitamin K_2 levels during anaerobic and aerobic growth. Circles indicate bacterial densities (upper graphs) and total vitamin K_2 (lower graphs) in S. aureus U-71 grown anaerobically in the absence of DPA (○) or in the presence of 74 μM DPA (●). Growth (▲, upper curve) and vitamin K_2 (▲, lower curve) of cells grown anaerobically and then aerated (↓) as in Fig. 2. DPA (74 μM) was added at the initiation of aeration.*

From studies of the incorporation and turnover, the pathways of carotenoid metabolism in *S. aureus* U-71 appear to follow the usual sequence of phytoene desaturation through ζ-carotene, cyclization to δ-carotene, and hydroxylation to form the rubixanthins with an alternate pathway of hydration to phytofluenol, followed by cyclization to the rubixanthins (9). The hydroxylation of δ-carotene to form rubixanthins appears to involve molecular oxygen and a mixed oxidase system and is responsible for the major portion of the rubixanthin (10). The anaerobic pathway involves hydroxylation to the phytofluenols and functions to a limited extent during both anaerobic and aerobic growth (9, 10).

Phytoene, the initial 40-carbon carotenoid, accumulates in many microorganisms in the presence of DPA (6, 11, 14, 16). In anaerobically growing *S. aureus*, DPA had little effect on the formation of phytoene, the phytofluenols, or ζ-carotene (Fig. 1). ζ-Carotene synthesis was inhibited 33% and the rubixanthin synthesis by 22% in the presence of DPA during anaerobic growth (Fig. 1). Phytoene synthesis was inhibited slightly during aerobic growth (Fig. 3), as in other strains of *S. aureus* (19). The most marked effect of DPA was on the aerobic synthesis of the cyclic carotenoids (Fig. 3). The aerobic synthesis of δ-carotene was inhibited 20-fold and the rubixanthins 50-fold by DPA (Fig. 2, 3).

About 2% of the DPA was recovered in the lipid extract (3 μmoles per g, dry weight). This is about the same concentration of DPA accumulated in the membranes of *Bacillus megaterium* (16). This DPA concentration approximates the vitamin K_2 content of the membrane and is 20

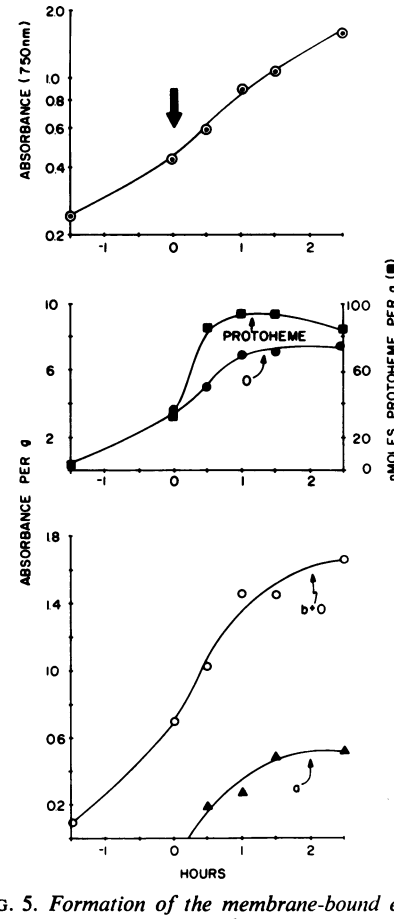

FIG. 5. *Formation of the membrane-bound electron transport system in the switch from anaerobic to aerobic growth. Aeration was initiated at indicated time (↓, upper curve) of anaerobically growing cultures as in Fig. 2. Protoheme was assayed as the reduced-minus-oxidized pyridine hemochrome (5). Cytochrome oxidase o was estimated from the reduced-minus-reduced CO-saturated difference spectra and cytochromes b + o and a were estimated from the reduced-minus-oxidized difference spectrum (5). Cells were reduced in the presence of 20 mM sodium L-lactate.*

times the highest level of carotenoids found in this strain of *S. aureus*.

The induction of the electron transport system after aerating anaerobic cultures required about 2 hr (Fig. 5). In this period, there was at least a 20-fold increase in δ-carotene and the rubixanthins (Fig. 2). When DPA was added at the onset of aerobic growth, the total carotenoid and vitamin K_2 level was depressed 50%, and the synthesis of the rubixanthins was depressed sixfold in the 2-hr period. This inhibition by DPA occurred without any detectable change in the formation or function of the electron transport system under these conditions of growth (Fig. 5). DPA had no effect on the formation of cytochrome *a* which supposedly contains an isoprenoid side chain on its heme. A fully formed electron transport system contains 90 nmoles of protoheme per g (dry weight) distributed between cytochrome *b* and cytochrome oxidase *o*. In the presence of DPA, the total carotenoid concentration was depressed from 154 nmoles to 60 nmoles per g, dry weight (rubixanthin from 75 to 1.1 nmoles per g, dry weight) and the vitamin K_2 from 2.0 to 1.1 μmoles per g (dry weight). These levels were apparently adequate for function at 37 C. The levels of DPA used in this study were considerably below those reported by Baker (1) to inhibit electron transport.

S. aureus has a high level of vitamin K_2 relative to the cytochrome content. In *Haemophilus parainfluenzae*, the molar ratio between 2-demethyl vitamin K_2 and cytochrome *b* is maintained at 15 to 1, even though the cytochrome *b* content of the membrane can vary over a wide range (21). The vitamin K_2 to cytochrome *b* ratio of *S. aureus* is 50 to 1 in the aerobic exponential phase or 25 to 1 when grown with DPA. The high ratio between vitamin K_2 and the cytochrome *b* and the fact that vitamin K_2 was formed during anaerobic growth in which no functional cytochromes were formed suggested that vitamin K_2 might have functions in the membrane other than in electron transport.

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 Institute of General Medical Sciences.

R. K. Hammond 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.

LITERATURE CITED

1. Baker, J. E. 1963. Diphenylamine inhibition of electron transport in plant mitochondria. Arch. Biochem. Biophys. 103:148–155.
2. Bishop, D. H. L., K. D. Pandya, and H. K. King. 1962. Ubiquinone and vitamin K in bacteria. Biochem. J. 83:606–614.
3. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.
4. Cho, K. Y., W. A. Corpe, and M. R. J. Salton. 1964. Effect of diphenylamine on the fatty acid composition of some bacterial lipids. Biochem. J. 93:26c–28c.
5. Frerman, F. E., and D. C. White. 1967. Membrane lipid changes during formation of a functional electron transport system in *Staphylococcus aureus*. J. Bacteriol. 94:1868–1874.
6. Goodwin, T. W., M. Jamikorn, and J. S. Willmer. 1953. Studies on carotenogenesis. 7. Further observations concerning the action of diphenylamine in inhibiting the synthesis of β-carotene in *Phycomyces blakesleeanus*. Biochem. J. 53:531–538.
7. Hammond, R. K., and D. C. White. 1969. Separation of vitamin K_2 isoprenologues by reversed phase thin layer chromatography. J. Chromatogr. 45:446–452.
8. Hammond, R. K., and D. C. White. 1969. Formation of vitamin K_2 isoprenologues by *Staphylococcus aureus*. J. Bacteriol. 100:573–578.
9. Hammond, R. K., and D. C. White. 1970. Carotenoid formation by *Staphylococcus aureus*. J. Bacteriol. 103:191–198.
10. Hammond, R. K., and D. C. White. 1970. Inhibition of carotenoid hydroxylation in *Staphyloccous aureus* by mixed-function oxidase inhibitors. J. Bacteriol. 103:607–610.
11. Jensen, S. L., G. Cohen-Bazire, T. O. M. Nakayama, and R. Y. Stanier. 1958. The path of carotenoid synthesis in a photosynthetic bacterium. Biochim. Biophys. Acta 29:477–498.
12. Kakutani, Y. 1967. Detection of some isoprenoids and the influence of diphenylamine on the biosynthesis of isoprenoid by *Sporobolomyces shibatanus*. J. Biochem. (Tokyo) 61:193–198.
13. Olson, J. A., and H. Knizley, Jr. 1962. The effect of diphenylamine on carotenoid, sterol and fatty acid synthesis in *Phycomyces blakesleeanus*. Arch. Biochem. Biophys. 97: 138–145.
14. Rilling, H. C. 1965. A study of inhibition of carotenoid synthesis. Arch. Biochem. Biophys. 110:39–46.
15. Salton, M. R. J., and A. F. M. Ethisham-Ud-Din. 1965. The localization of cytochromes and carotenoids in isolated bacterial membranes and envelopes. Aust. J. Exp. Biol. Med. Sci. 43:255–264.
16. Salton, M. R. J., and M. D. Schmitt. 1967. Effects of diphenylamine on carotenoids and menaquinones in bacterial membranes. Biochim. Biophys. Acta 135:196–207.
17. Slechta, L., O. Gabriel, and O. Hoffmann-Ostenhof. 1958. Separation of carotenoid synthesis from fat synthesis in *Rhodotorula gracilis* by diphenylamine. Nature (London) 181:268–269.
18. Sugimura, T., and H. Rudney. 1962. The effect of aerobiosis and diphenylamine on the content of ubiquinone in *Rhodospirillum rubrum*. Biochim. Biophys. Acta 62:167–170.
19. Suzue, G. 1959. Studies on carotenogenesis by *Micrococcus pyogenes var aureus*. J. Biochem. (Tokyo) 46:1497–1504.
20. White, D. C. 1963. Factors affecting the affinity for oxygen of cytochrome oxidases in *Haemophilus parainfluenzae*. J. Biol. Chem. 238:3757–3761.
21. White, D. C. 1965. Synthesis of 2-dimethyl vitamin K_2 and the cytochrome system in *Haemophilus*. J. Bacteriol. 89:299–305.
22. White, D. C., and F. E. Frerman. 1967. Extraction, characterization, and cellular localization of the lipids of *Staphylococcus aureus*. J. Bacteriol. 94:1854–1867.