JOURNAL OF BACTERIOLOGY, Nov. 1969, p. 573-578
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003197
Vol. 100, No. 2
Printed in U.S.A.

Formation of Vitamin K_2 Isoprenologues by *Staphylococcus aureus*

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Received for publication 18 August 1969

In *Staphylococcus aureus*, vitamin K_2 isoprenologues can be labeled with ^{14}C-acetate, mevalonate, glycerol, α-naphthol, and menadione. All the radioactivity from α-naphthol is in the ring nucleus. Eighty-six per cent of the radioactivity from mevalonate is in the side chain. The labeled isoprenologues of vitamin K_2 can be separated and recovered quantitatively. The rates of incorporation and turnover after pulse-chase experiments indicate that the isoprenologues do not lose radioactivity once they are formed. The kinetics of incorporation of radioactivity into the ring nucleus of each isoprenologue are the same. The alkylation of the ring does not involve a simple sequential addition of isoprenoid units of the same specific activity. The incorporation of mevalonate shows that shorter side chain isoprenologues have a higher specific activity per five carbon unit than the longer side chain isoprenologues. Perhaps the alkyl precursors are from different pools since the kinetics of incorporation into all isoprenologues are different.

Vitamin K_2 isoprenologues are 2-methyl-1,4-napthoquinones with polyisoprenoid side chains, are found in the membranes of many microorganisms (9), and appear to be involved in the formation and function of the electron transport system (4, 9). The naphthalene ring system is formed from chorismic acid through a branch of the shikimic acid pathway (3). The isoprenoid side chains are formed from mevalonate (13). *Staphylococcus aureus* contains isoprenologues with 0 to 45 carbon atoms in the side chain (K_2-0 through K_2-45, with K_2-45 indicating a vitamin K_2 isoprenologue with 45 carbon atoms at the 3 position of the naphthalene ring) and the proportions of the isoprenologues change during changes in the growth conditions (4). In this study, the synthesis of the ring from acetate and the alkylation of α-naphthol (7) or 2-methyl-1,4-naphthoquinone (K_2-0) were used to study the metabolism of the vitamin K_2 isoprenologues during aerobic growth.

MATERIALS AND METHODS

Materials. Dibenzylethylenediamine - DL - mevalonate-*5-^3H* or *-2-^{14}C* and glycerol-*2-^{14}C* were supplied by New England Nuclear Corp., Boston, Mass. α-Naphthol-*1-^{14}C* and 2-methyl-^{14}C-*1,4*-naphthoquinone were supplied by Amersham/Searle, Des Plaines, Ill. Acetate-*2-^{14}C* was purchased from Calbiochem, Los Angeles, Calif. Silica Gel G and Keiselguhr G (Brinkman Instruments, Inc., Westbury, N.Y.) and 99% olefin-free hexadecane (Matheson Coleman and Bell, Cincinnati, Ohio) were used for thin-layer chromatography (TLC). Highest purity commercial solvents were utilized without further purification.

Growth of the bacteria. The strain, medium, and harvesting conditions of the bacteria have been described (4). To study incorporation efficiencies of the precursors, cells were grown for six to eight doublings in the presence of 5 μc of ^{14}C in 250-ml Erlenmeyer flasks containing 50 ml of medium shaken at 60 cycles per min. For incorporation experiments, the bacteria were grown in 1,500 ml of medium for seven to eight doublings in the presence of 20 μc of isotope in 2,500-ml, low-form Erlenmeyer flasks. The flasks were shaken in a gyratory shaker at 37 C. Periodic samples were withdrawn into an equal volume of ice; the cells were separated from the media by centrifugation, and the lipids were extracted. For turnover experiments, the cultures were grown with 5 to 25 μc of radioactive precursor per 200 ml of media for two to three doublings. The culture was centrifuged at 37 C; the cells were then washed by the addition of medium over the bacterial pellet, resuspended in 1,800 ml of warm, nonradioactive medium, and incubated. Periodic samples were removed. The addition of unlabeled precursor at the beginning of the chase of pulse-chase experiments had no effect on the turnover.

Extraction of the lipids, purification of the vitamin K_2, and separation of the isoprenologues. The lipids were extracted from the cell suspension by a modified Bligh and Dyer procedure (2). The recovery in the chloroform phase of radioactive K_2-0 [8.5×10^4 counts/min] added to a lipid extract from *S. aureus* in a mixture of chloroform, methanol, and water (1:2:0.8) was 81%, after making the proportions of the extraction mixture 1:1:0.8. At most, 20% of the

K_2-0 is lost during the isolation procedure. The vitamin K_2 was purified by TLC on Silica Gel G (4). Thin layers of Keiselguhr G (50 μm in thickness) were impregnated with hexadecane by ascending chromatography with 5% hexadecane in chloroform (v/v). The vitamin K_2 isoprenologues were separated by ascending chromatography with acetone-water (95:5, v/v) saturated with hexadecane (10). This method separates the isoprenologues (Hammond and White, J. Chromatog., *in press*). The quinones were localized by spraying first with 1% $NaBH_4$ in 50% ethyl alcohol and then with 0.2% aqueous neotetrazolium. The quinones appeared as red spots without heating. Absorption spectra of vitamin K_2 isoprenologues were determined in isooctane in the Cary 15 spectrophotometer with a molar extinction coefficient of 19.28×10^3 at 248 nm.

Assay of radioactivity. Radioactivity was measured in a Packard scintillation spectrometer (model 2311; Packard Instrument Co., Inc., Downers Grove, Ill.) in a scintillation fluid of 9.28 M 2,5-bis[2(5-tertbutylbenzoxazoyl)]-thiophene (BBOT) in toluene. Radioactive samples were dried in the scintillation vials before the scintillation fluid was added. Quinones containing only ^{14}C were assayed under conditions such that the efficiency of counting was 82%. Quinones containing ^{14}C and ^{3}H were counted with an efficiency of 42% for ^{14}C and 8% for ^{3}H.

RESULTS

Efficiencies of incorporation. *S. aureus* incorporates a number of compounds into vitamin K_2 (Table 1). Mevalonate, α-naphthol and K_2-0 were most efficiently incorporated into vitamin K_2. Incorporation of phenylalanine and shikimic acid could not be demonstrated unless phenylalanine assay medium (Difco) was used. About 87 to 90% of the radioactivity from mevalonate is found in the side chain, and 100% of the radioactivity from α-naphthol is found in the ring. During the growth cycle, the proportions of isoprenologues in cells grown with K_2-0 (1.02 μmoles), α-naphthol (3.66 μmoles), and mevalonate (0.03 μmole) are essentially the same as in cultures grown without added precursors.

Proportion and characterization of the isoprenologues. Isoprenologues were separated by reversed phase TLC. The chromatographic mobilities corresponded to authentic standards in two systems, and the ratio of ^{14}C in the ring to ^{3}H in the side chain increased as chain length decreased in isoprenologues from cells grown in the presence of α-naphthol-*1*-^{14}C and mevalonate-*5*-^{3}H. Each isoprenologue, K_2-5 through K_2-45, had the characteristic absorption maxima at 243, 248, 260, 269, and 320 to 325 nm in isooctane. K_2-0 isolated from cells grown with or without added K_2-0 had absorption maxima at 244, 251, 263, and 320 to 325 nm with a shoulder at 249 nm. The K_2-0 had the same chromatographic mobility and absorption spectrum as authentic K_2-0.

The proportions of the isoprenologues of vitamin K_2 change during the exponential phase of growth (Fig. 1). During this period of growth the total amount of vitamin K_2 remained constant at 2.0 ± 0.1 μmoles per g (dry weight). The proportions of isoprenologues were assayed either spectrophotometrically or by the proportion of the total radioactivity in the ring. The major isoprenologue (K_2-40) increases from 36 to 70% of the total vitamin K_2. This represents an increase of 0.68 μmoles of K_2-40 per g (dry weight) of cells. In this same period, the proportions of K_2-0, -5, -25, -30, and -35 decrease. These isoprenologues decrease a total of 0.34 μmoles of vitamin K_2 per g (dry weight), which is half of the increase of K_2-40 in the same period of growth.

Incorporation of α-naphthol-1-^{14}C and mevalonate-5-^{3}H. The rates of incorporation of α-naphthol into the ring of each isoprenologue are comparable (Fig. 2). At any time during growth, the proportions of ^{14}C in the ring of each isopre-

TABLE 1. *Incorporation of ^{14}C into vitamin K_2 in Staphylococcus aureus*

Substrate added	CPM added	CPM recovered in K_2	Percentage recovered in K_2
Acetate-*2*-^{14}C	7.9×10^6	3.6×10^2	0.005[a]
Glycerol-*2*-^{14}C	1.7×10^7	6.0×10^3	0.035
Mevalonate-*2*-^{14}C	7.6×10^6	1.6×10^4	0.211
Shikimate-*1,6*-^{14}C	4.1×10^6	3.0×10^2	0.007[b]
Phenylalanine-UL-^{14}C	9.0×10^6	1.5×10^2	0.002[b]
α-Naphthol-*1*-^{14}C	1.6×10^7	5.0×10^4	0.313[c]
Methyl-^{14}C-menadione	8.2×10^6	7.0×10^5	8.550[c]

[a] Cultures were grown for six to eight doublings in the presence of 5 μc of ^{14}C in 250-ml Erlenmeyer flasks containing 50 ml of culture shaken at 60 cycles per min. The cells were harvested; the lipids were extracted, and the vitamin K_2 was purified and assayed for radioactivity.

[b] These cells were grown in phenylalanine assay medium (Difco).

[c] Lipid substrates dissolved in dimethylsulfoxide and added to the medium. Final dimethylsulfoxide concentration was 0.1% (v/v).

nologue (Fig. 2) are similar to the proportions in Fig. 1. This suggests a single α-naphthol pool of constant specific activity. Saturation of the pool occurs after 10 to 15 min. In contrast, the rates of incorporation of mevalonate into the side chain of each isoprenologue are different (Fig. 3). The

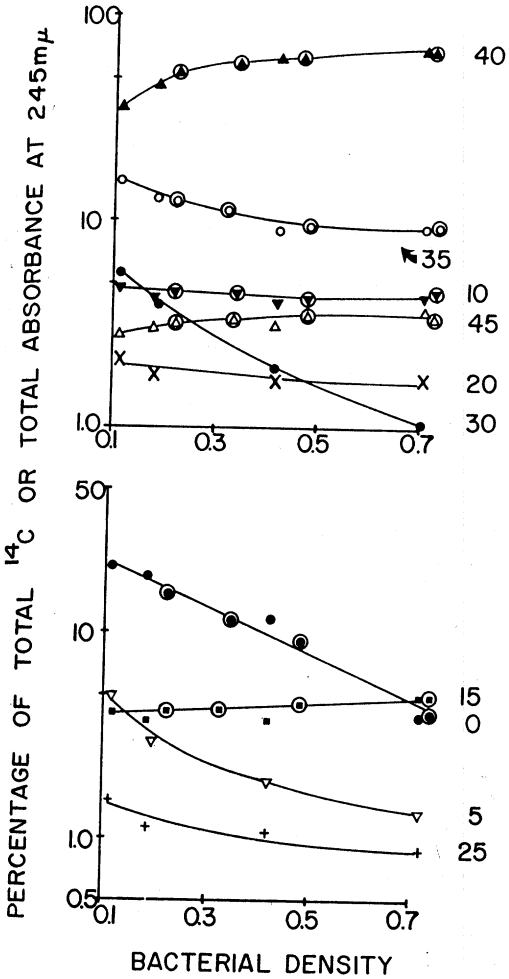

FIG. 1. *Proportions of vitamin K_2 isoprenologues in S. aureus. Cultures were grown for seven to eight doublings in the presence of 20 μc of methyl-^{14}C-menadione in 1,500 ml of medium. The cells were harvested, extracted, the vitamin K_2 purified, the isoprenologues separated and assayed for radioactivity. Isoprenologue content was calculated from the proportion of total ring ^{14}C found in each isoprenologue. The proportions of isoprenologues were also determined spectrophotometrically using a molar extinction coefficient of 19.28 × 10^3 at 248 nm (circled symbols). Symbols for upper graph: △, K_2-45; ▲, K_2-40; ○, K_2-35; ●, K_2-30; ×, K_2-20; and ▼, K_2-10. Symbols for lower graph: +, K_2-25; ■, K_2-15; ▽, K_2-5; and ●, $K_2$0. Bacterial density was measured as the absorbance at 750 nm (4).*

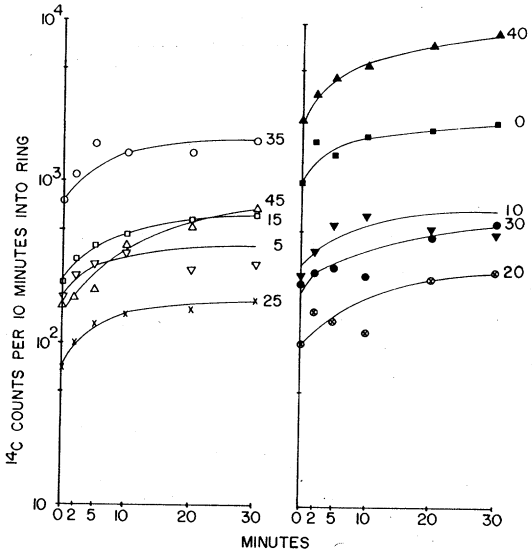

FIG. 2. *Incorporation of α-naphthol-1-^{14}C into vitamin K_2 isoprenologues in S. aureus. Cultures were grown for seven to eight doublings in the presence of 20 μc of α-naphthol-1-^{14}C in 1,500 ml of medium. The isoprenologues were isolated as in Fig. 1 and assayed for radioactivity. Symbols: △, K_2-45; ▲, K_2-40; ○, K_2-35; ●, K_2-30; ×, K_2-25; ⊗, K_2-20; □, K_2-15; ▼, K_2-10; ▽, K_2-5; and ■, K_2-0. Right hand ordinate dimensions are the same as the left hand ordinate dimensions.*

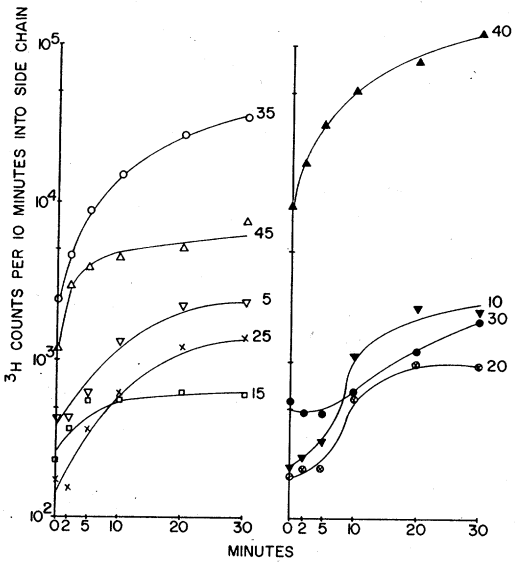

FIG. 3. *Incorporation of mevalonate-5-^{3}H into vitamin K_2 isoprenologues in S. aureus. Cultures were grown and assayed as in Fig. 2 and the symbols are as in Fig. 2.*

precursor pools of the isoprenologues seem to saturate at different times. The isoprenologues with longer side chains do not have a consistently higher total radioactivity. The tritium radioactivity per five carbon unit for each isoprenologue

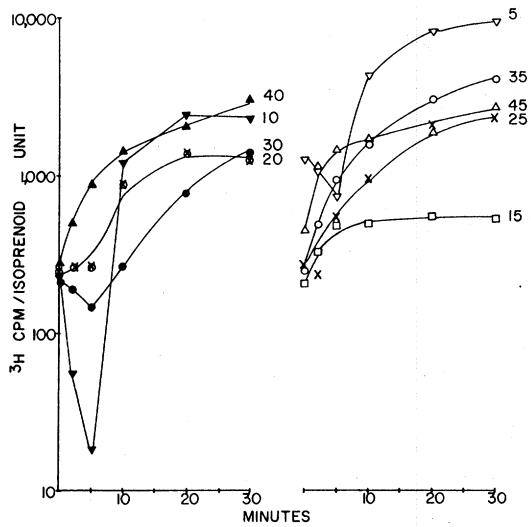

FIG. 4. *Specific activity of ^3H in each five carbon isoprenoid unit of the vitamin K_2 isoprenologues in S. aureus. The specific activity per isoprenoid unit was calculated using the total ^3H per isoprenologue (Fig. 3) and the proportion of each isoprenologue calculated from the α-naphthol-1-^{14}C in the ring (Fig. 2). Symbols: △, K_2-45; ▲, K_2-40; ○, K_2-35; ●, K_2-30; ×, K_2-25; ⊗, K_2-20; □, K_2-15; ▼, K_2-10; ▽, K_2-5; and ■, K_2-0.*

TABLE 2. *Recovery of vitamin K_2 in growing Staphylococcus aureus incubated with ^{14}C-labeled isoprenologues of vitamin K_2*

Isoprenologue added	CPM[a] added	CPM recovered in media	CPM recovered in vitamin K_2
K_2-45, -40	125,000	123,000	10[b]
K_2-25, -30, -35	23,000	23,000	20
K_2-10, -15, -20	23,000	21,000	50
K_2-0, -5	19,000	15,000	500[c]

[a] CPM, counts/min.

[b] Cultures were grown for five to six doublings in the presence of labeled vitamin K_2 isoprenologues in 50-ml cultures. The isoprenologues were added in 0.5 ml of dimethylsulfoxide. The cells were harvested, the lipids were extracted, and the vitamin K_2 was purified and assayed for radioactivity.

[c] The radioactivity recovered in the vitamin K_2 from this culture was found in K_2-0, 22%; K_2-5, 36%; K_2-10, 14%; K_2-20, 16%; remaining isoprenologues 12%.

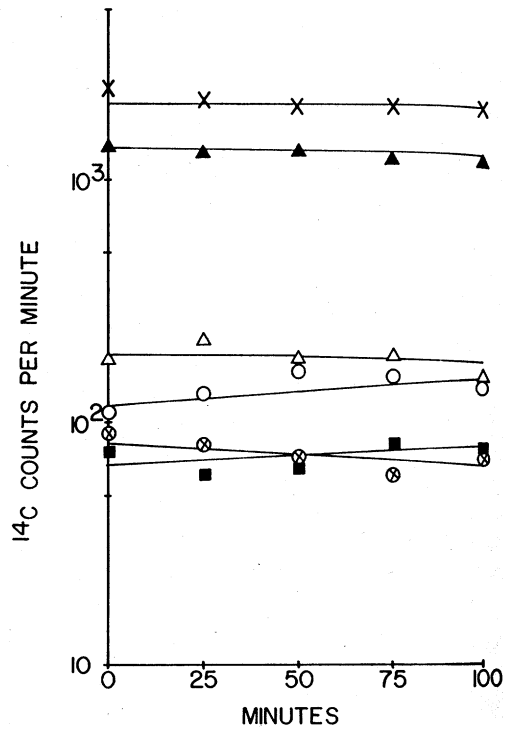

FIG. 5. *Turnover of vitamin K_2 isoprenologues labeled with acetate-2-^{14}C in S. aureus. Cultures were grown for two to three doublings in the presence of 25 μc of acetate-2-^{14}C per 200 ml of medium. The culture was centrifuged at 37 C and the bacterial pellet resuspended in 1,800 ml of warm, nonradioactive medium. Incubation was continued and samples were removed and the isoprenologues isolated and assayed as in Fig. 1. Symbols: ×, total K_2; △, K_2-45; ▲, K_2-40; ○, K_2-35; ⊗, K_2-20; and ■, K_2-0.*

is illustrated in Fig. 4. The five carbon unit in the shortest isoprenologue, K_2-5, has a very high level of radioactivity compared to an average five carbon unit of K_2-10 to -45. K_2-10 has a higher specific radioactivity per five carbon unit than K-15, -20, -25, -30, and -45. When vitamin K_2 was degraded in permanganate (1), all of the ^{14}C from α-naphthol-*1*-^{14}C and none of the ^3H from mevalonate-*5*-^3H was recovered in the phthalic anhydride and phthalic acid derived from the naphthalene ring.

Turnover of vitamin K_2 isoprenologues. Vitamin K_2 isoprenologues labeled in both the ring and side chain from acetate-*2*-^{14}C demonstrate no appreciable turnover of the five major isoprenologues (Fig. 5). Isoprenologues labeled in the ring or methyl group with α-naphthol-*1*-^{14}C or 2-methyl-^{14}C-*1,4*-naphthoquinone demonstrate no turnover during 2.5 bacterial doublings. Shorter pulses (5 to 15 min) with these com-

pounds again showed no turnover during one doubling (40 min).

Vitamin K_2 isoprenologues labeled in the side chain with mevalonate-*2-*^{14}C demonstrate no turnover during 2.5 doublings with the possible exceptions of K_2-10 and -25 (Fig. 7). If the relatively slow turnover of K_2-10 and -25 is real, it can account for less than 1% of the K_2-40 synthesized per doubling. A shorter pulse (10 min) of mevalonate showed the same results.

Reactivity of labeled vitamin K_2 isoprenologues. Addition of labeled vitamin K_2 isoprenologues in dimethylsulfoxide to growing cultures of *S. aureus* showed no incorporation or metabolism of K_2-10 to -45 (Table 2). The K_2-10 to -45 were recovered in the medium after centrifugation. Approximately 0.4% of the K_2-0 to -5 was incorporated into K_2-0 to -45. The ratio of K_2-0 to K_2-5 added to the cultures was 4.7 to 1. The ratio of K_2-0 to K_2-5 recovered from the cells was 1.6 to 1, indicating that most, if not all, the incorporation was of the K_2-0.

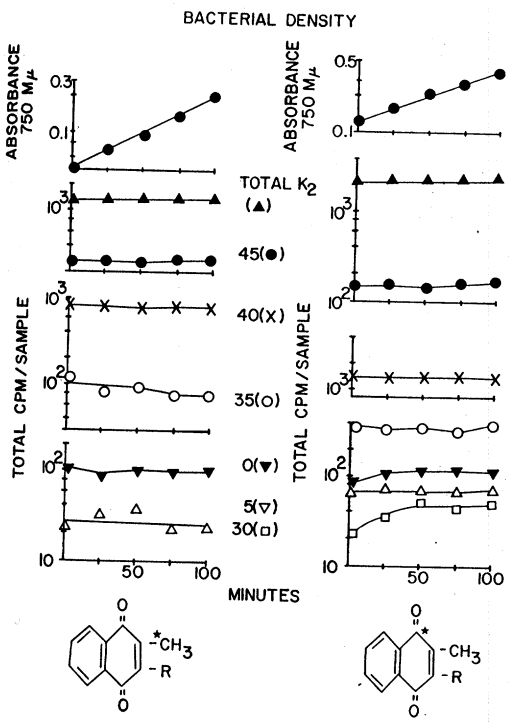

FIG. 6. *Turnover of vitamin K_2 isoprenologues labeled with α-naphthol-1-*^{14}C *or methyl-*^{14}C*-menadione in S. aureus. Cultures were grown either in the presence of 5 μc of methyl-*^{14}C*-menadione (left graphs) or 20 μc of α-naphthol-1-*^{14}C *(right graphs) per 200 ml of culture and treated as in Fig. 5. The upper graph in each case illustrates growth during the chase phase of the experiments. The symbols are defined in the middle column.*

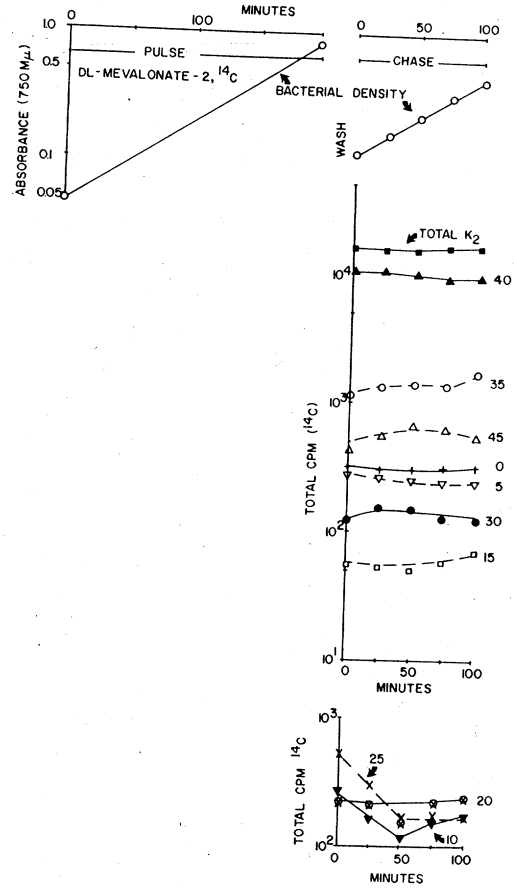

FIG. 7. *Turnover of vitamin K_2 isoprenologues labeled with mevalonate-2-*^{14}C *in S. aureus. Cultures were grown for 3.5 doublings in the presence of 20 μc of mevalonate-2-*^{14}C *per 200 ml of medium as illustrated in the top graphs. Vitamin K_2 isoprenologues were extracted, purified and assayed as in Fig. 5 (bottom graphs). Symbols: △, K_2-45; ▲, K_2-40; ○, K_2-35; ●, K_2-30; ×, K_2-25; ⊗, K_2-20; □, K_2-15; ▼, K_2-10; ▽, K_2-5; +, K_2-0; and ■, total K_2.*

DISCUSSION

Vitamin K_2 isoprenologues with 0 to 45 carbon atoms in the side chain can be separated using the hexadecane-impregnated, thin-layer plates. K_2-0 (menadione) was identified by its chromatographic mobility and absorption spectrum. It is usually not thought of as a normal constituent of biological material (12). K_2-5, -10, and -15 have not been reported previously in *S. aureus*. Isoprenologues from K_2-20 to K_2-45 have been reported in *S. aureus* (6).

The study of the metabolism of the vitamin K_2 isoprenologues in *S. aureus* was possible because of the development of the rapid, efficient method for the separation of the isoprenologues involving

TLC with hexadecane-impregnated Keiselguhr G and the fact that all the vitamin K_2 isoprenologues are labeled in the ring with α-naphthol and K_2-0, or in the side chain with mevalonate. The labeling of the ring nucleus and the side chain with these precursors is reasonably efficient (Table 1). Addition of these components during growth does not affect the proportions of the isoprenologues, indicating that these compounds are incorporated directly into vitamin K_2. The poor efficiency of shikimate as a precursor of vitamin K_2 probably reflects the impermeability of this strain of *S. aureus*. In phenylalanine assay medium, growth of this strain is proportional to added phenylalanine. Addition of shikimate in this medium has no effect on growth.

Vitamin K_2 isoprenologues labeled in either the ring nucleus or the side chain do not undergo any detectable turnover during 2.5 doublings of cultures in exponential growth (Fig. 5–7). The kinetics of incorporation of ^{14}C from α-naphthol into the ring are the same for each isoprenologue, indicating that the ring is formed from a single precursor pool (Fig. 2). However, the kinetics of incorporation of ^{14}C or ^{3}H from mevalonate into the side chains of each isoprenologue are different (Fig. 3). The specific activity of ^{3}H per isoprenoid unit is not equal in each isoprenologue (Fig. 4). This suggests multiple precursor pools of different specific activity as the source of the side chains. The proportion of K_2-40 increases and the proportions of K_2-0, -5, -25, -30, and -35 decrease during exponential growth. The increase in K_2-40 represents twice the decrease in the other isoprenologues combined (Fig. 1). These data, plus the facts that the isoprenologues are stable once they are formed and the specific activities of the side chains are different, are inconsistent with the hypothesis that the side chains are formed by a simple sequential addition of isoprenoid units of constant specific activity to the shorter isoprenologues.

Vitamin K_2 isoprenologues with longer side chains were not incorporated into cells of *S. aureus*. The labeled isoprenologues are not taken up by the cells since they can be quantitatively recovered in the medium after the incubation. Few microorganisms are known to incorporate lipids (11) and some strains of *Bacteroides melaninogenicus* (*Fusiformis nigrescens*) incorporate vitamin K_2 (5, 8).

S. aureus contains 10 isoprenologues of vitamin K_2. Perhaps the isoprenologues perform different functions or are at different locations in the cell. It is possible that the alkylating enzyme is relatively nonspecific to side chain length. The alkylating enzyme of ubiquinone, however, is relatively specific since a single isoprenologue of ubiquinone is usually found in a single organism. Nearly all bacteria contain at least several isoprenologues of vitamin K_2 when the lipids are carefully examined.

Further understanding of the formation and function of the isoprenologues of vitamin K_2 must await the isolation of the alkylating enzyme system.

ACKNOWLEDGMENTS

This investigation was supported by contract 12-14-100-951(73) with the Agricultural Research Service, U.S. Department of Agriculture, administered by the Eastern Utilization and Development Division, Philadelphia, Pa., and U.S. Public Health Service grant GM-10285 of the 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.

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