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Incorporation of Amino Acid-Derived Carbon into Tylactone by *Streptomyces fradiae* GS14

JOE E. DOTZLAF,* LINDA S. METZGER, AND MARK A. FOGLESONG
Eli Lilly & Company, Indianapolis, Indiana 46285

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Washed cells from 72-h cultures of *Streptomyces fradiae* GS14 were used to examine the distribution of radiolabel from ^{14}C-amino acids and related compounds into tylactone, CO_2, and cells. Test compounds were categorized according to products of their oxidative degradation. Those compounds known to produce propionyl-coenzyme A by direct catabolic oxidation were designated as group I. Group II included those compounds oxidized to methylmalonyl-coenzyme A via succinyl-coenzyme A and the tricarboxylic acid cycle. Group III contained compounds known to be oxidized to acetoacetyl-coenzyme A. The total amount of label recovered after 60 min ranged from 3 to 65%. Although label from all test compounds except proline (group II) and lysine (group III) was incorporated into tylactone after 60 min, label from group I and group III compounds was incorporated at levels five times greater than label from group II compounds. From 55 to 75% of the recovered label from propionate (I), asparagine (II), glutamine (II), glutamate (II), α-ketoglutarate (II), and succinate (II) was recovered as $^{14}CO_2$. From 75 to 95% of the recovered label from the remaining compounds tested was located in the cells. Based on the data, a pathway for the role of amino acids in the biosynthesis of tylactone is proposed.

Tylactone (Fig. 1) is the initial lactone precursor to tylosin, an antibiotic produced commercially by *Streptomyces fradiae* (1–3). Ōmura et al. (6), using ^{13}C enrichment, showed that tylactone was formed from five propionate molecules, two acetate molecules, and one butyrate molecule. Subsequent inhibition studies with cerulenin, an inhibitor of fatty acid synthetase, showed that tylactone and related macrolide aglycone molecules were assembled in a manner similar to fatty acid biosynthesis (5, 7–9). The data of Ōmura et al. (5–9) suggested that the precursors for tylactone biosynthesis were incorporated in the following manner: propionate as one propionyl-coenzyme A (CoA) (primer) and four methylmalonyl-CoA molecules, acetate as two malonyl-CoA molecules, and butyrate as one ethylmalonyl-CoA molecule.

In this study, the role of amino acids as sources for the tylactone precursors was examined by using a *tylA* mutant of *S. fradiae* (1). This strain was blocked in the production of the tylosin sugars and therefore produced tylactone as its product (1, 3). Radiolabeled test compounds were categorized based on generally accepted catabolic pathways. Group I included those compounds that produced propionyl-CoA directly. Compounds catabolized to methylmalonyl-CoA via succinyl-CoA and the tricarboxylic acid (TCA) cycle were assigned to group II. Group III included those compounds known to be oxidized to acetoacetyl-CoA.

Results from this investigation clearly confirmed the role of amino acids as sources for the tylactone precursors.

(A preliminary report of the present work was presented previously [J. E. Dotzlaf, L. S. Metzger, and M. A. Foglesong, Abst. Annu. Meet. Am. Soc. Microbiol. 1983, O63, p. 250].)

MATERIALS AND METHODS

Strain and culture conditions. For all experiments, a tylactone-producing strain of *S. fradiae* C4 (*tylA*) isolated by Baltz and Seno (1) and designated GS14 was used. Cells were grown in a complex medium as described previously (1).

Preparation of washed cell suspensions. After 72 h of incubation, the cells from 300 ml of broth were removed by centrifugation at 16,000 × *g* for 5 min in a Sorvall RC-5 centrifuge. Cell pellets were washed twice with 300 ml of 0.1 M potassium phosphate buffer, pH 7.0. Approximately 6.5 g of wet cells were suspended in the buffer to a volume of 50 ml, and portions were diluted 10-fold with buffer to yield final cell suspensions containing 1.2 to 1.7 mg of dry cells per ml.

Uptake and distribution reactions. Reactions were carried out in 35-ml flint glass bottles (Kimble, Toledo, Ohio) that were closed with gray butyl rubber stoppers (West Rubber Co., Phoenixville, Pa.) fitted with plastic center wells (Kontes Scientific Glassware/Instruments, Vineland, N.J.) and secured with aluminum crimp seals (West Rubber Co.). The center wells contained 0.3 ml of hyamine hydroxide (New England Nuclear Corp., Boston, Mass.) as a CO_2 absorbant. Each bottle held 5 ml of cell suspension; controls contained 5 ml of heat-killed cell suspension (100°C, 10 min). After sealing, the bottles were fitted to a plastic carrier and attached to a water bath shaker maintained at 30°C and 200 rpm.

Reactions were initiated by injecting 20 μl of the appropriate reaction solution. Reaction solutions supplied 0.021 μmol (1.0 μCi) of ^{14}C-labeled test compound in addition to 0.67 μmol of unlabeled sodium acetate and 0.67 μmol of unlabeled isoleucine or leucine. Preliminary experiments indicated that without the inclusion of acetate and isoleucine or leucine, significant labeling of the tylactone would not occur (unpublished data). These unlabeled compounds served two functions: to supply complementary tylactone precursors (malonyl-CoA and methyl-CoA or ethylmalonyl-CoA) and to dilute sufficiently any unwanted labeled acetyl-CoA or methylmalonyl-CoA generated by the test compounds (Fig. 2). Unless otherwise stated, duplicate reactions were run for 15, 30, and 60 min with duplicate controls run for 60 min. The reactions were terminated, and any dissolved CO_2 was released by injecting 0.3 ml of 1.0 N HCl. Forty reactions were done with each cell suspension, and

* Corresponding author.

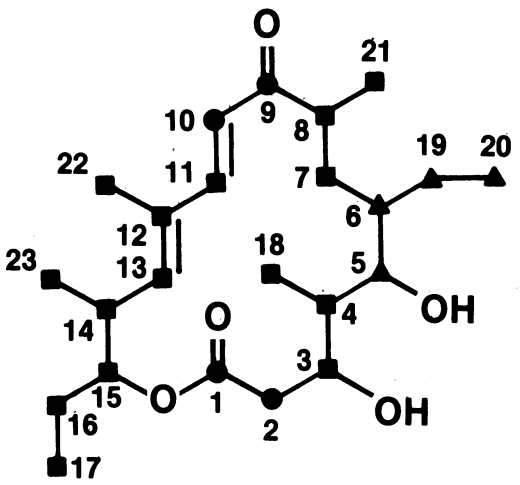

FIG. 1. Structure of tylactone, showing subunits derived from propionate (■), from acetate (●), and from butyrate (▲).

isoleucine was tested each time to verify the integrity of the cell suspension.

Distribution analysis of ^{14}C from the test compounds. (i) $^{14}CO_2$ determination. The center wells were transferred to counting vials containing 10 ml of Aquasol-2 (New England Nuclear) scintillation cocktail. The mixture was acidified with 0.2 ml of glacial acetic acid, and the radioactivity was measured in a Nuclear Chicago scintillation counter.

(ii) ^{14}C-labeled cell determination. The bottle contents and two 4-ml deionized water washes were centrifuged at 2,500 × *g* for 15 min. The pellet was washed once with 8 ml of deionized water and suspended in about 4 ml of deionized water. After the suspension volumes were determined, 0.5-ml portions were mixed with 10 ml of Aquasol-2, and the radioactivity was measured.

(iii) [^{14}C]tylactone determination. The cell wash supernatants and remaining cell suspensions were adjusted to pH 9.5 with 5 N NaOH and were extracted twice with 65 ml of ethyl acetate. The solvent phases were dehydrated with Na_2SO_4 and evaporated to dryness by using a Buchi Rotavapor-R (Brinkmann Instruments, Inc., Westbury, N.Y.). The residues containing the [^{14}C]tylactone were taken up in 10 ml of Aquasol-2 and counted for radioactivity.

Preliminary whole broth labeling experiments showed that approximately 95% of the ethyl acetate-extractable label was associated with tylactone (unpublished data). Tylactone production in the washed cell system was verified by high-pressure liquid chromatography with a modification of the procedure described by Kennedy (4).

Chemicals. The test compounds and their group designations are listed in Table 1. All were uniformly labeled with ^{14}C except propionate, which was labeled only at carbon 1. Succinate was purchased from Research Products International Corp., Mount Prospect, Ill. The remaining test compounds were obtained from New England Nuclear. All unlabeled reactants were purchased from Sigma Chemical Co., St. Louis, Mo.

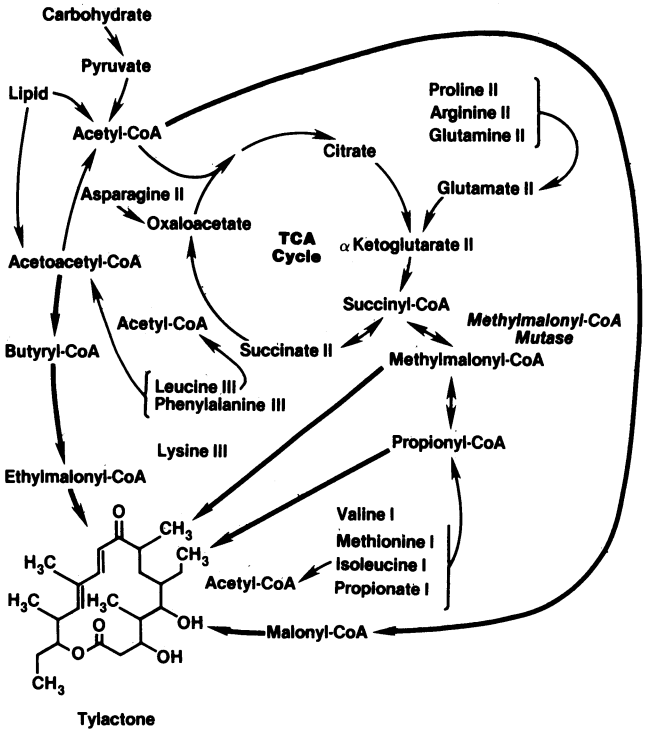

FIG. 2. Proposed pathway for the biosynthesis of tylactone. Compounds tested are designated by Roman numerals which correspond to their group classification. Heavy arrows indicate probable major routes to tylactone.

RESULTS

Total label recovered. The total label recovered (as percentage of available label) for each compound increased with time. However, distribution into tylactone, CO_2, and cells (as percentage of total recovery) remained nearly constant with time. Therefore, unless stipulated, only 60-min data will be discussed. Figure 3 depicts the total recovery data. Except for methionine (group I), the data showed group I > group III > group II.

Group I label distribution. The group I amino acids showed similar patterns of distribution into tylactone, CO_2, and cells and averaged approximately 3, 7, and 90%, respectively (Fig. 4). The ratios of tylactone to CO_2 (Table 1), which may indicate the proportioning of catabolic intermediates between tylactone and the TCA cycle, were consistently high for this group (0.302 to 0.492).

TABLE 1. Ratios of tylactone to CO_2 for group I, II, and III amino acids and related compounds

Compound	Group	Tylactone:CO_2[a]		
		60 min	120 min	180 min
Valine	I	0.302		
Methionine	I	0.485		
Isoleucine	I	0.492	0.514	0.514
Proline	II	0	0.014	0.025
Arginine	II	0.040		
Asparagine	II	0.009		
Glutamine	II	0.010		
Glutamate	II	0.016		
Leucine	III	0.610		
Phenylalanine	III	0.770		
Lysine	III	0	0	0
Propionate	I	0.150		
α-Ketoglutarate	II	0.024		
Succinate	II	0.041		

[a] Where no values are reported, no tests were made.

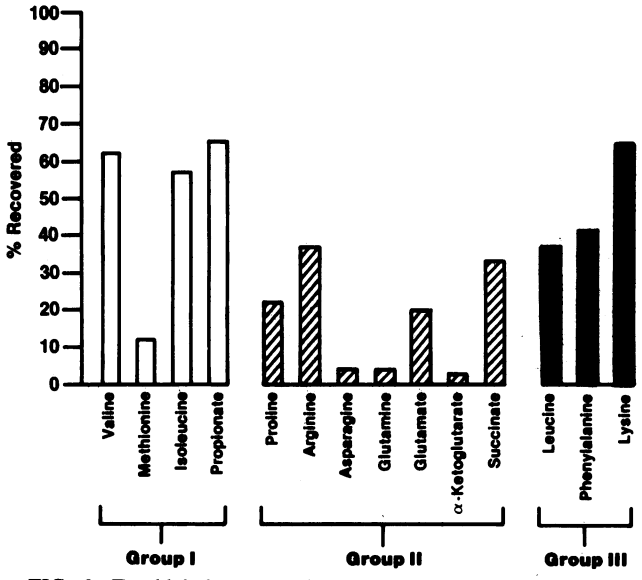

FIG. 3. Total label recovered per milligram of dry cells for group I, II, and III test compounds, as percentage of available label, after 60 min. The values represent averages of duplicate samples from at least two experiments.

Group II label distribution. Proline and arginine from group II showed greater labeling in cells than in CO_2, whereas the reverse was true for asparagine, glutamine, and glutamate (Fig. 4). However, label associated with tylactone was low across the group and undetectable for proline (Fig. 4).

The ratios of tylactone to CO_2 for these compounds (Table 1) were much lower than for group I and ranged from 0.009 to 0.040. Because proline showed no detectable label in tylactone after 60 min, reactions for this compound were extended to 180 min. Label from proline in CO_2 and cells increased with time through 180 min (Fig. 5). Label from proline was detected in tylactone after 120 min (Fig. 5). The ratios of tylactone to CO_2 for proline at 120 and 180 min (0.012 and 0.024, respectively) were consistent with those for the other group II compounds (Table 1). Isoleucine incorporations into CO_2, cells, and tylactone peaked at 120 min and remained level through 180 min (Fig. 5).

Group III label distribution. The group III compounds showed similar labeling patterns in CO_2 and cells (Fig. 4). The labeling of tylactone (Fig. 4) and the ratios of tylactone to CO_2 (Table 1) for leucine and phenylalanine resembled those for group I. Lysine, like proline (group II), showed no detectable label in tylactone after 60 min (Fig. 4). However, unlike proline, lysine showed no incorporation into tylactone in reactions extended to 180 min.

Non-amino acid label distribution. Figure 6 shows the distribution data for non-amino acid representatives of group I (propionate) and group II (α-ketoglutarate and succinate). The labeling of tylactone, CO_2, and cells (Fig. 6), as well as the ratios of tylactone to CO_2 (Table 1) for the group II representatives, were consistent with those for asparagine (group II), glutamine (group II), and glutamate (group II) (Fig. 4 and Table 1). Propionate incorporation into tylactone was consistent with the group I amino acids but inconsistent with their incorporation into CO_2 and cells (Fig. 4 and 6). Propionate was incorporated into CO_2 and cells in a fashion similar to α-ketoglutarate and succinate (Fig. 6).

DISCUSSION

Ōmura et al. (6) described a 5:2:1 ratio of propionate-acetate-butyrate incorporation into tylactone. The fact that five propionate molecules are required to synthesize each tylactone molecule indicates the importance of propionate as a potential limiting precursor. As discussed earlier, the five propionate molecules were probably incorporated as one propionyl-CoA (primer) and four methylmalonyl-CoA molecules (5, 7–9). Since the interconversion of propionyl-CoA and methylmalonyl-CoA has been demonstrated in *S. fradiae* (10), one can postulate common sources for these two tylactone precursors. Our results clearly show that these precursors can be derived from amino acids both directly (group I) and indirectly via the TCA cycle (group II) (Fig. 2).

The high ratios of tylactone to CO_2 (Table 1) for the group I amino acids and propionate indicate that large portions of their catabolic intermediates (i.e., propionyl-CoA and methylmalonyl-CoA) were directed to tylactone in the washed cell system (Fig. 2). In contrast, the group II amino acids and related compounds had substantially lower ratios of tylactone to CO_2 (Table 1).

The conversion of the TCA cycle intermediate succinyl-CoA to the tylactone precursor methylmalonyl-CoA by the enzyme methylmalonyl-CoA mutase would enable group II compounds to label tylactone (Fig. 2). Furthermore, the

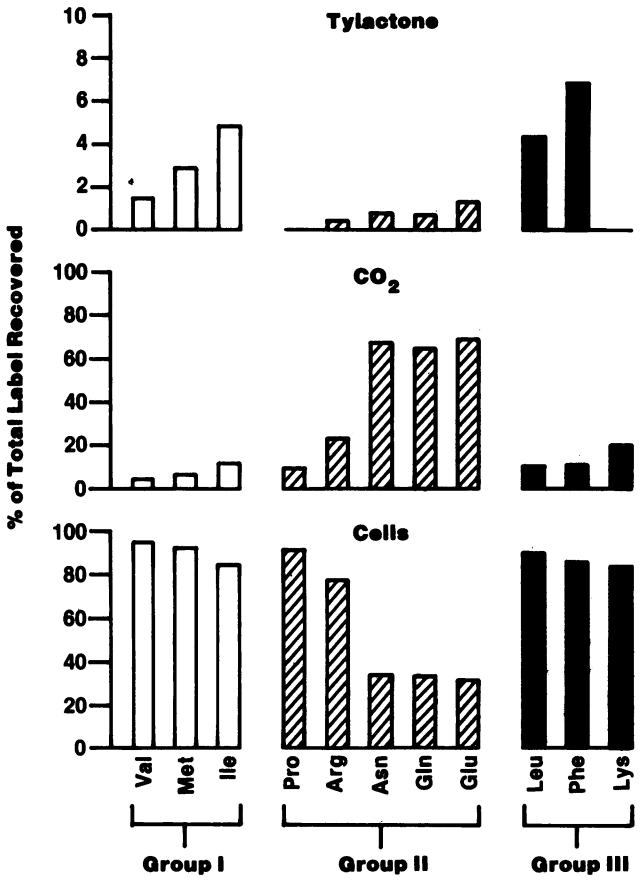

FIG. 4. Label distribution into tylactone, CO_2, and cells from group I, II, and III amino acids, as percentage of total label recovered, after 60 min. Approximately 80% of the cell label was precipitated with 20% trichloroacetic acid. The nonprecipitated label for each reaction was assumed to consist of unused test compound, its catabolic intermediates, or both.

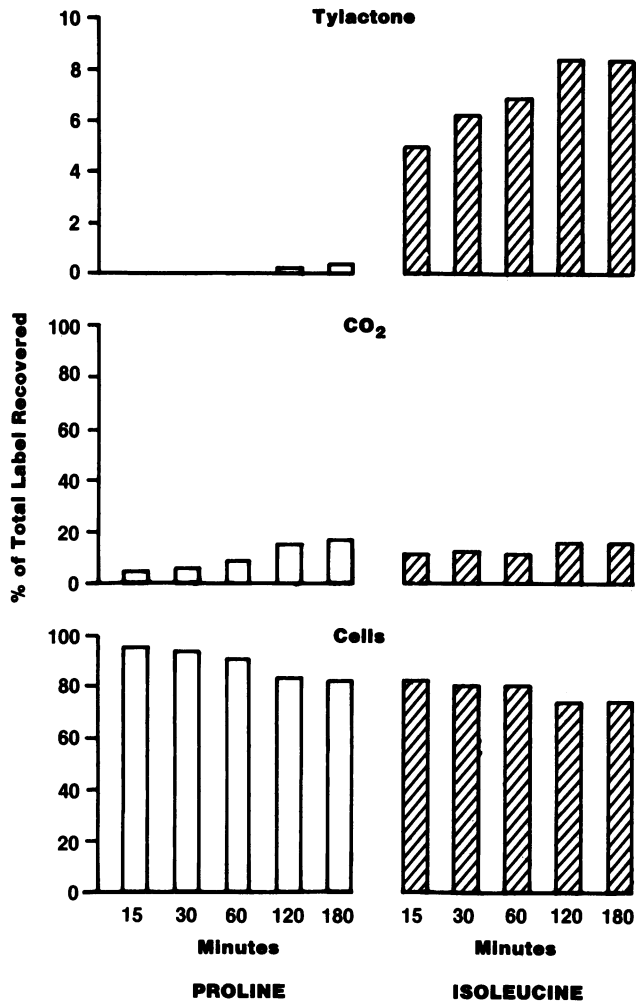

FIG. 5. Label distribution into tylactone, CO_2, and cells from proline (group II) and isoleucine (group I), as percentage of total label recovered, with time.

reversible nature of this enzyme would allow group I compounds to enter the TCA cycle (Fig. 2). The data demonstrate that the enzyme functioned in both directions during the washed cell studies. However, the differences in the ratios of tylactone to CO_2 (Table 1) between groups I and II indicate that the preferred direction of the enzyme was towards succinyl-CoA. This was also reflected in the high level of $^{14}CO_2$ generated from the group I compound propionate (Fig. 6). In both instances, the limiting reactant was the radiolabeled test compound since unlabeled acetate and leucine (the proposed precursors to malonyl-CoA and ethylmalonyl-CoA, respectively) were present at 32-fold greater concentrations.

Contrary to the rest of the group II compounds, arginine and proline labeled the cells more than they did CO_2 (Fig. 4). This suggests that arginine and proline, although rapidly taken up by the washed cells as indicated by the total recovery data (Fig. 3), were converted to TCA cycle intermediates (Fig. 2) more slowly than the other group II compounds. The fact that the ratios of tylactone to CO_2 (Table 1) for arginine and proline were in agreement with the values for the other group II compounds indicates that once converted to TCA cycle intermediates, arginine and proline were utilized by the cells just as the rest of the group II compounds were.

The group III compounds leucine and phenylalanine also exhibited high ratios of tylactone to CO_2 (Table 1), indicating that their common catabolic intermediate, aceto-acetyl-CoA, was the probable intermediate in ethylmalonyl-CoA production (Fig. 2). The limited literature concerning the incorporation of C_4 units into tylactone is somewhat confusing. Ōmura et al. (6) not only showed incorporation of [1-^{13}C]butyrate at C-5 of tylactone (Fig. 1), as expected, but they also detected enrichment at C-3, 7, 11, 13, and 15, carbons normally enriched by [1-^{13}C]propionate. In concurrent experiments with [3-^{13}C]ethylmalonate (originally published as [4-^{13}C]ethylmalonate [6]), they showed an enrichment at C-19 as well as at C-4, 8, 12, 14, and 16 (Fig. 1), which was in agreement with the [1-^{13}C]butyrate data (6). Subsequent work led Ōmura and his co-workers (8, 9) to propose separate pathways for the conversion of butyrate and ethylmalonate to methylmalonyl-CoA for incorporation into the C_3 subunit portions of tylactone. While exploring the possible pathways leading to methylmalonyl-CoA, Ōmura et al. (8, 9) utilized [1,3,1′-^{13}C]ethylmalonate and found enrichment at C-5 and C-19 (Fig. 1) as well as at C-11/12, C-13/14, and C-15/16 in the manner described for [3-^{13}C]ethylmalonate. Curiously, though, there did not appear to be enrichment at C-3/4 and C-7/8, as would be expected (6, 8, 9).

The complex incorporation patterns reported for C_4 units (6, 8, 9) dictate caution when interpreting the group III labeling data presented here. Nevertheless, the fact that the unlabeled C_2 (acetate) and C_3 (isoleucine) subunit precursors were present at concentrations 32 times greater than the group III test compounds suggests that leucine and phenylalanine indeed labeled tylactone as the C_4 (butyrate) subunit.

The lack of tylactone labeling by lysine (group III) indicates that, although actively metabolized, as indicated by its high $^{14}CO_2$ generation (Fig. 4), lysine was probably not

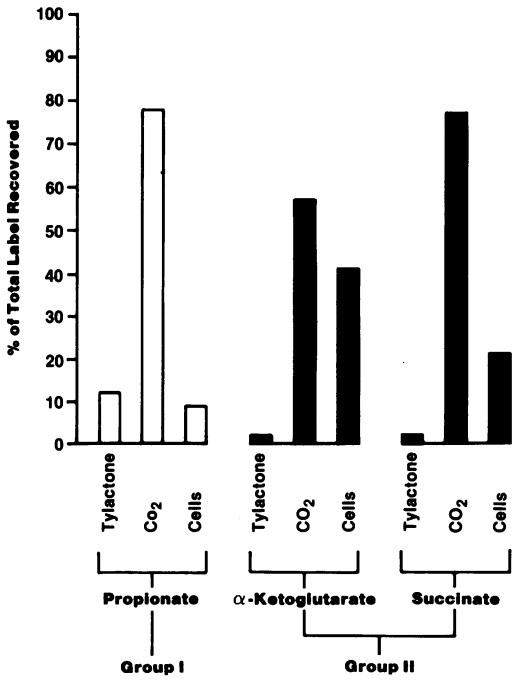

FIG. 6. Label distribution into tylactone, CO_2, and cells from propionate (group I), α-ketoglutarate (group II), and succinate (group II), as percentage of total label recovered, after 60 min.

catabolized to acetoacetyl-CoA. The labeling of CO_2 and cells by lysine resembled the patterns exhibited by arginine and proline of group II (Fig. 4). Because of this similarity, lysine was tested as a group II compound in reactions carried out to 180 min. No detectable [^{14}C]tylactone was recovered from these reactions. The data suggest that $^{14}CO_2$ generated from lysine was the result of a decarboxylation without further catabolism of the product.

The evidence reported here clearly shows the involvement of amino acids in the biosynthesis of tylactone by *S. fradiae* GS14. The data also imply possible roles for carbohydrate and lipid in tylactone biosynthesis. For instance, the group II data, showing the involvement of the TCA cycle and the methylmalonyl-CoA mutase enzyme, imply that any carbon entering the TCA cycle is a potential source for methylmalonyl-CoA and propionyl-CoA generation. In addition, acetoacetyl-CoA, the probable source for ethylmalonyl-CoA, might be from the interrupted β-oxidation of lipid. Finally, acetyl-CoA, the probable source for malonyl-CoA, can be generated from the catabolism of various amino acids, lipid, and carbohydrate. These possibilities are depicted in Fig. 2.

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