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# METABOLISM OF MEVALONIC ACID BY *LACTOBACILLUS PLANTARUM*

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ABSTRACT

DURR, I. F. (American University of Beirut, Beirut, Lebanon), AND A. N. SHWAYRI. Metabolism of mevalonic acid by *Lactobacillus plantarum*. J. Bacteriol. **88**:361-366. 1964.—*Lactobacillus plantarum* strain 8014-$H_2$, unlike other lactobacilli studied, does not require mevalonic acid for growth, but growing and resting cells utilize it only for the synthesis of nonsaponifiable lipids. Upon the incubation of washed cells with mevalolactone-*2*-$C^{14}$, the label appeared in the lipids but not in $CO_2$. On the other hand, when mevalolactone-*1*-$C^{14}$ was used, the label appeared in $CO_2$ but not in lipids. For every mole of $CO_2$ liberated, 1 mole of radioactive carbon was introduced in the lipids, suggesting that terpenic polymers were synthesized. Amino acids did not stimulate the utilization of mevalonic acid. Starved cells could not synthesize nonsaponifiable lipids from mevalonic acid unless glucose was supplied. Sodium fluoride (0.14 M), 2,4-dinitrophenol (0.003 M), *p*-hydroxymercuribenzoate (0.0013 M), potassium phosphate (0.1 M), and ammonium formate (0.04 M) were potent inhibitors. Cells metabolized only one isomer of DL-mevalonic, and utilized the salt form at least twice as efficiently as the lactone. Optimal synthesis of lipids from mevalonic acid occurred aerobically, at pH 5 and 30 C.

Mevalonic acid, a growth factor for a number of lactobacilli, is a central intermediate in the synthesis of several terpenes and sterols (Wright, 1961). Whereas the metabolism of mevalonic acid is well delineated in animals and yeast, little is known about the mechanism of its metabolism by lactobacilli. Thorne and Kodicek (1962) reported that mevalonic acid-dependent strains of lactobacilli converted this compound into nonsaponifiable lipids. They also reported that several nutrients were required for the incorporation of mevalonic acid. Since these authors used mevalonic acid-dependent strains, it was difficult to ascertain whether the reported nutrients were specifically involved in the metabolism of mevalonic acid, or needed for the optimal growth of the microorganism. Therefore we sought to find a lactobacillus which does not require mevalonic acid for growth. Investigations on the growth requirements of *L. plantarum* strain 8014-$H_2$ indicated that this microorganism, unlike the other lactobacilli studied, grows equally well on a medium deficient in mevalonic acid. Yet resting or growing cells of this microorganism convert one isomer of DL-mevalonic acid into nonsaponifiable lipids. Consequently, washed suspensions of *L. plantarum* have been advantageously used to study the requirements for the metabolism of mevalonic acid independent of conditions necessary for the optimal growth of the microorganism. This dissociation is also desirable, since conditions of growth significantly alter the nature of the synthesized lipids (Asselineau and Lederer, 1960).

## MATERIALS AND METHODS

*Bacteriological. L. plantarum* 8014-$H_2$ was obtained from the American Type Culture Collection. Stock cultures were maintained by growing the microorganism in tubes containing 10 ml of the semisynthetic medium of Skeggs et al. (1956). The tubes were kept at 30 C for 24 hr and were subsequently stored at 10 C. These cultures were renewed weekly. When cells were needed, 3 liters of the above medium were inoculated with 20 ml of the culture, and incubated at 30 C for 30 hr. The medium was stirred gently by a magnetic stirrer. Cells were harvested by centrifugation at 4,500 $\times$ *g* for 15 min, and washed twice with cold distilled water. The yield of cells was about 3 g of wet cells per liter of growth medium. *L. plantarum* grew equally well when the medium was supplemented with different amounts of mevalonic acid. Unlike *L. plantarum*, several lactobacilli fail to grow on the medium of Skeggs et al. (1956), unless mevalonic acid is added.

*Reagents*. Chemicals for the growth medium

were obtained from Difco, and Nutritional Biochemicals Corp., Cleveland, Ohio. Radioactive DL-mevalolactone was obtained from the Radiochemical Centre, Amersham, England. All other chemicals were obtained from Merck, Darmstadt, Germany, and Sigma Chemical Co., St. Louis, Mo.

*Assay.* Washed wet cells (400 mg) were incubated in air with either DL-mevalolactone-*1-$C^{14}$* or DL-mevalolactone-*2-$C^{14}$*. Extra additions were as indicated under the appropriate sections; the final volume was 5 ml, and the incubation time was 3 hr at 37 C in a Dubnoff shaker. The reaction was stopped by the addition of 0.3 ml of 3 N $H_2SO_4$. Respiratory $CO_2$ was collected, and its radioactivity was measured as described by Bloom, Stetten, and Stetten (1953). The cells were collected by centrifugation, and washed six times with 5 ml of cold distilled water. The cells were then suspended in 15 ml of water; a 0.1-ml sample was plated on a stainless-steel planchet, and counted in a thin-window counter. Under these conditions of plating, errors due to self absorption were not encountered. In control experiments, cells were inactivated by autoclaving for 15 min prior to incubation.

*Extraction of lipids.* Cells (83 g, wet weight) harvested from 33 liters of medium were washed twice with cold water and suspended in 1 liter of water containing 500 $\mu$moles of DL-mevalonic acid ($6 \times 10^6$ counts per min). The incubation was carried out at 30 C for 5 hr, and the cells were washed four times with 1 liter of water. The cells incorporated 10% of the added radioactive mevalonate. The lipids were then extracted as described by Hofmann, Lucas, and Sax (1952), and were found to contain all the incorporated radioactivity.

*Potassium and sodium mevalonate.* These salts were prepared as described by Fumagalli et al. (1962); 0.032 $\mu$mole of DL-mevalonic-*2-$C^{14}$* acid (30,000 counts per min) in the form of the lactone, or its equivalent of the salts, was used. The reaction mixture contained 20 $\mu$moles of sodium citrate buffer (pH 5.8). The incubation was carried out for 15 min at 37 C. Other details were as described above.

*Buffers and pH.* Since it was accidentally found that phosphate, but not citrate, ion inhibited the synthesis of lipids from mevalonic acid, the latter was used as a buffering system by titrating 1 M citric acid with 3 N NaOH to the desired pH by use of a glass electrode. For studies on optimal pH conditions, DL-mevalolactone-*2-$C^{14}$* (0.64 $\mu$mole, 100,000 counts per min) was incubated with 400 $\mu$moles of the buffer for 1 hr at 37 C. Subsequently, a water suspension of the cells was added, and the reaction was carried out as described above.

*Inhibitors.* The pH of the incubation mixture was adjusted to 5 with 1 N KOH, and the cells were preincubated with the inhibitor for 10 min at 25 C. Subsequently, 0.3 $\mu$mole of DL-mevalolactone-*2-$C^{14}$* (273,000 counts per min) was added and treated as described above.

## RESULTS

*Nature of the lipids.* Cells were initially extracted, as described by Hofmann et al. (1952), with acetone and ether until the extracts were free from radioactivity. The lipids so extracted are designated as free lipids. Subsequently, the cells were autoclaved with sulfuric acid to extract the bound lipids. Saponification and ether extraction of both the free and the bound fractions indicated that lipids derived from labeled mevalonic acid were of the nonsaponifiable type. The total radioactivity recovered in the free nonsaponifiable lipids was 3.4 times that in the bound fraction. The total radioactivity in the free and bound nonsaponifiable lipids was equal to the radioactivity fixed by the cells. A detailed chemical analysis of these lipids will be reported in a subsequent communication.

*Stoichiometry of mevalonic acid metabolism.* When resting cells were incubated with mevalolactone-*2-$C^{14}$*, no radioactivity was detected in the respired $CO_2$. However, the cells became highly radioactive. On the other hand, when mevalolactone-*1-$C^{14}$* was used, the label appeared in the respired $CO_2$, but not in the cells. Table 1 shows that for every mole of labeled carbon incorporated from mevalolactone-*2-$C^{14}$* into the lipids, 1 mole of radioactive carbon was liberated as $CO_2$ from mevalolactone-*1-$C^{14}$*. Table 1 also shows that after 6 hr of incubation at 37 C there was no further utilization of mevalonic acid. It may be noted that about 20% of DL-mevalonic acid was incorporated by the cells under these conditions. Addition of glucose after 12 hr of incubation did not stimulate the metabolism of mevalonic acid.

*Time studies.* Data shown in Table 1 indicate that the rate of mevalonic acid metabolism considerably declines after 3 hr of incubation at 37 C,

whereas Thorne and Kodicek (1962) observed a lag of 3 hr before *L. casei* could utilize mevalonic acid. Figure 1 shows the rate of mevalonic-*1*-$C^{14}$ acid metabolism determined by the labeled $CO_2$ liberated during short intervals of time. Whereas the rate of $CO_2$ production was fairly rapid and linear during the first 2 hr and considerably declined thereafter, only a slight lag of not more than 10 min could be detected in the metabolism of mevalonic acid by *L. plantarum* (Fig. 1).

*Reactive form of mevalonic acid.* Since mevalonic acid can exist as a salt and as a lactone, it became of interest to determine which form the cells would utilize more efficiently. In 15-min incubations, the rate of synthesis of nonsaponifiable lipids from sodium or potassium mevalonate was at least twice that from the lactone.

Wolf et al. (1956) demonstrated that DL-mevalonic acid has half the biological activity of the natural isomer in supporting the growth of *L. acidophilus*. When resting cells of *L. plantarum* were repeatedly incubated with DL-mevalonic-*2*-$C^{14}$ acid, only half of the added amount was converted into lipids. Recovery of the unmetabolized isomer, and subsequent incubation with excess cells, failed to show any further uptake, thereby suggesting that *L. plantarum* metabolizes only one isomer of mevalonic acid.

*Concentration of mevalonic acid.* Figure 2 shows that the rate of synthesis of nonsaponifiable lipids is not detectable when the concentration of DL-mevalonic acid is lower than $6 \times 10^{-6}$ M, but linearly increases as the concentration of mevalonic acid is raised. However, at a concentration of 0.0012 M DL-mevalonic acid, the rate of synthesis approaches zero-order kinetics.

TABLE 1. *Stoichiometry of mevalonic acid metabolism by Lactobacillus plantarum*

| Time | Counts per min | |
|---|---|---|
| | C-1* | C-2* |
| *hr* | | |
| 0 | 0 | 0 |
| 3 | 77,000 | 78,000 |
| 6 | 97,450 | 97,500 |
| 9 | 96,300 | 97,680 |

* C-1 and C-2 refer to $CO_2$ from mevalolactone-*1*-$C^{14}$, and cell-fixed radioactivity from mevalolactone-*2*-$C^{14}$, respectively; 0.53 μmole of radioactive DL-mevalolactone was used (500,000 counts per min).

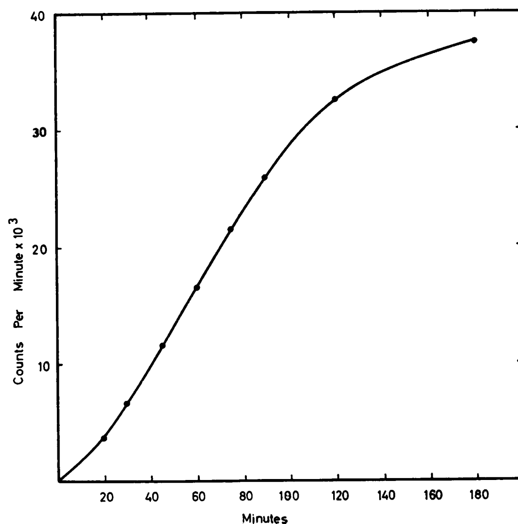


FIG. 1. *Rate of mevalonic acid decarboxylation. The concentration of* DL-*mevalolactone-1-*$C^{14}$ *was* $4.6 \times 10^{-4}$ M *with a specific activity of* $18.5 \times 10^4$ *counts per min per μmole.*

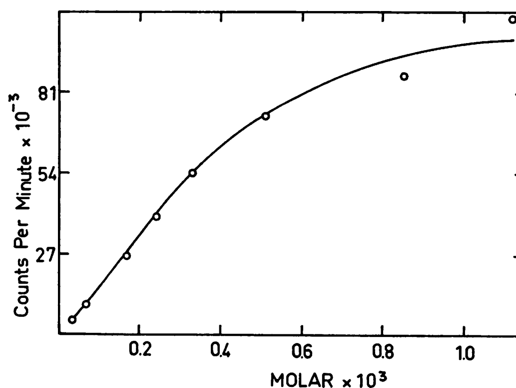


FIG. 2. *Effect of concentration of mevalonic acid on synthesis of nonsaponifiable lipids. Specific activity of* DL-*mevalolactone-2-*$C^{14}$ *was* $3 \times 10^5$ *counts per min per μmole.*

*Optimal pH and temperature.* Figure 3 shows that, when resting cells were incubated with labeled mevalonic acid in the pH range of 3 to 8.6, maximal incorporation into nonsaponifiable lipids occurred at pH 5, and greatly declined at neutral and alkaline pH. When the incubations were carried out at different temperatures (Table 2), optimal synthesis of lipids occurred at 30 C, which is also the optimal temperature for the growth of this microorganism.

*Inhibitors.* Table 3 shows the effect of several

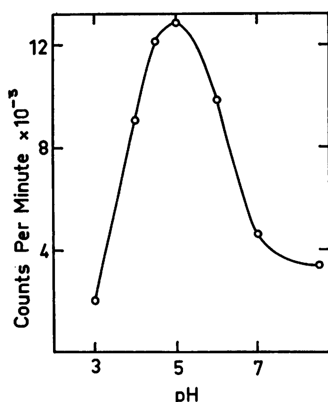


FIG. 3. *Effect of pH on synthesis of nonsaponifiable lipids.*

TABLE 2. *Effect of temperature on the incorporation of mevalonic acid into lipids of Lactobacillus plantarum**

| Temp | Counts per min |
|---|---|
| *C* | |
| 0 | 750 |
| 20 | 2,960 |
| 30 | 3,820 |
| 37 | 3,500 |

* Cells were preincubated for 15 min to attain the desired temperature. Subsequently, DL-mevalonic-*2*-$C^{14}$ acid (0.24 μmole, 250,000 counts per min) was added, and the reaction continued for 1 hr.

TABLE 3. *Inhibitors of mevalonic acid metabolism*

| Inhibitor* | Percentage of control |
|---|---|
| None (control) | 100.0 |
| Sodium fluoride (0.14) | 14.4 |
| *p*-Hydroxymercuribenzoate (0.0013) | 10.8 |
| 2,4-Dinitrophenol (0.009) | 0.62 |
| 2,4-Dinitrophenol (0.003) | 7.8 |
| Potassium acid phosphate (0.1) | 38.1 |
| Ammonium formate (0.04) | 46.7 |
| Potassium chloride (0.04) | 93.5 |
| Sodium malonate (0.14) | 80.0 |

* Numbers in parentheses represent the final molarity of the inhibitor.

compounds on the metabolism of mevalonic acid. Sodium fluoride, 2,4-dinitrophenol, and *p*-hydroxymercuribenzoate markedly inhibited the biosynthesis of nonsaponifiable lipids from mevalonic acid. Phosphate and formate ions unexpectedly inhibited this pathway. Citrate and malonate were not inhibitory within a wide range of pH, nor could they reverse the inhibition caused by phosphate and formate ions. It may be pointed out that the addition of an amino acid mixture consisting of methionine, leucine, isoleucine, valine, serine, and glutamic acid did not stimulate the utilization of mevalonic acid beyond that of the control.

*Effect of glucose and anaerobiosis.* The inhibitory effect of sodium fluoride and 2,4-dinitrophenol suggested that a source of energy was required for the utilization of mevalonic acid. Since unsupplemented resting cells can metabolize mevalonic acid, it may be assumed that the needed energy is derived from the metabolism of endogenous substrates. Hence, starving these cells should block the synthesis of the nonsaponifiable lipids. This is borne out by the results of the following experiment. Resting cells were gently shaken at 5 C for 24 hr. Subsequently, when these cells were incubated with 0.8 μmole of DL-mevalolactone-*2*-$C^{14}$ (750,000 counts per min) under the standard conditions of assay, only 0.075% of DL-mevalonic acid was converted into cellular lipids. However, the addition of 100 mg of glucose caused a 64-fold stimulation.

A preliminary report on the identity of the nonsaponifiable lipids derived from mevalonic acid indicated that one of them was a hydroxylated compound of a large molecular weight (Thorne and Kodicek, 1963). The possibility that the synthesis of such compounds, analogous to that of lanosterol (Tchen and Bloch, 1957), might require molecular oxygen was investigated. When resting cells were incubated under an atmosphere of nitrogen or $CO_2$, the synthesis of nonsaponifiable lipids was depressed by 20 to 50% of that under aerobic conditions.

## DISCUSSION

Evidence is provided that resting-cell suspensions of *L. plantarum* efficiently incorporate one isomer of DL-mevalonic acid into nonsaponifiable lipids. This stereoselectivity is apparently typical of microorganisms which metabolize mevalonic acid (Wagner and Folkers, 1961). The utilization of mevalonic acid by resting cells of *L. plantarum* proceeds independently of exogenous sources of carbon and nitrogen. The previously reported (Thorne and Kodicek, 1962) inability of *L. casei* and *L. arabinosus* to metabolize mevalonic acid

except in the presence of nitrogenous and other substances probably reflects basic requirements fundamental for their growth, rather than for the specific metabolism of this compound. This is further supported by the fact that the addition of several amino acids to resting cells of *L. plantarum* did not stimulate the metabolism of mevalonic acid beyond that of the control.

The inhibitory effect of sodium fluoride and 2,4-dinitrophenol, and the stimulatory effect of glucose, on starved cells suggest that a source of energy is required for the metabolism of mevalonic acid. The failure of malonate to inhibit the metabolism of mevalonic acid is perhaps due to some permeability barriers. Thus, when malonate-*2*-$C^{14}$ was incubated with cells of *L. plantarum*, no radioactivity was detected intracellularly. The impermeability of certain bacterial cells to malonate is well documented (Krampitz, 1961).

The mechanisms by which phosphate and formate ions inhibit the synthesis of nonsaponifiable lipids is not yet understood. The inhibitory effect of some anions on the synthesis of lipid polymers is well known. Macrae and Wilkinson (1958) reported that borate and tris (2-amino-2-hydroxymethylpropane, 1:3 diol) blocked the synthesis of polyhydroxybutyrate in washed suspensions of *Bacillus cereus*. Whether such anions interfere with the transport mechanisms of the precursors or with the synthesis of the polymers remains to be investigated.

The fact that the salt of mevalonic acid is twice as efficiently metabolized as the lactone is not surprising, since intermediates usually exist in the ionized form (Davis, 1958). Since both forms of mevalonic acid are in equilibrium, the reported efficiency of the salt must be a minimal value. However, these considerations do not preclude the possibility that the bacterial cell may be more permeable to the lactone than to the salt of mevalonic acid. Fumagalli et al. (1962) found that rat liver incorporated the lactone into cholesterol more efficiently than the potassium salt, but the reverse was true of the brain. Obviously, a quantitative evaluation of membrane permeability to mevalonic acid, its salt, and lactone will be necessary to assess the role of transport mechanisms in controlling the metabolism of this compound.

Studies on the stoichiometry of mevalonic acid metabolism showed that for every mole of labeled carbon dioxide liberated from mevalolactone-*1*-$C^{14}$, 1 mole of radioactive carbon was incorporated from mevalolactone-*2*-$C^{14}$ into nonsaponifiable lipids. This stoichiometry suggests that the decarboxylation of mevalonic acid most likely leads to the formation of the biologically active isoprene unit from which terpenes and sterols are derived. The finding that fatty acids as well as other saponifiable compounds were not labeled shows that the conversion of mevalonic acid to the bacterial lipids occurs without prior cleavage into short-chain fatty acids.

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