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# Incorporation of Radioactive Acetate into Diacetyl by *Streptococcus diacetilactis*

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*Streptococcus diacetilactis* was grown in a partially defined, lipoic acid-free medium containing radioactive acetate with and without addition of 0.1% unlabeled sodium pyruvate. Labeled carbon was incorporated into diacetyl, but neither the amount of diacetyl produced nor its specific activity was influenced by addition of pyruvate. Acetoin had low specific activity, indicating that it was a mixture of radioactive and nonradioactive acetoin. The specific activity of acetoin was lower when pyruvate, a precursor of unlabeled acetoin, was added to the medium, which indicated that the radioactive acetoin was produced from radioactive diacetyl by diacetyl reductase. Results substantiate condensation of acetyl-coenzyme A with hydroxyethylthiamine pyrophosphate as the in vivo mechanism for synthesis of diacetyl.

Lactic streptococci are unable to synthesize lipoic acid and in its absence require acetate for growth (5, 6, 12). The activation of acetate to acetyl-coenzyme A (acetyl-CoA) requires CoA and adenosine triphosphate. Although animal tissues, yeast, and some bacteria activate acetate by involving a single enzyme, acetyl-CoA synthetase (9), most bacteria, including *S. diacetilactis* (5), involve two enzymes, acetate kinase (10) and phosphotransacetylase (17). With lipoic acid in the medium, lactic streptococci do not require acetate and form acetyl-CoA from pyruvate by decarboxylation and formation of hydroxyethylthiamine pyrophosphate and transfer of the acyl group of the latter to CoA (4, 5). Because in vitro results indicate that *S. diacetilactis* and several other microorganisms synthesize diacetyl by condensation of acetyl-CoA with hydroxyethylthiamine pyrophosphate and rearrangement (3, 15), the acetyl-CoA incorporated into diacetyl by microorganisms that require lipoic acid should not come from pyruvate or pyruvate precursors in media devoid of lipoic acid or substances that contain it (e.g., yeast extract). According to the mechanism of Speckman and Collins (15), any diacetyl synthesized in such a medium should depend on the production of acetyl-CoA from acetate.

This investigation was designed to study in vivo formation of diacetyl in a medium devoid of lipoic acid. A preliminary investigation (16) had shown that amounts of diacetyl and acetoin sufficient for the study are produced from glucose in the partially defined medium of Harvey and Collins (8) by *S. diacetilactis*, an organism that produces reduced nicotinamide adenine dinucleotide oxidase (2, 4, 15).

## MATERIALS AND METHODS

*S. diacetilactis* 18-16 was propagated routinely at 22 C in sterile litmus milk and subcultured in the partially defined, lipoic acid-free medium of Harvey and Collins (8), as modified by Collins and Bruhn (5) except that we used 1% glucose. Acetoin and diacetyl were separated by salting-out chromatography (14) and determined quantitatively by the Westerfeld procedure (20). Radioactivity was measured in a Nuclear-Chicago scintillation counter (model 70034; efficiency approximately 75%) with samples in 10 ml of Bray solution (11). Sodium acetate was obtained from Calbiochem (Los Angeles, Calif.). Chromatographically homogeneous sodium acetate-*1*-$^{14}C$ (specific activity, 8.6 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.).

## RESULTS AND DISCUSSION

*S. diacetilactis* 18-16 was grown for 16 h at 22 C under static conditions in 200 ml of basal medium (pH 6.5) without lipoic acid but containing 0.2% unlabeled sodium acetate and two $\mu$Ci of sodium acetate-*1*-$^{14}C$. Cells were harvested by centrifugation (6,000 $\times$ *g* for 10 min) and washed twice with 10 ml of 0.5 M phosphate buffer, pH 6.5. A sample of spent medium combined with cell washes was fractionated by salting-out column chromatography. A sample of each fraction was assayed for acetoin and diacetyl and analyzed for radioactivity.

Acetoin and diacetyl were formed, and both compounds were radioactive (Fig. 1). Specific activity of the diacetyl was high ($7.2 \times 10^7$ counts per min per $\mu$mol), which indicated that in absence of lipoic acid diacetyl was synthesized by incorporation of acetyl-CoA from acetate. Considerably more acetoin than diacetyl was produced (Fig. 1), but the specific activity of the acetoin was low ($2.8 \times 10^4$ counts per min per $\mu$mol), which suggested that most of the acetoin was unlabeled and produced from glucose via pyruvate and $\alpha$-acetolactate by mechanisms that do not involve acetyl-CoA (3, 4, 13, 15). Nevertheless, some of the acetoin was radioactive and probably produced from radioactive diacetyl by the physiologically irreversible action of diacetyl reductase (13, 15, 18).

To determine if the radioactive acetoin had been produced from diacetyl, we repeated the above experiment with and without addition of 0.1% sodium pyruvate, with the media adjusted to pH 5.5 to enhance entry of pyruvate into the bacteria. We did not attempt an experiment with added citrate, because cleavage of the citrate by citritase would have yielded acetate (7) and resulted in dilution of the radioactivity of the diacetyl produced.

In the medium containing pyruvate, nine times as much acetoin was produced (Fig. 2), but the specific activity of the acetoin ($4.1 \times 10^3$ counts per min per $\mu$mol) was only one-eighth of that found when pyruvate was omitted ($3.3 \times 10^4$ counts per min per $\mu$mol). It was apparent that the added pyruvate, which was not required as a hydrogen acceptor in the formation of lactic acid (4, 8), had served as a precursor of acetoin, increased the production of unlabeled acetoin, and decreased the specific activity of the total acetoin by diluting the radioactive acetoin that was produced irreversibly from radioactive dia-

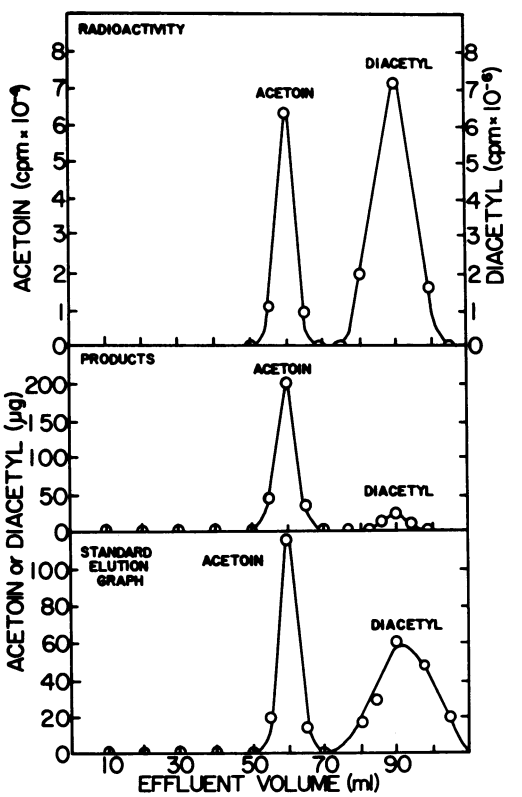


FIG. 1. *Radioactivity of acetoin and diacetyl produced in 16 h at 22 C by Streptococcus diacetilactis 18-16 at pH 6.5 in a lipoic acid-free medium containing $^{14}$C-acetate. Products were separated by salting-out chromatography and determined quantitatively by the Westerfeld method (20). The standard elution graph was determined with a mixture of acetoin and diacetyl (150 μg of each).*

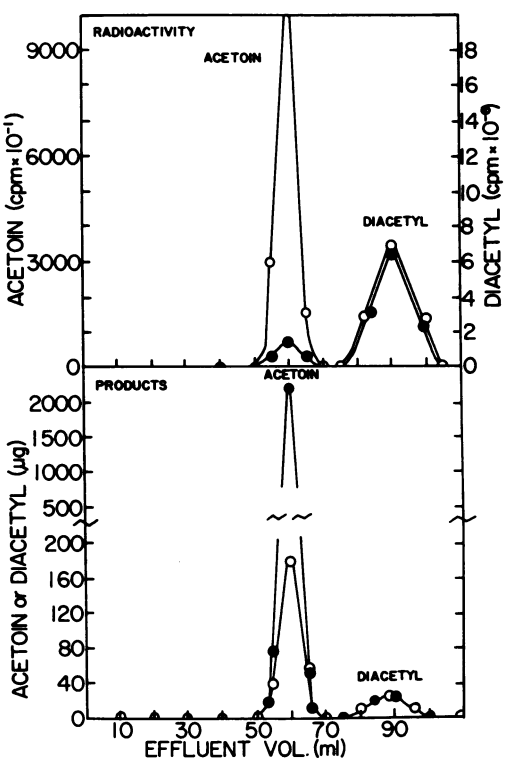


FIG. 2. *Radioactivity of acetoin and diacetyl produced in 16 h at 22 C by Streptococcus diacetilactis 18-16 at pH 5.5 in a lipoic acid-free medium containing $^{14}$C-acetate with and without addition of 0.1% sodium pyruvate. Products were separated by salting-out chromatography and determined quantitatively by the Westerfeld method (20). Symbols: ●, pyruvate added; ○, pyruvate not added.*

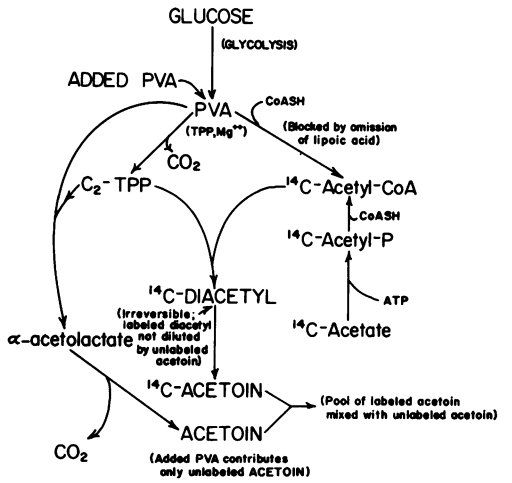


FIG. 3. *Experimental rationale for observed labeling of acetoin and diacetyl produced by Streptococcus diacetilactis 18-16 in a lipoic acid-free medium containing $^{14}$C-acetate with and without added pyruvate.*

cetyl by diacetyl reductase. Simultaneously, *S. diacetilactis* produced identical amounts of diacetyl with and without added pyruvate (Fig. 2), and the specific activities of the diacetyl produced were approximately equal ($6.6 \times 10^7$ and $6.9 \times 10^7$ counts per min per $\mu$mol, respectively). With the formation of acetyl-CoA from pyruvate blocked by the omission of lipoic acid, the limiting step in diacetyl synthesis was availability of acetyl-CoA from acetate, and the added pyruvate did not increase diacetyl production.

The labeling patterns and experimental rationale are summarized in Fig. 3. Although these experiments were run with *S. diacetilactis*, the findings apparently apply to other microorganisms that produce diacetyl. Suomalainen and Ronkainen (19) found yeast to produce diacetyl from 0.05 M pyruvate only if acetyl-CoA was added to their test system, and Branen and Keenan (1) found *Lactobacillus casei* to produce radioactive diacetyl when grown in a medium containing $^{14}$C-labeled acetate. Their results and those of this study indicate that the mechanism for diacetyl synthesis determined from in vitro studies (3, 15) is operative in vivo for a variety of diacetyl-producing microorganisms.

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