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Biosynthesis of Diacetyl in Bacteria and Yeast

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Both diacetyl and acetoin were produced by cell-free extracts and cultures of *Pseudomonas fluorescens*, *Aerobacter aerogenes*, *Lactobacillus brevis*, and *Saccharomyces cerevisiae* 299, whereas only acetoin was produced by cell-free extracts and cultures of *Streptococcus lactis*, *Serratia marcescens*, *Escherichia coli*, and *S. cerevisiae* strains 513 and 522. Cell-free extracts that produced diacetyl did not produce it from acetoin; they produced it from pyruvate, but only if acetyl-coenzyme A was was added to the reaction mixtures. Production of diacetyl by *S. cerevisiae* 299 was prevented by valine, inhibited by sodium arsenite, and stimulated by pantothenic acid. Valine did not prevent the production of acetoin. *E. coli* and the three strains of *S. cerevisiae* did not decarboxylate α-acetolactate but did use acetaldehyde in the production of acetoin from pyruvate. The other organisms produced acetoin from pyruvate via α-acetolactate.

Juni (6, 7) elucidated certain differences in the mechanisms by which bacteria and yeast and mammalian tissues form acetoin (3-hydroxy-2-butanone) from pyruvate. The bacteria he studied formed acetoin by decarboxylation of α-acetolactate, which was formed by enzymatic condensation of two molecules of pyruvate, probably by formation of an acetaldehyde-thiamine pyrophosphate (TPP) complex from pyruvate and condensation of the complex with another molecule of pyruvate. Free acetaldehyde is not involved in this mechanism (6, 8, 11, 18). The yeast and mammalian tissues formed acetoin by a different mechanism, probably by condensation of the acetaldehyde-TPP complex with free acetaldehyde formed from pyruvate (7).

It has been assumed that diacetyl (2,3-butanedione) is formed by oxidation of acetoin in bacteria (9, 17) and in yeast (10, 16, 21). Speckman and Collins (18), however, recently reported that *Streptococcus diacetilactis* and *Leuconostoc citrovorum* do not form diacetyl from acetoin; instead, they form diacetyl by condensation of the acetaldehyde-TPP complex with acetyl coenzyme A (CoA).

This study was done to determine whether the mechanism for diacetyl biosynthesis found by Speckman and Collins (19) is used by other diacetyl-producing microorganisms and to verify the mechanisms that have been described for acetoin production. All bacteria and yeast in the study formed acetoin by the expected mechanisms. Those that formed diacetyl were found to form it by the new mechanism, not from acetoin.

MATERIALS AND METHODS

Organisms and culturing procedures. The following organisms which produced CO_2 and acetoin plus diacetyl from pyruvate were used in this study: *Pseudomonas fluorescens*, *Aerobacter aerogenes*, *Serratia marcescens*, *Escherichia coli*, *Streptococcus lactis* C_2, *Lactobacillus brevis*, *Sacchromyces cerevisiae* var. *ellipsoideus* (UCD Enology strain no. 513, Distillers', and no. 522, Montrachet), and *S. cerevisiae* (UCD Food Science and Technology strain no. 299). *S. cerevisiae* strain NCYC 1200, obtained late in the study and not included, produced an amount of diacetyl similar to that produced by strain 299.

S. lactis was propagated routinely in sterile skim milk fortified with 0.75% nonfat milk solids. For obtaining cells, it was grown at 26 C for 15 hr in lactose-citrate broth (3), *p*H 6.8. *L. brevis* was propagated in tomato-enriched medium (6% Difco-Rogosa SL broth dissolved in filtered tomato juice) and, for obtaining cells, was grown at 21 C for 40 hr in orange-lactose broth (commercial orange juice with 0.5% lactose added, steam-sterilized, with the *p*H not adjusted). The media used for propagating and obtaining cells of the other bacteria were, respectively, Difco Nutrient Agar and Nutrient Broth, *p*H 7.0. To obtained cells of these species, *P. fluorescens* was grown at 21 C for 72 hr, *A. aerogenes* at 32 C for 35 hr, *S. marcescens* at 32 C for 30 hr, and *E. coli* at 37 C for 36 hr. *S. cerevisiae* was grown for 2.5 days at 21 C in YED medium (yeast extract, 2%; glucose, 2%; KH_2PO_4, 0.5%; *p*H 5.95) or (for certain indicated experiments) in ASD medium (ammonium sulfate, 0.1%; KH_2PO_4, 0.5%; glucose, 2%; *p*H 5.85). The glucose content of the YED medium was increased to 20% for certain indicated experiments.

Cell-free extracts. Each species was grown in 4-liter Erlenmeyer flasks. Each flask contained 2 liters of

broth and was shaken every 3 to 5 hr. The content of acetoin plus diacetyl was followed by analysis with the Westerfeld test (22). The cells were harvested by centrifugation when the content of acetoin plus diacetyl stopped increasing. They were washed twice with cold 0.2 M phosphate-cysteine buffer at the *p*H of the original growth medium (0.001 M with respect to L-cysteine) and were resuspended in the same buffer. The cells were disrupted by ballistic disintegration with a Mickel tissue disintegrator operated at maximal amplitude for 1 hr (1.5 hr for yeast) in a room maintained at 4 C, and crude extract was separated from the disrupted cells by centrifugation at 2 C for 30 to 40 min in a refrigerated centrifuge at 23,000 × *g*. To purify the crude extract, 1 μmole of ethylenediaminetetraacetate (EDTA) was added per mg of protein in the cell-free extract to remove metal ions; then 15 ml of the extract (containing about 10 mg of protein per ml) was dialyzed for 12 hr against 100 volumes of 0.2 M phosphate-cysteine buffer at the *p*H of the original growth medium. CoA was removed from dialyzed extracts by treatment with an anion-exchange resin (acid-washed Dowex 1-X2). One volume of the extract was stirred slowly for 5 min with one-quarter volume of the resin. The *p*H was checked periodically and was maintained between 7 and 7.3 (5.8 to 6.0 for yeast) by dropwise addition of 0.2 M phosphate-cysteine buffer. The resin was sedimented by centrifugation for 3 min at 5,000 × *g*, and the supernatant fluid was removed with a Pasteur pipette.

For experiments with *S. cerevisiae* 522, extracts were prepared by the method of Juni (7) modified as follows. Freshly harvested cells were carefully washed and lyophilized. A 10-g amount of the lyophilized cells was crushed with a mortar and pestle, and the resulting paste was extracted with 40 ml of 0.13 M phosphate buffer, *p*H 5.5. The crude extract (43.5 ml) was cooled to −1 C in an ice-salt bath, and 9 ml of cold (−18 C) 95% ethyl alcohol was added, dropwise, with stirring. A precipitate which formed for 1 hr at −1 C was removed by centrifugation. Then 34.8 ml of cold 95% alcohol was added to the supernatant fluid, and the mixture was allowed to precipitate at −18 C for 12 hr. The precipitate was removed by centrifugation and resuspended in 10 ml of distilled water. Insoluble material, removed from the suspension by centrifugation, was discarded. Finally, the suspension was dialyzed as indicated above, and CoA was removed.

Analytical procedures. The diacetyl and acetoin produced by all organisms except *S. cerevisiae* 299 were separated by salting-out chromatography with a column (1.9 × 27 cm) packed with Dowex 1-X8 anion-exchange resin slurried in 3.0 M $(NH_4)_2SO_4$, as described by Speckman and Collins (19), and were determined quantitatively by the Westerfeld method (22). With *S. cerevisiae* 299, except in the experiment involving radioactive pyruvate or acetyl-CoA, diacetyl and acetoin were determined either by the Westerfeld method after separation with the 1.9 × 27 cm column (Fig. 2) or by the gas chromatographic method of Jennings and Nursten (5; results in Table 1), except that the column was packed with 10% silicone oil SF9560 (Varian Aerograph, Walnut Creek, Calif.) and operated at 65 C. For the experiment involving radioactive pyruvate or acetyl-CoA (Fig. 3), acetoin and diacetyl were separated with a (3.5 × 54 cm) column packed with Dowex 1–X8 anion-exchange resin slurried in saturated Na_2SO_4, as described by Speckman and Collins (19), and were determined by radioactivity or the Westerfeld test (22). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer with 0.5% 2,5-diphenyloxazole (PPO), 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 5% naphthalene in a total volume of 20 ml of liquid containing 38% toluene, 38% dioxane, and 24% ethyl alcohol. A fraction collector (Redi-Rak, Stockholm, Sweden) was used to collect and distribute effluent during chromatographic separations.

A Beckman spectrophotometer, model DB, was used for all spectrophotometric determinations, and *p*H was measured with a Radiometer (Copenhagen, Denmark) *p*H meter, model 22. A Klett-Summerson colorimeter, model 800-3, was used with filter no. 54 for Westerfeld analysis, and with filter no. 29 or 66 for measuring the turbidity of cells during growth. Protein was determined by the biuret method (2), and citrate by the method of Marier and Boulet (12). Decarboxylation was measured by the conventional Warburg manometric technique. Diacetyl reductase activity was measured indirectly by measuring oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) spectrophotometrically at 340 mμ.

Sodium pyruvate, calcium d-pantothenate, CoA, TPP, NAD, $NADH_2$, sodium pyruvate-*2*-^{14}C (specific activity, 10 mc per mmole), and sodium pyruvate-*3*-^{14}C (specific activity, 4.6 × 10^4 counts per min per μmole) were obtained from Calbiochem (Los Angeles, Calif.). Acetyl-CoA-*1*-^{14}C (specific activity, 70 mc per mmole) was obtained from New England Nuclear Corp. (Boston, Mass.). Isotopically labeled acetoin (specific activity, 5.9 × 10^4 counts per min per μmole) was prepared by the action of cell-free extracts of *S. diacetilactis* 18–16 on sodium pyruvate-*3*-^{14}C (18). Acetyl-CoA was synthesized from acetic anhydride and CoA (20). Acetaldehyde, acetoin, and diacetyl were obtained from Eastman Organic Chemicals Co. (Rochester, N.Y.). Acetoin, obtained as the crystalline dimer, was washed with ether until free from diacetyl, as shown by both column and gas chromatography. α-Acetolactate was prepared from its ester (obtained from K & K Laboratories, Jamaica, N.Y.), as described by Speckman and Collins (18), by saponifying with two equivalents of cold 0.025 N NaOH, added slowly to the system held in a water bath at 5 C and continuously purged with a stream of nitrogen.

RESULTS

Production of acetoin or diacetyl and acetoin by growing cultures. Six species of bacteria and three yeast strains reported to produce diacetyl or acetoin were tested for production of the compounds. Each produced acetoin, but five (*S. lactis*, *S. marcescens*, *E. coli*, and *S. cerevisiae* 522 and 513) did not produce detectable diacetyl,

even though one of them (*S. marcescens*) produced large amounts of acetoin. Figure 1 gives results obtained with growing cultures of four of these five. Results with *S. cerevisiae* 513 were similar to those obtained with *S. cerevisiae* 522.

Streptococcus lactis produced acetoin rapidly during the logarithmic growth phase, and converted the acetoin in the growth medium to 2,3-butylene glycol (2,3-butanediol). (A cell-free extract of *S. lactis* was found able to reduce acetoin in the presence of $NADH_2$.) *Serratia marcescens* produced acetoin during and after the logarithmic growth phase, but *E. coli* produced detectable quantities only after the maximal stationary growth phase had been achieved. Apparently these two organisms did not reduce appreciable quantities of acetoin.

Saccharomyces cerevisiae 522 produced more acetoin when the glucose content of YED medium was increased to 20%, and almost 10 times as much in the ASD medium (the medium without yeast extract), though growth in ASD was retarded considerably, and the final cell population was much lower than it was in the more complete medium. Figure 1 includes a comparison of results for *S. cerevisiae* in YED medium (without additional glucose) and ASD medium. The larger amounts of acetoin with additional glucose and in ASD medium possibly reflected, at least in part, differences in the amounts of pyruvate available for acetoin synthesis (4). Detectable diacetyl production by *S. cerevisiae* 522 or 513 was not induced by replacing glucose with maltose, increasing the concentration of glucose up to 40%, or decreasing the *p*H of the medium to 4.65.

P. fluorescens, *A. aerogenes*, *L. brevis* (in orange-lactose broth), and *S. cerevisiae* 299 formed both diacetyl and acetoin. *L. brevis* did not produce detectable quantities of diacetyl or acetoin in tomato-enriched medium, but cell-free extracts of cells harvested from this medium produced both compounds. The uninoculated medium used for comparison in the determinations of acetoin and diacetyl gave discolored controls, and it is possible that small amounts of acetoin and diacetyl were undetected. This medium contained only 0.3% citrate, compared with 1% in the orange-lactose broth. Thus, it seems probable that *L. brevis*, like *S. diacetilactis* (3, 4), does not produce enough excess pyruvate from carbohydrate to result in the production of appreciable acetoin and diacetyl. *P. fluorescens*, *A. aerogenes*, *S. marcescens*, *E. coli*, and *S. cerevisiae*, however, produced acetoin or acetoin and diacetyl in broths that contained no citrate.

S. cerevisiae 299 began producing diacetyl only when the *p*H of the growth medium dropped below 4.9 (Fig. 2). The organism produced both diacetyl and acetoin at 10 and 20 C in YED medium containing 20% glucose (Fig. 2A and Aa) and at 20 C in ASD medium (Fig. 2Ba), but pro-

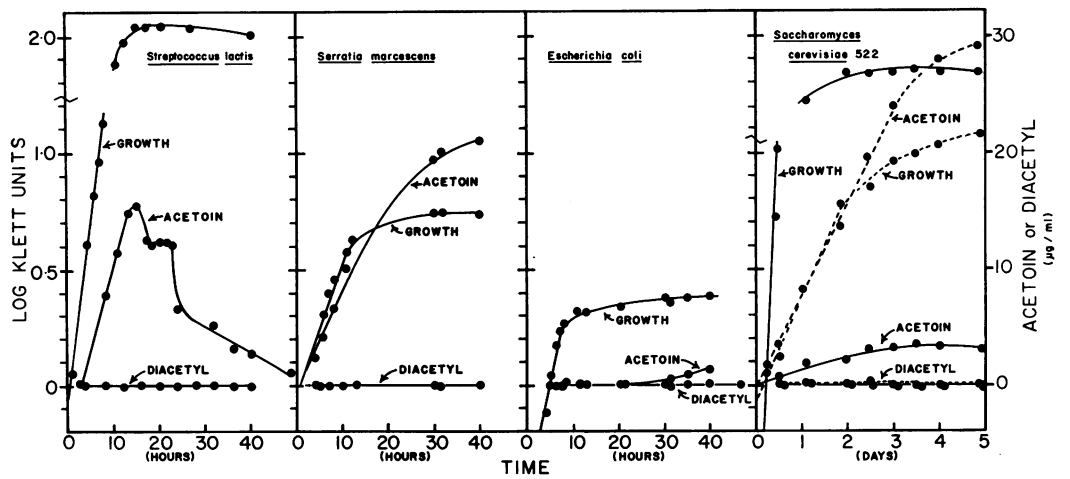

FIG. 1. *Relationship between growth and acetoin production by Streptococcus lactis C_2, Serratia marcescens, Escherichia coli, and Saccharomyces cerevisiae 522. S. cerevisiae was tested in ASD medium (dotted lines) and in YED medium (solid lines). See Materials and Methods for composition of the media. Klett-Summerson filter no. 66 was used for determining the growth of S. cerevisiae, and filter no. 29 was used for the other organisms. Klett units corresponding to 1 mg (dry weight) per ml for S. lactis, S. marcescens, E. coli, and S. cerevisiae were 395, 235, 230, and 210, respectively. For determining these relationships, cells that were growing logarithmically were harvested by centrifugation, washed, and suspended in distilled water; curves relating cell mass (determined after drying to constant weight at 105 C) to Klett units were constructed.*

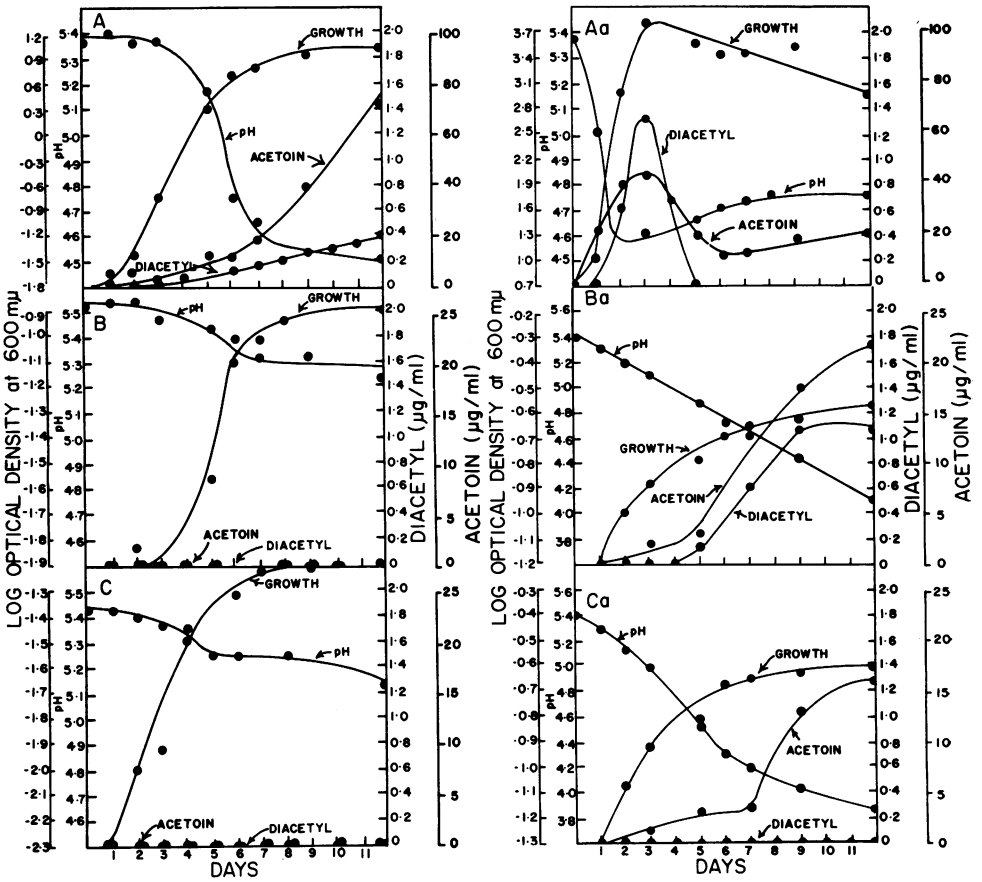

FIG. 2. *Diacetyl and acetoin production by Saccharomyces cerevisiae 299. (A and Aa) YED medium containing 20% glucose at 10 and 20 C, respectively. (B and Ba) ASD medium at 10 and 20 C, respectively. (C and Ca) ASD medium plus 0.1% DL-valine at 10 and 20 C, respectively. One milligram (dry weight) per milliliter of S. cerevisiae 299 corresponded to OD_{600} equals 4.70.*

duced neither compound at 10 C in ASD medium (Fig. 2B). This lack of production probably occurred because the *p*H remained above 4.9 or was related to differences in growth and fermentation that permitted only minimal amounts of excess pyruvate, or resulted from a combination of these conditions. The amounts of diacetyl formed in the media were related to cell population, as previously reported for yeast (15), but the amounts of diacetyl produced per unit of cells were increased by raising the glucose content of the YED medium to 20%, and they were greater in the ASD medium than in the YED medium containing 20% glucose (Fig. 2Aa and Ba).

Influence of DL-valine, calcium pantothenate, or sodium arsenite on diacetyl formation by S. cerevisiae 299. The addition of 0.1% DL-valine to ASD medium prevented the formation of diacetyl by *S. cerevisiae* 299, but did not prevent the formation of acetoin (Fig. 2Ba and Ca). Subsequently, we found that pantothenate stimulated the production of diacetyl and arsenite inhibited it. Addition of 0.02% calcium d-pantothenate to YED medium containing 20% glucose resulted in a 62% increase in the diacetyl content at the population maximum of *S. cerevisiae* 299, with a 33% increase in this maximum. Addition of sodium arsenite to give a concentration of 5 mM resulted in a 77% decrease in the diacetyl content, with a corresponding 43% decrease in the population maximum.

Production of acetoin or acetoin and diacetyl by cell-free extracts. The production of acetoin, or acetoin and diacetyl, by cell-free extracts of the organisms confirmed the results that had been obtained with growing cultures. Table 1 gives selected data for four organisms. Each of the

TABLE 1. *Production of acetoin or diacetyl and acetoin by cell-free extracts of bacteria and yeast*[a, b]

Organism	Substrate					Products	
	Pyruvate	α-Aceto-lactate	Acetyl-CoA	Acetaldehyde	Acetoin	Acetoin	Diacetyl
	μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Lactobacillus brevis	25.0	—	—	—	—	3.82	0
	—	—	—	—	1.9	1.82	0
	25.0	—	10.0	—	—	1.02	1.78
Escherichia coli	25.0	—	—	—	—	0.44	0
	—	—	—	—	11.4	8.62	0
	—	10.0	—	—	—	0.02	0
	25.0	—	12.5	—	—	0.91	0
	12.5	—	12.5	12.7	—	4.96	0
Saccharomyces cerevisiae 513	50.0	—	—	—	—	2.35	0
	—	10.0	—	—	—	0	0
	100.0	—	25.0	—	—	5.69	0
	50.0	—	—	50.0	—	5.45	
S. cerevisiae 299	27.2	—	—	—	—	Trace	0
	—	—	—	—	11.4	5.43	0
	—	10.0	—	—	—	0	0
	27.2	—	—	22.7	—	Trace	0
	27.2	—	25.0	—	—	Trace	0.14
	27.2[c]	—	—	—	—	1.01	0
	—[c]	10.0	—	—	—	0	0

[a] Reaction conditions and cofactors were as follows. *L. brevis*: Mg^{++}, 22.5 μmoles; TPP, 0.105 μmole; extract protein, 3.3 mg; 0.2 M phosphate buffer; *p*H 5.3; temperature, 30 C; total volume, 5.0 ml; reaction time, 16 hr. *E. coli*: Mg^{++}, 22.5 μmoles; TPP, 0.105 μmole; extract protein, 9 mg; 0.2 M phosphate buffer, *p*H 6.7; temperature, 37 C; total volume, 5.0 ml; reaction time, 17 hr. *S. cerevisiae* 513: Mg^{++}, 22.5 μmoles; TPP, 0.105 μmole; extract protein, 3.5 mg; 0.066 M acetate buffer; *p*H 5.5; temperature, 21 C; total volume, 6.0 ml; reaction time, 16 hr. *S. cerevisiae* 299: extracts prepared from cells grown on YED medium containing 20% glucose; Mg^{++}, 45 μmoles; TPP, 0.21 μmole; extract protein, 5.4 mg; 0.1 M succinate buffer; *p*H 4.65; temperature, 27 C; total volume, 7.0 ml; reaction time, 7 hr.

[b] Reaction times for maximal production of acetoin or diacetyl and acetoin were determined experimentally. Reaction mixtures containing boiled extract were used as controls.

[c] Phosphate buffer (0.05 M, *p*H 5.8) replaced succinate buffer.

extracts required TPP and Mg^{++} for the production of acetoin from pyruvate. The bacteria, except *E. coli*, produced acetoin from pyruvate or α-acetolactate; *E. coli* and yeast did not decarboxylate α-acetolactate, and acetaldehyde stimulated their production of acetoin from pyruvate. Cell-free extracts of those organisms that had been found to produce diacetyl as growing cultures (*P. fluorescens*, *A. aerogenes*, *L. brevis*, *S. cerevisiae* 299) were found to produce diacetyl. However, they did not produce diacetyl from acetoin or α-acetolactate, with or without the addition of NAD. They produced diacetyl from pyruvate, but only if acetyl-CoA was included in the reaction mixtures. Extracts of the five organisms that had been found unable to produce diacetyl as growing cultures did not produce diacetyl, with or without the inclusion of acetyl-CoA in the reaction mixtures.

The synthesis of diacetyl by a cell-free extract of *S. cerevisiae* 299 was studied with radioactive pyruvate and radioactive acetyl-CoA. The results in Fig. 3 show that the extract formed acetoin from pyruvate. However, it did not incorporate acetyl-CoA into acetoin or convert acetoin to diacetyl. Acetyl-CoA was incorporated into diacetyl, and the extract formed diacetyl from pyruvate only if acetyl-CoA was included in the reaction mixture.

DISCUSSION

The primary purpose of this investigation was to determine whether the mechanism described by Speckman and Collins for diacetyl formation by *S. diacetilactis* and *L. citrovorum* (18) is used by other microorganisms. Yeast seemed particularly important. Simultaneously, the study gave information verifying the formation of acetoin

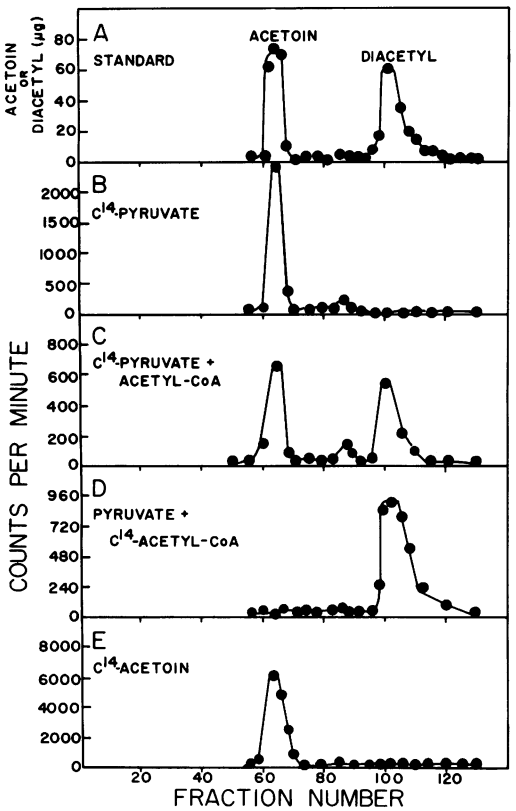

FIG. 3. *Incorporation of labeled pyruvate or labeled acetyl-CoA into diacetyl by dialyzed, resin-treated cell-free extracts of Saccharomyces cerevisiae 299. The standard elution graph (A) was determined with a mixture containing 100 µg each of acetoin and diacetyl. Reaction mixtures contained TPP, 0.21 µmole; Mg^{++}, 45 µmoles; extract protein, 8 mg; succinate buffer, pH 4.6; plus: (B) sodium pyruvate-2-^{14}C, 27.3 µmoles (8.05×10^4 counts per min per µmole); (C) sodium pyruvate-2-^{14}C, 27.3 µmoles (8.05×10^4 counts per min per µmole), and acetyl-CoA, 10 µmoles; (D) sodium pyruvate, 27.3 µmoles, and acetyl-CoA-1-^{14}C, 10.0 µmoles (21.0×10^4 counts per min per µmole); (E) acetoin-^{14}C, 11.5 µmoles (3.5×10^4 counts per min per µmole), and NAD, 10.0 µmoles. Total volume, 6.0 ml; temperature, 27 C; reaction time, 7 hr.*

by previously described mechanisms (6, 7, 18). Yeast did not convert α-acetolactate to acetoin by the mechanism normally used in bacteria (6). The production of acetoin by cell-free extracts of the yeast was stimulated by the addition of acetaldehyde, as would be expected from the work of Juni (7), which indicates that yeast and mammalian tissues form acetoin by condensation of free acetaldehyde with the acetaldehyde-TPP complex formed from pyruvate. The results with bacterial extracts, except that of *E. coli*, were consistent with the mechanism reported for bacteria by Juni (6), i.e., the formation of acetoin by decarboxylation of α-acetolactate formed from pyruvate. *Escherichia coli*, however, appears to use the mechanism normally used by yeast and mammalian tissues. Acetaldehyde stimulated the production of acetoin by cell-free extracts of this organism, and the extracts were unable to decarboxylate α-acetolactate. The minute amounts of acetoin that were formed in two of three experiments to test decarboxylation of α-acetolactate by *E. coli* are attributed to nonenzymatic decarboxylation during the 17-hr reaction (18). Additionally, cell-free extracts of each of the organisms that produced diacetyl were found to oxidize $NADH_2$, indicating the presence of diacetyl reductase.

Dialyzed, resin-treated cell-free extracts of each of the four organisms that produced diacetyl from pyruvate produced it only if acetyl-CoA was included in the reaction mixtures, suggesting that all diacetyl-producing microorganisms probably use the mechanism found by Speckman and Collins (18). The involvement of acetyl-CoA in the formation of diacetyl by *S. cerevisiae* 299 was substantiated in experiments with radioactive pyruvate and radioactive acetyl-CoA.

The new mechanism for diacetyl synthesis, and the failure of both growing cultures and cell-free extracts of several acetoin-producing organisms to produce diacetyl, place in question the results of a large number of published papers in which the sum of acetoin and diacetyl has been considered to indicate the production of diacetyl. The new mechanism is contrary to the previous assumption that bacteria (9, 17) and yeast (10, 16, 21) form diacetyl by oxidation of acetoin. In yeast, the inhibition of diacetyl by valine incorporated into the growth medium (13–15) has been considered to substantiate α-acetolactate and acetoin as intermediates in the biosynthesis of diacetyl, since α-acetolactate is an intermediate in the biosynthesis of valine and, in some microorganisms, acetoin. *A. aerogenes* has been shown to have two enzymes that catalyze the formation of α-acetolactate, and valine has been found to inhibit one of the enzymes, leaving enough activity to produce sufficient α-acetolactate to meet the demands for growth without wasteful formation of valine (23).

At least some of those organisms that are able to use α-acetolactate for forming pantothenic acid or valine, or both, are provided with a means of forming acetoin that does not involve α-acetolactate. Failure of valine to prevent the formation of acetoin by *S. cerevisiae* 299 is consistent with results showing that in this organism α-acetolactate does not serve as an intermediate in the

synthesis of acetoin. The inhibition of diacetyl formation by sodium arsenite, and the stimulation by pantothenic acid, substantiate the role of acetyl-CoA in the synthesis of diacetyl. Also, they indicate that involvement of α-acetolactate in the synthesis of diacetyl is determined by whether the organism in question is able to synthesize pantothenic acid.

The mechanism by which valine prevents the formation of diacetyl is not clear. One could suppose that inhibition of valine synthesis by feedback inhibition would suppress the formation of α-ketoisovaleric acid. Consequently, since α-ketoisovaleric acid also is an intermediate in the formation of pantothenic acid, formation of the latter would be suppressed. This, in turn, would suppress the formation of acetyl-CoA, and subsequently the formation of diacetyl, since pantothenate is a constituent of CoA. However, transamination of amino acids by yeast (1) is common, and one would expect *S. cerevisiae* to make sufficient α-ketoisovaleric acid by transamination of the valine added to the medium. *S. cerevisiae* 299 apparently has a partial requirement for pantothenic acid, since it was stimulated by calcium d-pantothenate. Possibly the amount of pantothenic acid formed from valine by the organism at low *p*H is small, the added valine causes enhancement of some of the organism's needs for CoA, and there is little excess CoA that can be directed to diacetyl synthesis.

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