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Enzyme Basis for pH Regulation of Citrate and Pyruvate Metabolism by *Leuconostoc oenos*

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Citrate and pyruvate metabolism by nongrowing cells of *Leuconostoc oenos* was investigated. ^{13}C nuclear magnetic resonance (NMR) spectroscopy was used to elucidate the pathway of citrate breakdown and to probe citrate or pyruvate utilization, noninvasively, in living cell suspensions. The utilization of isotopically enriched substrates allowed us to account for the end products derived from the metabolism of endogenous reserves. The effect of environmental parameters, e.g., pH, gas atmosphere, and presence of malate, on the end products of citrate utilization was studied. Approximately 10% of the citrate supplied was converted to aspartate which remained inside the cells. A metabolic shift with pH was observed, with acetoin production being favored at pH 4, whereas lactate and acetate production increased significantly at higher pH values. The information obtained with NMR was complemented with studies on the relevant enzyme activities in the metabolic pathway of citrate breakdown. The intracellular pH of the cells was strongly dependent on the external pH; this result, together with the determination of the pH profile of the enzymic activities, allowed us to establish the basis for pH regulation; lactate dehydrogenase activity was optimal at pH 7, whereas the acetoin-forming enzymes displayed maximal activities below pH 5. Citrate utilization was also monitored in dilute cell suspensions for comparison with NMR experiments performed with dense suspensions.

The activity of lactic acid bacteria in wine has a great influence on the composition and organoleptic properties of this product. Malolactic fermentation is encouraged primarily to lower wine acidity, but increased biological stability and subtle changes in flavor and aroma complexity are generally regarded as additional advantages (18). *Leuconostoc oenos* is the major species found in wine during the malolactic fermentation, since it is best adapted to the low pH (3 to 3.5) and high ethanol concentration that characterize wine after alcoholic fermentation (18, 39).

During vinification, citrate utilization by lactic acid bacteria is an important metabolic process because of the production of flavoring compounds, such as diacetyl, acetoin, butanediol (C_4 compounds), and acetate (12). In particular, the content in diacetyl is a parameter of critical importance to wine quality; small amounts (1 to 4 mg · liter^{-1}) are considered beneficial, but slightly higher contents (5 to 7 mg · liter^{-1}) give an undesirable butterlike flavor (8).

Detailed studies on the metabolic behavior of *L. oenos* are needed in order to control the final properties of wine. This remains a distant goal since a complete description of the mechanism and regulation of citrate metabolism is not available for lactic acid bacteria from wine and, though relevant work on this subject has been published (9, 19, 28), most of the data available are difficult to interpret. As evident from a recent review on citrate metabolism by lactic acid bacteria (15), nearly all the research effort invested so far has been concerned with dairy lactic acid bacteria.

Following uptake, citrate is converted to pyruvate in two nonredox reactions: citrate is first cleaved to oxaloacetate and acetate in a reaction catalyzed by citrate lyase, and oxaloacetate is subsequently converted to pyruvate by oxaloacetate decarboxylase (Fig. 1). The fate of pyruvate will depend on the cultivation conditions such as aeration, pH, and carbohydrate availability. The production of C_4 compounds is favored under carbohydrate limitation, low external pH, and aerobiosis (32). In *Leuconostoc* cells isolated from dairy sources, the formation of C_4 compounds was observed only in the absence of carbohydrate or at low pH (6, 30).

The metabolic pathway of citrate breakdown and diacetyl production by lactic acid bacteria has been the subject of considerable controversy. It was suggested that diacetyl was synthesized from the condensation of one molecule of hydroxyethylthiamine PP_i and one molecule of acetyl-coenzyme A (CoA) (31). Recent work with lactococci has shown that diacetyl and acetoin are produced from the intermediate metabolite α-acetolactic acid that decarboxylates spontaneously to diacetyl in the presence of oxygen and/or low pH (16, 25, 34).

The purpose of the present study is to contribute to the understanding of citrate metabolism and its regulation in *L. oenos*. Isotopic labeling coupled to ^{13}C nuclear magnetic resonance (NMR) spectroscopy was used to probe citrate and pyruvate utilization in living nongrowing cell suspensions and to elucidate the metabolic pathway leading to diacetyl production. The effects of environmental parameters, e.g., pH and oxygen availability, on the end products of citrate utilization were also investigated, and the enzymatic basis for metabolic regulation by pH was established.

MATERIALS AND METHODS

Organism and growth conditions. *L. oenos* GM (Microlife Techniques, Sarasota, Fla.) was routinely grown in FT80 medium (4), modified by omission of Tween 80, containing 8 g of glucose liter^{-1} and 2.4 g of citrate liter^{-1}. Cells were grown at pH 4.8 and 30°C in static 2-liter glass bottles until the late exponential

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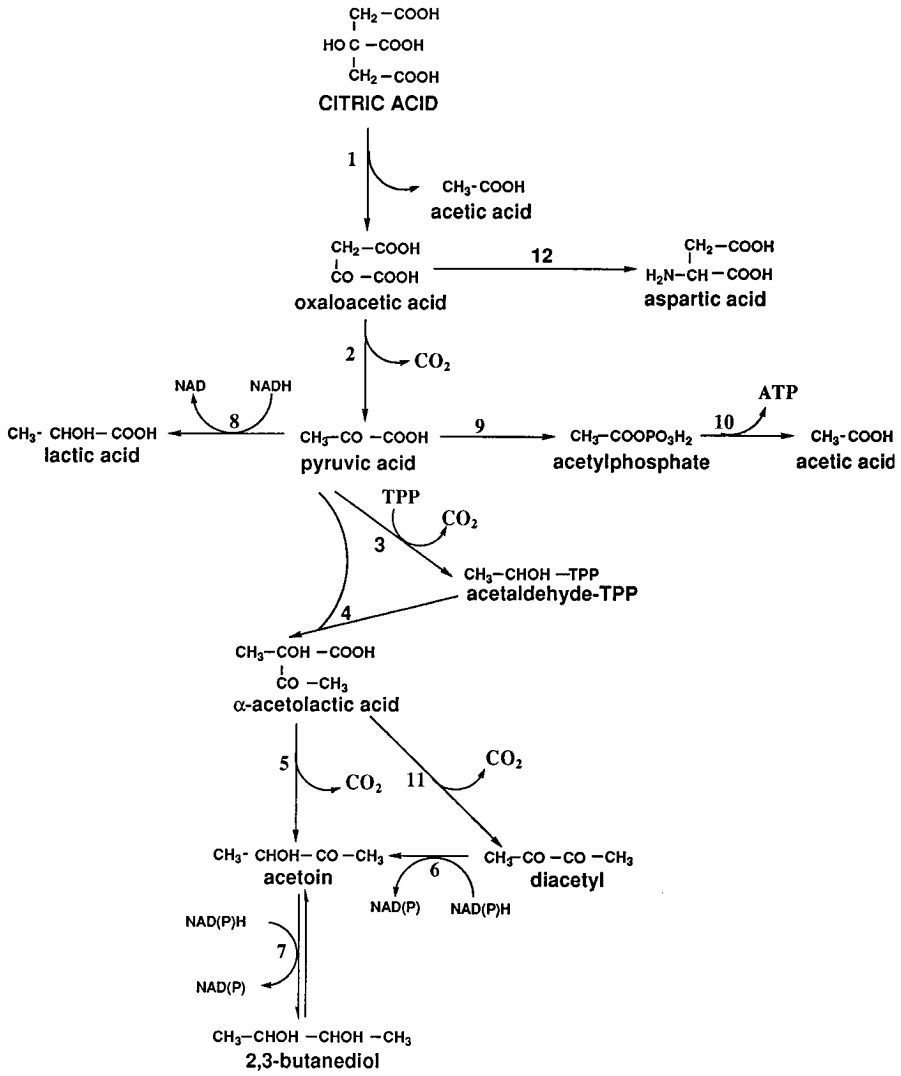

FIG. 1. Metabolic pathway of citrate breakdown by *L. oenos*. 1, citrate lyase; 2, oxaloacetate decarboxylase; 3, pyruvate decarboxylase; 4, α-acetolactate synthase; 5, α-acetolactate decarboxylase; 6, diacetyl reductase; 7, acetoin reductase; 8, lactate dehydrogenase; 9, pyruvate dehydrogenase complex; 10, acetate kinase; 11, nonenzymatic decarboxylative oxidation of α-acetolactate; 12, aspartate aminotransferase. TPP, thiamine PP_i.

growth phase. For the NMR experiments, the $MnSO_4$ concentration in the growth medium was decreased to 1 mg · liter^{-1}.

Sample preparation. (i) NMR experiments. Cells were harvested by centrifugation (2,000 × *g*, 10 min at 4°C), washed twice with 5 mM potassium phosphate (pH 5), and resuspended in 50 mM potassium phosphate with an adequate pH to approximately 15 g (dry weight) of cells · liter^{-1}. The cell suspension (3.5 ml) was immediately transferred to a 10-mm NMR tube, 5% (vol/vol) ^2H_2O was added to provide a lock signal, and the ^{13}C-labeled substrate (citrate or pyruvate) was provided. Once the substrate had been exhausted, the acquisition was stopped, cells were centrifuged (30,000 × *g*, 30 min at 4°C), and the supernatant was saved. The cell pellet was washed once with buffer and centrifuged again, and the two fractions were pooled. Supernatant solutions were kept at −20°C until analyzed.

(ii) Experiments with dilute cell suspensions. Cells were harvested and resuspended in 50 mM potassium phosphate with an adequate pH to approximately 3 g (dry weight) of cells · liter^{-1}. The cell suspension (30 ml) was incubated in a water bath at 30°C and stirred gently under a continuous flow of nitrogen. The substrates were added, and samples were taken at time intervals following the addition and immediately centrifuged (2,000 × *g*, 10 min at 4°C). The supernatant solutions were kept at −20°C until further analysis.

NMR spectroscopy. One-dimensional ^{13}C and ^1H NMR spectra were recorded with Bruker AMX-500 or AMX-300 spectrometers, as previously described (25). The inverse-detected heteronuclear multiple quantum coherence spectrum (2) was acquired after cell disruption in order to identify the resonances due to intracellular components. The spectrum was acquired on an AMX-500 spectrometer, with presaturation of the water signal and a spectral width of 10 kHz for F_1 and 10 kHz for F_2, collecting 4,096 by 256 datum points. A delay of 3.5 ms was used for evolution of $^1J_{CH}$. All the spectra were run at a probe head temperature of 30°C.

Measurement of end products. Unless stated otherwise, end products were determined in the supernatant solutions obtained as described above. Citrate, acetate, lactate, malate, acetoin, 2,3-butanediol, and diacetyl were measured by ^1H NMR spectroscopy that allowed a ready evaluation of the ^{13}C isotopic enrichment of the end products (25). For these measurements, cell supernatants were diluted fivefold in ^2H_2O, and formate was used as an internal concentration standard. The concentration of the products in the liquid supernatants was determined by comparing the intensities of their resonances to that of formate in a fully relaxed spectrum. It was checked that formate was not produced as a fermentation end product. ^{13}C-labeled aspartate was determined in fully relaxed ^{13}C NMR spectra by using the intensity of the resonance of acetate for comparison; acetate concentration was measured in the same sample by using the appropriate Boehringer Mannheim kit.

Extraction and identification of internal ^{13}C-labeled metabolites. Following the NMR experiments with addition of labeled citrate, the cell supernatants were obtained as described above and the cell pellet was subsequently resuspended up to a volume of 3.5 ml in the same buffer used for the experiments; cells were then passed three times through a French pressure cell at 20,000 lb/in^2 and centrifuged at 30,000 × *g* for 30 min at 4°C. The cell debris was washed once and centrifuged again, and the supernatant fractions were pooled. The cell extract obtained was kept at −20°C until analyses by ^{13}C and ^1H NMR.

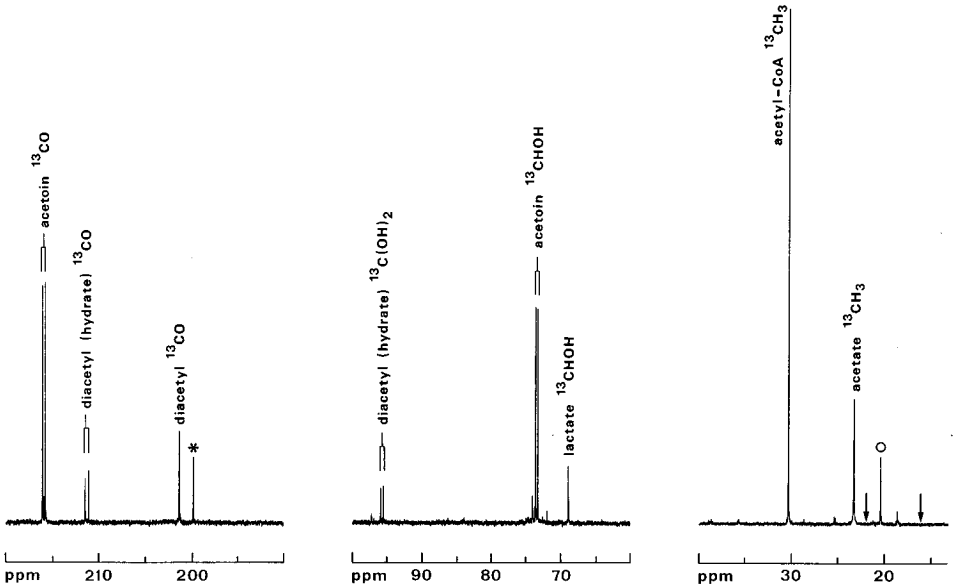

FIG. 2. ^{13}C NMR spectrum of a supernatant solution obtained after the cometabolism of [2-^{13}C]pyruvate and [2-^{13}C]acetyl-CoA by permeabilized cells of *L. oenos* under aerobic conditions. The arrows at higher field indicate the positions expected for the resonances of acetoin, diacetyl, and 2,3-butanediol labeled on the methyl groups. Symbols: ○, *N*-acetyl–CoA, impurity in the preparation of acetyl-CoA; *, unassigned carbonyl resonance.

Measurement of intracellular pH. The intracellular pH was determined from the distribution of [*carboxyl*-^{14}C]benzoic acid, by the silicon oil centrifugation technique (24). Cells were harvested and resuspended in 50 mM potassium phosphate at the adequate pH to a protein concentration of approximately 5 mg · ml^{-1}. The internal pH was measured in cells to which no substrate was added or during the metabolism of 20 mM pyruvate, at external pH values of 4.0, 5.2, and 6.5. An intracellular volume of 3.6 μl · mg of protein^{-1} was considered for the calculation of intracellular pH (27).

Preparation of cell extracts. Cells were cultivated until the mid-exponential phase of growth (with or without citrate in the medium), harvested, and suspended in 50 mM potassium phosphate. Cells were placed in an ice-water bath and disrupted with a Braun Lab-U sonicator at low power with a 0.9-cm-diameter tip three times for 6 min each (duty cycle, 40%). Cell debris was removed by centrifugation at 30,000 × *g* for 30 min at 4°C, and the cell extracts were stored at −20°C until analysis.

Enzyme assays. Citrate lyase (5), D-lactate dehydrogenase (3), diacetyl reductase, and acetoin reductase (33) were assayed as previously described. α-Acetolactate synthase assays were done in 1 ml of 50 mM potassium phosphate (pH 5.8)–0.5 mM $MgCl_2$–0.8 mM thiamine PP_i–1 to 100 mM sodium pyruvate, at 30°C. The reaction was started by the addition of cell extract (200 to 300 μg of protein) and stopped at the appropriate time by pH decrease imposed by addition of 6 N H_2SO_4 (16). Acetoin was then measured by the method of Veringa et al. (37). α-Acetolactate decarboxylase activity was measured as described previously (16), in 1 ml of 50 mM potassium phosphate (pH 5.8), at 30°C. Cell extract (200 to 300 μg of protein) was added, and the reaction was started by addition of α-acetolactate (1 to 15 mM). At the appropriate time, the reaction was stopped by addition of 1 M NaOH. α-Acetolactate disappearance was monitored by the method of Veringa et al. (37). All activities are expressed as initial rates. One unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of product under the assay conditions. The pH was checked at the end as well at the start of each assay and did not change by more than 0.1 U. All assays were performed at substrate saturation concentrations, except in the experiments aiming at the determination of kinetic parameters.

Miscellaneous. Cell suspensions were permeabilized as described previously (25). Protein was assayed by the method of Lowry et al. (20).

Chemicals. α-Acetolactate was obtained by saponification of the double ester ethyl 2-acetoxy-2-methylacetoacetate (Aldrich) as described by Veringa et al. (37). [3-^{13}C]pyruvate and [2-^{13}C]pyruvate (99% enriched) were obtained from Cambridge Isotope Laboratories, Cambridge, Mass.; [2,4-^{13}C]citrate (100%) and [2-^{13}C]acetyl-CoA (90%) were supplied by Advanced Research on Chemistry, Amsterdam, The Netherlands. [*carboxyl*-^{14}C]benzoic acid (50 mCi · mmol^{-1}) was supplied by NEN. All other chemicals were of reagent grade.

RESULTS

Elucidation of the metabolic pathway of citrate breakdown. Permeabilized cells were obtained as previously described (25) and placed immediately in the NMR tube. An aerobic atmosphere was established by bubbling oxygen through the cell suspension with an air-lift system (29), and the labeled substrates [2-^{13}C]pyruvate and [2-^{13}C]acetyl-CoA were added in equimolar amounts (12 mM), following the same strategy used for *Lactococcus lactis* (25, 34). Figure 2 shows the ^{13}C NMR spectrum of a supernatant solution obtained in an experiment in which the cometabolism of [2-^{13}C]pyruvate and [2-^{13}C] acetyl-CoA was monitored under aerobic conditions. The pathway involving condensation of acetyl-CoA with hydroxyethyl-thiamine PP_i would lead to labeling in the CH_3 and CO groups of diacetyl as well as in the CH_3 group and the CO or CHOH group of acetoin or 2,3-butanediol, respectively. However, the label from acetyl-CoA was found only in the methyl group of acetic acid. No diacetyl, acetoin, or 2,3-butanediol labeled on the methyl group was detected. The products formed were lactate, labeled on the CHOH group; acetoin, labeled on the CO and on the CHOH groups; and diacetyl, enriched on the CO groups. The resonances of the hydrated form of diacetyl, $CH_3{}^{13}C(OH)_2{}^{13}COCH_3$, were also detected (Fig. 2). The labeling pattern found on these metabolites is consistent with the condensation of one molecule of pyruvate and one molecule of hydroxyethyl-thiamine PP_i (Fig. 1).

Effect of pH on the pattern of end products from pyruvate. The metabolism of [3-^{13}C]pyruvate (70 μmol, corresponding to a final concentration of 20 mM) was monitored in vivo under several experimental conditions. Product formation from metabolism of [3-^{13}C]pyruvate at external pH values of 4.0, 5.0, or 6.2 was compared (Fig. 3). At pH 4.0 (bar A), 94% of the pyruvate was converted to acetoin, while at pH 6.2 (bar C) approximately equimolar amounts of acetate and lactate were produced and no acetoin was detected. An intermediate situation was found at pH 5.0 (bar B), where an acetate/lactate/acetoin molar ratio of 1:1:1 was observed and small amounts of 2,3-butanediol were detected. The rates of pyruvate consumption were also affected by pH: at pH 6.2 and 5.0, the rates were approximately 50 and 70 μmol · min^{-1} · g (dry weight) of

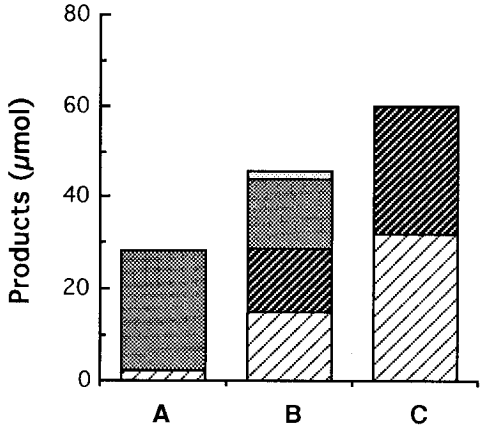

FIG. 3. Effect of pH on the end products derived from pyruvate metabolism as analyzed by NMR spectroscopy. Shown are patterns of end product formation from pyruvate at pH 4.0 (bar A), 5.0 (B), and 6.2 (C). Symbols: ▨, acetate; ▨, lactate; ■, acetoin; □, 2,3-butanediol.

cells^{-1}, respectively, whereas at pH 4.0 the rate decreased to approximately 2 μmol · min^{-1} · g (dry weight) of cells^{-1}.

Enzyme activities. The observed pH-induced metabolic shift suggested that the activities of the enzymes involved in pyruvate breakdown might be influenced by pH. To elucidate this question, the activities of key enzymes involved in pyruvate-citrate utilization were measured. The absence or the presence of citrate (15 mM) in the growth medium was tested, and the results are shown in Table 1. No major differences were observed, indicating that the enzymes assayed were constitutively expressed. Lactate dehydrogenase activity was fructose-1,6-bisphosphate independent; acetoin and diacetyl reductase used NADPH as a cofactor. α-Acetolactate decarboxylase showed a 10-fold-higher specific activity than α-acetolactate synthase. The affinity constants of lactate dehydrogenase and α-acetolactate synthase for pyruvate were 0.6 and 15 mM, respectively. To establish whether the pH-induced metabolic shift could be related to the pH dependency of the pyruvate-utilizing enzymes, the activities were determined at several pH values in extracts of cells grown on citrate-containing medium (Table 2). The α-acetolactate synthase and the α-acetolactate decarboxylase (acetoin-forming enzymes) showed higher specific activities at pH 5.0, with a sharp decrease in activity at pHs above 5.5. In contrast, lactate dehydrogenase showed a pH profile with maximal activity between pH 6.0 and 7.0.

Intracellular pH determination. The intracellular pH was determined in washed cell suspensions of *L. oenos*, both in the absence and in the presence of pyruvate (20 mM). The relation between the intracellular and the external pH is shown in Fig. 4. Intracellular pH is highly dependent on the external pH imposed regardless of the absence or the presence of external substrate. Moreover, for each value of external pH tested, the intracellular pH was not significantly affected by energization with pyruvate. It was also observed that *L. oenos* cells were able to maintain a pH gradient, alkaline inside, for at least 1 h in the absence of any external energy source, at all the external pH values assayed.

TABLE 1. Activities of several enzymes involved in citrate metabolism by *L. oenos*[a]

Enzyme	Citrate	
	+	−
Citrate lyase	0.34	0.11
α-Acetolactate synthase	0.13	0.13
α-Acetolactate decarboxylase	1.3	1.2
Lactate dehydrogenase	8.5	8.6
Acetoin reductase	0.46	0.25
Diacetyl reductase	0.41	0.41

[a] Crude extracts were obtained from cells grown in the absence (−) or in the presence (+) of 15 mM citrate in the growth medium. Activities are expressed in units per milligram of protein, and all the determinations were made in triplicate in at least two different extracts. Standard deviations in the values shown are lower than 0.03.

TABLE 2. Comparison of the pH profiles of enzymes involved in pyruvate metabolism by *L. oenos*[a]

Enzyme	pH				
	5.0	5.5	6.0	6.5	7.0
Lactate dehydrogenase	4.3	6.3	8.9	8.6	8.6
α-Acetolactate synthase	0.13	0.13	0.10	0.05	0
α-Acetolactate decarboxylase	1.25	0.96	0.48	0.16	0

[a] Crude extracts were obtained from cells grown in medium containing 15 mM citrate. Activities are expressed in units per milligram of protein, and all the determinations were made in triplicate in at least two different extracts.

Effect of aeration on the pattern of end products from pyruvate. The effect of the gas atmosphere on the metabolism of pyruvate (75 μmol, corresponding to a final concentration of 20 mM) at pH 5.0 was also monitored by NMR. Aerobic or anaerobic conditions were established by bubbling oxygen or argon, respectively, through the cell suspension. Under aerobic conditions, pyruvate was consumed at a rate of 60 μmol · min^{-1} · g (dry weight) of cells^{-1} and in the absence of oxygen at a rate of 70 μmol · min^{-1} · g (dry weight) of cells^{-1}. The pattern of products obtained from pyruvate was dependent on the presence of oxygen (Fig. 5). The most striking differences were the threefold reduction in the conversion of pyruvate to lactate and the production of diacetyl under aerobic conditions. Diacetyl production was directly observed from the ^{13}C NMR spectra acquired during the consumption of pyruvate by living cells (data not shown); diacetyl was also detected in the supernatant solution obtained after complete exhaustion of pyruvate (Fig. 5). The three NMR resonances found at approximately 23 ppm were assigned to diacetyl and

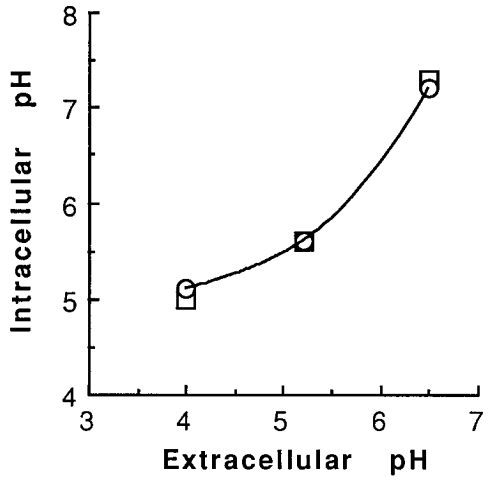

FIG. 4. Intracellular pH of *L. oenos* cells as a function of external pH. ○, no substrate added; □, 20 mM pyruvate added.

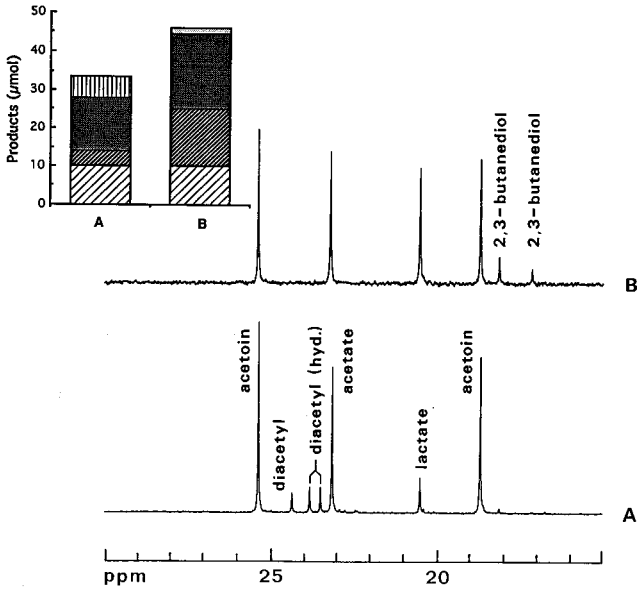

FIG. 5. Effect of aeration on the pattern of end products from pyruvate metabolism by *L. oenos* at pH 5.0. After pyruvate exhaustion, cell supernatants were obtained and analyzed by ^{13}C and ^{1}H NMR. The ^{13}C NMR spectra of the end products obtained under oxygen (A) or argon (B) atmosphere are shown. The inset shows the pattern of end products from pyruvate under oxygen (bar A) or argon (bar B). Carbon recoveries were 80% (bar A) and 85% (bar B). Symbols: ▨, acetate; ▨, lactate; ■, acetoin; ▥, diacetyl; □, 2,3-butanediol.

its hydrated form [$^{13}CH_3C(OH)_2CO^{13}CH_3$] on the basis of the chemical shifts and comparison with the spectrum of the pure compound. Under anaerobic conditions, acetate, lactate, acetoin, and 2,3-butanediol were produced in a molar ratio of approximately 1:1:1:0.1 (Fig. 5).

Citrate metabolism as monitored by ^{13}C NMR. The metabolism of [2,4-^{13}C]citrate (18 μmol, corresponding to a final concentration of 6 mM) by resting cells of *L. oenos* at pH 5.0 was probed in vivo, under anaerobic conditions (Fig. 6). Citrate was consumed at a rate of 100 $\mu mol \cdot min^{-1} \cdot g$ (dry weight) of $cells^{-1}$. Acetate, lactate, and acetoin were produced in a molar ratio of 5:1:0.5, and acetoin was slowly converted to 2,3-butanediol following citrate exhaustion (data not shown). The intermediate metabolite in the pathway of acetoin formation, α-acetolactate, was not detected in any of these in vivo experiments or in the ^{13}C NMR spectra of the supernatant solutions. α-Acetolactate was also not detected in any of the experiments in which the metabolism of pyruvate was monitored. The conversion of citrate to the end products was quantified from the analyses of both carbon and proton spectra of the supernatant solutions. Similar to findings with *L. lactis* (25), significant amounts of unlabeled acetate and lactate (on average, 10 $\mu mol \cdot min^{-1} \cdot g$ [dry weight] of $cells^{-1}$) were detected from the proton spectra of the supernatant solutions. The ^{13}C recovery in these experiments was rather low, taking into account that the percentage of enrichment of the citrate supplied was 99%. On average, not more than 60% of the label supplied was recovered as end products in the cellular supernatants. In contrast, when labeled pyruvate was supplied, the recoveries obtained were significantly higher, varying between 80 and 90%. These data suggest that when citrate was provided a significant amount of label remained inside the cells. To elucidate this question, cell suspensions were allowed to metabolize [2,4-^{13}C]citrate until substrate exhaustion, internal metabolites were extracted as described in Materials and Methods, and the resulting cell extract was analyzed by NMR. A single prominent resonance at 36.9 ppm observed in the ^{13}C NMR spectrum was tentatively assigned to the CH_2 group of aspartic acid on the basis of the pH dependence of its chemical shift. The assignment was confirmed in a ^{1}H-^{13}C correlation heteronuclear multiple quantum coherence experiment (data not shown): the resonance at 36.9 ppm in the ^{13}C dimension correlated with a complex pattern of resonances at approximately 2.8 ppm in the proton dimension, which were firmly

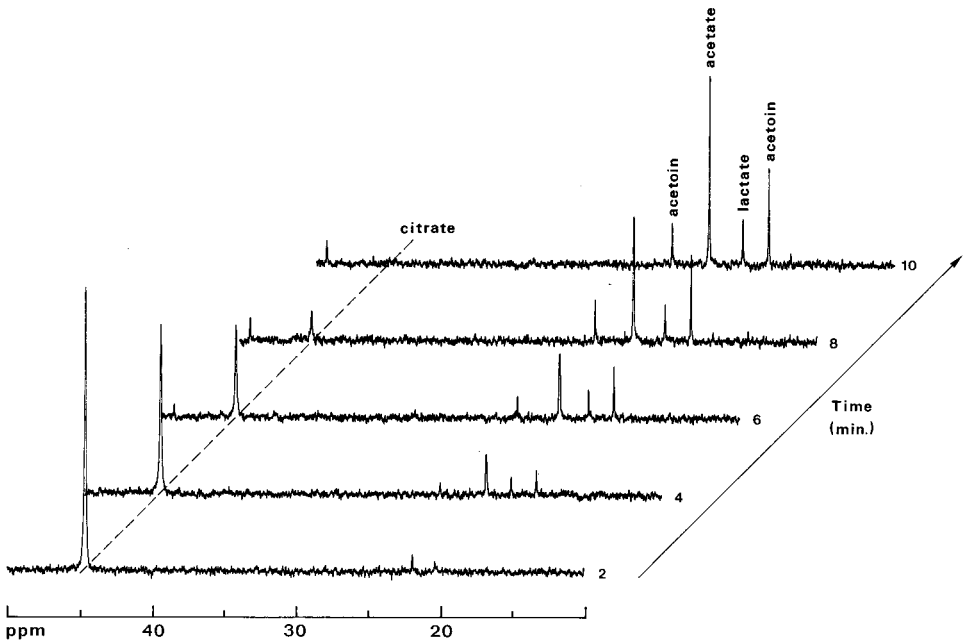

FIG. 6. ^{13}C NMR spectra showing the time course for the consumption of [2,4-^{13}C]citrate (30 μmol, 10 mM final concentration) by a cell suspension (15 g [dry weight] of $cells \cdot liter^{-1}$) of *L. oenos* at 30°C. Each spectrum represents 45 s of accumulation.

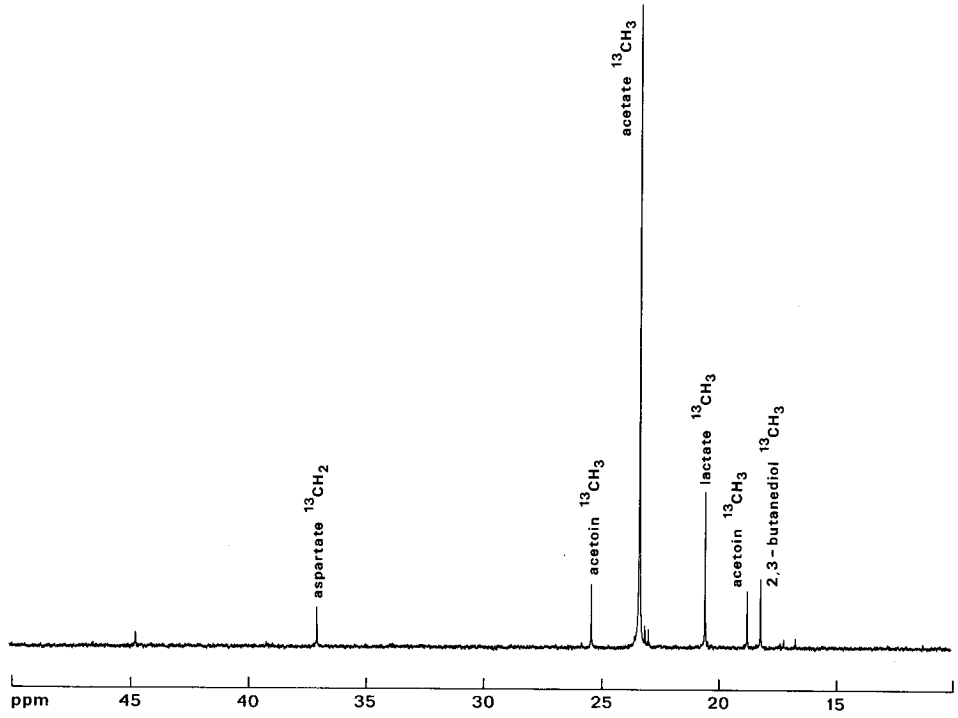

FIG. 7. ^{13}C NMR spectrum of a liquid supernatant obtained after the disruption of a cell suspension of *L. oenos* that was allowed to metabolize 30 μmol of [2,4-^{13}C]citrate.

assigned to aspartate by addition of a small amount of the pure compound.

With the information that part of the label derived from citrate remained in the cell as aspartate, the following procedure was used in order to obtain a more accurate ^{13}C balance: after the complete exhaustion of [2,4-^{13}C]citrate (18 μmol, 6 mM final concentration), cells were broken in a French press and centrifuged at 30,000 × *g* at 4°C for 15 min and the supernatant was collected and analyzed by ^{13}C NMR (Fig. 7). The label from citrate was recovered in the methyl groups of acetate (15 μmol), lactate (5.5 μmol), acetoin (1.6 μmol), and 2,3-butanediol (2.3 μmol) as expected and in the CH_2 group of aspartate (1.6 μmol). A ^{13}C recovery of 86 to 90% was found in this case.

Citrate metabolism as monitored in dilute cell suspensions. The metabolism of citrate (5 mM) by *L. oenos* at pH 3.5, 5.0, and 6.2 was monitored as described in Materials and Methods, and the results are shown in Fig. 8 for two pH values. The end products were acetate, lactate, and acetoin. The relative proportion of these products was 11:1:3 (molar ratio) at pH 3.5 and 10:1:2 at pH 5.0; a significant increase in the production of lactate was observed at pH 6.2, and the molar ratio was 4:1:0.2. The rates of citrate utilization by dilute cell suspensions at the different pH values tested were similar (approximately 160 $\mu mol \cdot min^{-1} \cdot g$ [dry weight] of $cells^{-1}$). Carbon recoveries in these experiments varied between 80 and 110%. However, it has to be taken into account that part of the acetate and lactate produced in these experiments was derived from endogenous reserve compounds and not from the citrate supplied.

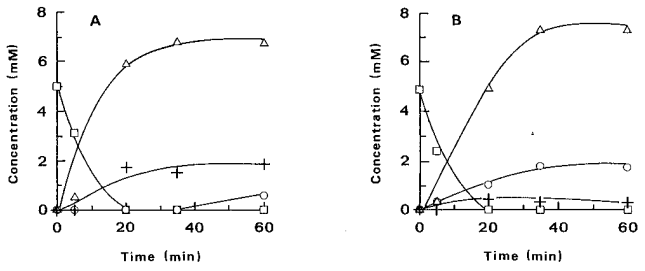

FIG. 8. Time course of citrate (5 mM) consumption and end product production by dilute cell suspensions (3 g [dry weight] of $cells \cdot liter^{-1}$) of *L. oenos* at pH 3.5 (A) or pH 6.2 (B) and 30°C. Substrate and products were determined by ^1H NMR as described in Materials and Methods. Symbols: □, citrate; △, acetate; +, acetoin; ○, lactate.

The metabolism of citrate in the presence of 30 mM L-malate was also studied, and no effect on the rate of citrate utilization or on the pattern of end products from citrate was found (data not shown).

DISCUSSION

The application of NMR coupled to the utilization of ^{13}C-enriched substrates allowed us to establish fermentation balances of citrate and pyruvate in *L. oenos*, to identify aspartate as an unexpected product of citrate metabolism, and to elucidate the metabolic pathway of diacetyl production. The use of isotopic labeled substrates was essential to make correct mass balances, since during the metabolism of labeled citrate these cells produced on average 10 μmol of unlabeled acetate plus $lactate \cdot min^{-1} \cdot g$ (dry weight) of $cells^{-1}$, derived from the metabolism of endogenous carbon reserves. Therefore, it was necessary to be able to distinguish between the amount of end products derived from the added substrates and the amount derived from endogenous compounds.

The results presented here show that the intermediate compound in the synthesis of acetoin and diacetyl is α-acetolactate, since the labeling pattern in those compounds is consistent with derivation from condensation of two molecules of pyru-

vate (Fig. 1). These data for *L. oenos* add information to recent reports on the metabolic pathway of citrate breakdown in *L. lactis* obtained by using either NMR (36, 38) or other analytical approaches (16) which showed that α-acetolactate is also the precursor of acetoin, diacetyl, and 2,3-butanediol in that dairy organism.

In our experiments with *L. oenos*, α-acetolactate was not detected. In contrast, this metabolite was transiently observed when the metabolism of pyruvate or citrate was probed by in vivo NMR in *L. lactis* (25). Comparison of the activities measured for the synthase and decarboxylase of α-acetolactate (Table 1) may shed some light on this question. In *L. oenos*, the decarboxylase activity was approximately 10-fold higher than the synthase activity, which means that, in vivo, the equilibrium is shifted towards the formation of acetoin and that, therefore, accumulation of α-acetolactate is unlikely; in fact, it never occurs to levels detectable by NMR. An inverse situation was found for the activities of the equivalent enzymes in *L. lactis*, i.e., α-acetolactate synthase was approximately 25-fold more active than the decarboxylase (16), explaining the transient accumulation of α-acetolactate observed during in vivo NMR experiments with these cells.

Detection and measurement of diacetyl in a direct way were not reported earlier, and this study shows that this compound is produced, probably by chemical oxidative decarboxylation of α-acetolactate, at physiological pH values (pH 5), provided that oxygen is supplied. Diacetyl was readily detected also in the supernatant solutions obtained in these experiments (Fig. 5). Comparison of the patterns of end products from pyruvate under aerobic-anaerobic conditions (Fig. 5) shows that less lactate is produced under aerobiosis; this observation can be explained by the activity of NAD(P)H oxidases that use oxygen as an electron acceptor, thereby allowing regeneration of $NAD(P)^+$ (35).

The pattern of end products derived from pyruvate was highly dependent on pH, with acetoin production being favored at low pH. A higher conversion of citrate or pyruvate to acetoin at low pH was previously reported in both homofermentative (23, 32) and heterofermentative (7, 10) lactic acid bacteria. The intracellular pH of *L. oenos* is not constant in the extracellular pH range studied (Fig. 4). It has been reported that fermentative organisms, including lactic acid bacteria, do not maintain a constant intracellular pH (17, 22), and this fact was considered to be an adaptive response to low pH values through mechanisms which are not energy consuming (13). The pH profiles of lactate dehydrogenase and of the acetoin-forming enzymes, together with the data on measurements of intracellular pH, suggest that the observed pH-induced metabolic shift is caused by changes in the internal pH that affect the activity of the enzymes responsible for pyruvate conversion. Fitzgerald et al. (11) explained the increased production of acetoin production at low pH in *Leuconostoc mesenteroides* as a result of the decrease in the K_m of lactate dehydrogenase for pyruvate. However, the influence of internal pH changes on the other enzymes of pyruvate metabolism was not taken into account in that study.

Similar patterns of end products were obtained for the metabolism of citrate and pyruvate by *L. oenos* (after correction for acetate resulting from citrate cleavage). However, the rates of consumption differed significantly, the rate of pyruvate utilization being much lower, especially at low pH values (approximately 50-fold lower at pH 4). This was also found in studies with *L. lactis* (25) and is probably due to lack of an efficient transport system for pyruvate in these organisms.

The low ^{13}C recoveries obtained in the experiments in which labeled citrate was supplied, both for *L. lactis* (25) and *L. oenos*, were puzzling since reasonably good recoveries were observed with either pyruvate or glucose. It seemed as though citrate was diverted to the synthesis of cellular components in a step prior to pyruvate formation. During recent studies aiming at the elucidation of citrate transport by *L. oenos* (26), it was observed that approximately 20% of the radioactive label provided in citrate remained inside the cells (25a). Subsequently, it was observed that the internalized labeled compound migrated with the same R_f value as aspartate on thin-layer chromatography (21). By using ^{13}C NMR, aspartate was identified as the product of citrate metabolism that remained inside the cells (Fig. 7). The labeling pattern on aspartate indicates that oxaloacetate formed from citrate is the precursor of aspartate. The conversion of oxaloacetate to aspartate proceeds in bacteria in a single-step reaction catalyzed by a transaminase, and this amino acid is the precursor of other amino acids, e.g., asparagine, methionine, and threonine (14). At this point, it cannot be ruled out that in *L. oenos* aspartate is used to synthesize amino acids, leading to dilution of the label in other cellular components, such as proteins, not detected by NMR. Interestingly, in a recent study concerning the variation of nutritional requirements of *L. oenos*, it was reported that aspartate was no longer an essential amino acid for growth provided that citrate and malate were added to the growth medium (1). Our data show that at least 10% of the citrate supplied was converted to aspartate that was not excreted to the extracellular medium.

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