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Decarboxylation of α-Keto Acids by *Streptococcus lactis* var. *maltigenes*[1]

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Decarboxylation rates for a series of C-3 to C-6 α-keto acids were determined in the presence of resting cells and cell-free extracts of *Streptococcus lactis* var. *maltigenes*. The C-5 and C-6 acids branched at the penultimate carbon atom were converted most rapidly to the respective aldehydes in the manner described for α-carboxylases. Pyruvate and α-ketobutyrate did not behave as α-carboxylase substrates, in that O_2 was absorbed when they were reacted with resting cells. The same effect with pyruvate was noted in a nonmalty *S. lactis*, accounting for CO_2 produced by some "homofermentative" streptococci. Mixed substrate reactions indicated that the same enzyme was responsible for decarboxylation of α-keto-isocaproate and α-ketoisovalerate, but it appeared unlikely that this enzyme was responsible for the decarboxylation of pyruvate. Ultrasonic disruption of cells of the malty culture resulted in an extract inactive for decarboxylation of pyruvate in the absence of ferricyanide. Dialyzed cell-free extracts were inactive against all keto acids and could not be reactivated.

Streptococcus lactis var. *maltigenes* (2) produces 3-methylbutanal, the principal compound responsible for the malty defect in milk (8). In the presence of resting cells of this organism, leucine is transaminated and the α-ketoisocaproic acid formed is decarboxylated to the aldehyde, part of which is reduced to the alcohol (16). Certain other amino acids and α-keto acids react similarly in the presence of this organism, but only traces of the respective aldehydes are produced by *S. lactis* and *S. cremoris* (13, 15). A taxonomic study revealed that the ability to decarboxylate such α-keto acids was the most significant characteristic in differentiating *S. lactis* var. *maltigenes* from other lactic streptococci (5). An earlier suggestion, that the decarboxylation mechanism in this organism resembles that present in yeast (14), prompted the present attempt to characterize in greater detail the enzyme(s) involved.

MATERIALS AND METHODS

Organisms. S. lactis var. *maltigenes* M_1 and *S. lactis* L_2 maintained as frozen skim-milk cultures were employed.

Cell crops. Cells were obtained in quantity by growth in biphasic cultures (23). Trypticase Soy Broth (BBL) was used for both phases, and the stationary layer was solidified with 2% agar. Active skim-milk culture (0.4%) was used as inoculum, and incubation was at 21 to 25 C for 20 hr. The liquid layer was strained through gauze and centrifuged. Collected cells were washed twice with water.

Cell concentration was determined turbidimetrically at 540 mμ. Nitrogen content of both resting cells and cell-free extracts was determined by digestion and nesslerization, and was correlated with dry weight in the case of resting cells and protein concentration (25) in the case of cell-free extracts.

Cell-free extracts. Cell-free extracts were prepared by treating suspensions [1.5 g (dry weight) of cells in 25 ml of 0.067 M phosphate buffer, *p*H 6.0] with a Branson Sonifier (model S-75) for 2.5-min periods at 15 C. Disruption of 85 to 90% of the cells was obtained after 12 to 15 min (1).

After ultrasonic treatment, the suspensions were centrifuged at room temperature for 15 min at 30,000 × *g*, and the supernatant fluid was decanted. Such cell-free extracts contained an average of 34.0 mg of protein per ml. Extract not used immediately was held frozen until needed.

As required, 10-ml samples of cell-free extract were placed in cellulose casings (Union Carbide Corp., Food Products Div., Chicago, Ill.) which had been boiled in 1.0 mM ethylenediaminetetraacetic acid for 5 min and rinsed thoroughly with distilled water (24). In different trials, the extracts were dialyzed against 0.067 M phosphate at *p*H 6.0 or 7.0 and 0.1 M phosphate at *p*H 7.0 or 8.0. Also used were polyvinyl-

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pyrrolidone (Plasdone; General Aniline & Film Corp., New York. N.Y.) at a concentration of 37.5% (w/v) (11) in 0.01 M phosphate buffer (*p*H 8.0) and 0.01 M cysteine in the same buffer but freed from O_2 by sparging with N_2 (19). Dialyses were conducted at 4 C; when 0.01 M cysteine was employed, the extract was frozen in the casing prior to dialysis and allowed to thaw during dialysis.

Manometric methods. Decarboxylation of α-keto acids was investigated by conventional manometric techniques (24). Reaction rates are expressed as Q_{CO_2} and Q_{O_2}, the microliters of CO_2 and O_2 exchanged per milligram of dry weight per hour, or as Q_{CO_2} (N) and Q_{O_2} (N), the microliters of CO_2 and O_2 exchanged per milligram of nitrogen per hour. All rates were measured in an atmosphere of air, and corrections were made for endogenous values.

Analyses of reaction products. At the conclusion of every manometric experiment, the reaction mixtures were analyzed for the presence of acid and neutral carbonyl compounds (3, 7). The volatile products in the headspace over the reaction mixture, resulting from decarboxylation of α-ketoisocaproic acid by resting cells of *S. lactis* var. *maltigenes*, were identified by gas-liquid chromatography (16). Products from the reaction of pyruvate with resting cells were assayed for the presence of acetate (4), acetyl phosphate (12), and acetoin and biacetyl (17).

RESULTS

In a typical experiment with resting-cell suspensions (Table 1), about 0.5 mole of O_2 was utilized per mole of CO_2 evolved from pyruvate and α-ketobutyrate. The amount of O_2 utilized during decarboxylation of α-ketoisocaproate, α-ketoisovalerate, and α-ketovalerate was negligible. The decarboxylation of α-keto-β-methylvalerate and α-ketocaproate (Fig. 1) also did not involve O_2. To be certain that oxidative decarboxylation was not the result of a contaminant, isolates from the malty culture were tested for production of malty aroma in skim-milk culture. Cells of these isolates decarboxylated both α-ketoisocaproate and pyruvate (Table 2). A nonmalty *S. lactis* (L-2) culture was included in this experiment. The decarboxylation rate of α-ketoisocaproate by resting cells of L-2 was only 18 to 30% that of the malty strains, but pyruvate decarboxylation by L-2 was 69 to 82% that of the malty strains.

TABLE 1. *Comparison of decarboxylation rates of α-keto acids by resting cells of Streptococcus lactis var. maltigenes*[a]

α-Keto acid	Q_{CO_2}	Q_{O_2}
Isocaproic	6.4	—
Isovaleric	4.2	—
Valeric	1.9	—
Butyric	6.2	3.2
Pyruvic	11.3	6.4

[a] Each flask contained: 0.0025 N neutralized substrate, 0.5 ml of resting cells [9.8 mg (dry weight) per flask], 1.0 ml of McIlvaine's buffer (*p*H 4.6), and 1.0 ml of water; total volume of reaction mixture was 3.0 ml; 0.2 ml of water or 20% KOH was added to the center well as required. Reaction time was 60 min; temperature, 30 C.

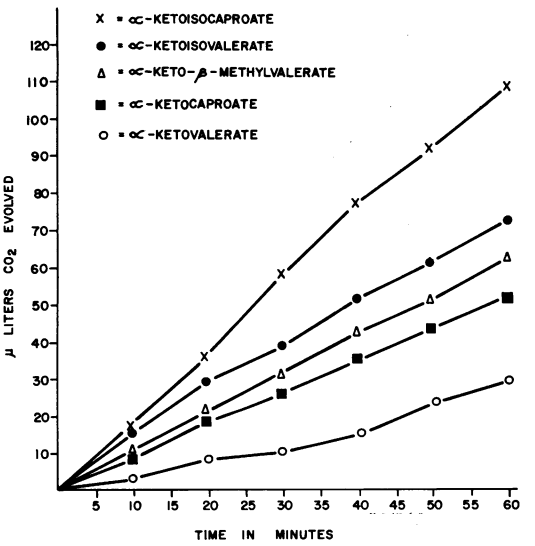

FIG. 1. *Rates of decarboxylation of α-keto acids by Streptococcus lactis var. maltigenes resting cells. Each flask contained 0.0025 N neutralized keto acid, 0.5 ml of resting cells [12.5 mg (dry weight) per flask], 1.0 ml of McIlvaine's buffer (pH 5.2), and 1.0 ml of water. Total reaction volume was 3.0 ml, and temperature was 30 C.*

The enzyme responsible for decarboxylation of α-ketoisocaproate in both resting cells and cell-free extracts was most active in the *p*H range of 5.2 to 5.8. Resting-cell preparations were active over a broader range. The optimal *p*H for the decarboxylation of pyruvate was 4.0 to 4.6 with resting cells, whereas oxidation, which was measured simultaneously, was optimal at *p*H 5.2. Evolution of CO_2 from pyruvate in the presence of cell-free extracts and $K_3Fe(CN)_6$ was most rapid at *p*H 5.8.

Decarboxylation of α-ketoisocaproate by cell suspensions was most rapid at 50 C, but, when cell extracts were the enzyme source, decarboxylation was most rapid at 40 C. Pyruvate decarboxylation, tested only with resting-cell preparations, was most active at 45 C. Oxygen uptake increased proportionately with CO_2 evolution at 25 to 35 C and then remained constant until 45 C. Both oxidation and decarboxylation ceased at 50 C. Although optimal temperatures for these reactions were somewhat higher, a temperature of 30

TABLE 2. *Comparison of decarboxylation rates by resting cells of isolates from Streptococcus lactis var. maltigenes culture M-1 and nonmalty S. lactis L-2*[a]

α-Keto acid	Culture	Malty aroma in milk culture	Q_{CO_2}
Isocaproic acid	L-2	−	3.2
	M-1	+	10.6
	M-1-1[b]	+	12.9
	M-1-2	+	14.8
	M-1-3	+	17.5
	M-1-4	+	10.9
Pyruvic acid	L-2	−	8.8
	M-1	+	11.8
	M-1-1	+	12.8
	M-1-2	+	12.7
	M-1-3	+	11.8
	M-1-4	+	10.7

[a] Each flask contained 13.2 mg (dry weight) of cells; *p*H of reaction mixture, 5.2. See Table 1 for other experimental details.
[b] Single colony isolate.

TABLE 3. *Effect of substrate concentration on decarboxylation of α-keto acids by Streptococcus lactis var. maltigenes*[a]

α-Keto acid	Substrate concn	Resting cells (mg per flask)	Q_{CO_2}	Q_{O_2}
	N			
Isocaproic	2.5×10^{-3}	18.8	10.6	—
	1.0×10^{-2}	18.8	10.0	—
	2.0×10^{-2}	18.8	10.8	—
	4.0×10^{-2}	18.8	9.9	—
Caproic	2.5×10^{-3}	18.8	3.5	—
	2.0×10^{-2}	18.8	4.4	—
Isovaleric	2.5×10^{-3}	13.5	11.4	—
	2.0×10^{-2}	13.5	11.5	—
Pyruvic	1.0×10^{-3}	29.5	4.4	2.8
	2.5×10^{-3}	29.5	9.2	6.4
	1.0×10^{-2}	29.5	20.6	6.4
	2.0×10^{-2}	29.5	33.1	6.6

[a] The *p*H of the reaction mixture was 5.2. See Table 1 for other experimental details.

C was used throughout this study to permit better comparison with experiments reported by others (6, 9, 10, 21)

Decarboxylation rates for several α-keto acids in the presence of resting cells are given in Table 3. With the exception of those for pyruvic acid, the decarboxylation rates were independent of the concentrations employed, indicating that substrate was present in excess of enzyme capacity. A little more than 0.5 mole of O_2 was consumed per mole of CO_2 evolved when concentrations of pyruvate were 0.0025 N or less, but the oxidation rate did not increase at higher pyruvate concentrations.

TABLE 4. *Effect of enzyme concentration on rate of decarboxylation of α-ketoisocaproic acid by Streptococcus lactis var. maltigenes*[a]

Prepn	Concn (mg of N per flask)	$Q_{CO_2}(N)$
Resting cells	0.37	79.3
	0.74	82.0
	1.84	82.8
	3.68	84.2
Cell-free extracts	0.32	84.5
	0.64	72.0
	1.60	68.0
	3.20	69.3

[a] The *p*H of the reaction mixtures was 5.2. See Table 1 for other experimental details.

Reaction rates were nearly proportional to resting-cell and cell-free extract concentrations when different concentrations of resting cells and cell-free extracts were tested for their effect on α-ketoisocaproate decarboxylation (Table 4).

Figure 1 illustrates the course of decarboxylation of those α-keto acids not utilizing O_2 in the presence of resting cells. Under the conditions used, the most reactive substrate was α-ketoisocaproate, followed by α-ketoisovalerate. In replicate experiments, the longer-chain acid was usually the more reactive, and, in those experiments in which a more rapid reaction with α-ketoisovalerate occurred, the difference was small. The unbranched acids were less reactive, and rates slowed as the chain length decreased.

A similar experiment with cell-free extracts is illustrated in Fig. 2. Ferricyanide was not included in any of the reaction mixtures, and, as a result, pyruvate decarboxylation was reduced considerably (*see* Table 7). The respiratory quotient (RQ) under these conditions was only slightly different from that found with resting-cell preparations. In the presence of cell-free extracts, α-ketoisovalerate was always the most reactive substrate, followed by α-ketoisocaproate. Shorter, unbranched acids again were less reactive.

Paper chromatography of the 2,4-dinitrophenylhydrazones prepared from reaction mixtures indicated formation of the expected aldehyde from all substrates with the exception of pyruvate, and only a small amount of propionaldehyde resulted from α-ketobutyrate decarboxylation. Acetoin, biacetyl, acetate, and acetyl phosphate were not detected after decarboxylation of 0.0025 N pyruvate in the presence of resting cells. Positive reactions for acetoin and biacetyl and a negative reaction for acetate were obtained

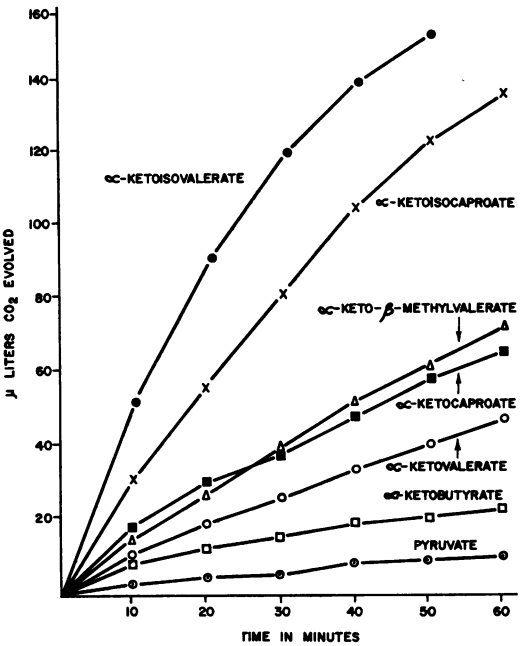

FIG. 2. *Rates of decarboxylation of α-keto acids by cell-free extracts of Streptococcus lactis var. maltigenes. Each flask contained 0.0025 N neutralized keto acid, 0.5 ml of cell-free extract, (2.9 mg of N per flask), 1.0 ml of McIlvaine's buffer, (pH 5.2), and 1.0 ml of water. Total reaction volume was 3.0 ml, and temperature was 30 C.*

after reaction with 0.02 N pyruvate. Gas-liquid chromatography of headspace volatiles from the α-ketoisocaproate reaction with resting cells revealed the presence of 3-methylbutanol as well as 3-methylbutanal.

Results of mixed substrate reactions (Table 5) indicated little difference in reaction rates when α-ketoisocaproate and α-ketoisovalerate were decarboxylated separately or together. Simultaneous decarboxylation of α-ketoisocaproate and pyruvate occurred at a rate nearly double that of the single substrates. The oxidation rate during simultaneous decarboxylation of pyruvate and α-ketoisocaproate was little more than half that obtained with pyruvate alone.

In the presence of cyanide (Table 6), the rate of pyruvate decarboxylation was only two-thirds that of the control, but oxidation was not affected. Since pyruvate forms the cyanohydrin under these conditions, the effective concentration would be lowered, resulting in a curtailed decarboxylation rate. Both decarboxylation and oxidation were restricted in the presence of arsenite. Dimedone caused an increase in the decarboxylation rate with a concomitant decrease in oxidation.

TABLE 5. *Decarboxylation reactions with mixed substrates in the presence of resting cells of Streptococcus lactis var. maltigenes*[a]

α-Keto acid[b]	Q_{O_2}	Q_{CO_2}	RQ
Pyruvic	6.7	11.6	1.73
Isocaproic	—	10.6	—
Isovaleric	—	11.4	—
Pyruvic + isocaproic	3.7	19.0	5.13
Isocaproic + isovaleric	—	11.0	—

[a] Each flask contained 13.8 mg (dry weight) of cells, and the *p*H of the reaction mixture was 5.2. See Table 1 for other experimental details.

[b] The substrate concentration for each of the acids was 2.5×10^{-3} N.

TABLE 6. *Decarboxylation of pyruvate by resting cells of Streptococcus lactis var. maltigenes in the presence of inhibitors or dimedone*[a]

Pyruvate Concn	Agent present	Concn	Q_{CO_2}	Q_{O_2}
N				
2.5×10^{-3}	None	—	10.3	5.0
2.0×10^{-2}	None	—	32.0	6.50
2.0×10^{-2}	NaCN	5×10^{-3} M	22.3	7.60
2.0×10^{-2}	$NaAsO_2$	2×10^{-4} M	20.2	1.45
2.0×10^{-2}	Dimedone	Saturated	44.1	2.80

[a] Each flask contained 14.8 mg (dry weight) of cells; *p*H of reaction mixture, 5.2. See Table 1 for other experimental details.

A comparison of activity at several stages in the preparation of cell-free extracts is given in Table 7. Reaction rates for extracts were based on the dry weight of resting cells corrected for efficiency of cell disruption (90%) and volume loss resulting from separation of cells and debris from the supernatant fluid. A decrease of about 70% in both decarboxylation and oxidation of pyruvate resulted from ultrasonic treatment, and an additional 7% was lost during centrifugation. Oxidation and decarboxylation of pyruvate were not increased by addition of lipoic acid and Mg^{++} and thiamine pyrophosphate (TPP) to the reaction mixture, but $K_3Fe(CN)_6$ completely restored decarboxylation. Yeast extract or heat-inactivated *S. lactis* var. *maltigenes* cells did not restore activity to the extracts. Dialysis resulted in complete inactivation of cell-free extracts for all substrates and activity was not restored by addition of any of the above factors. Addition of freeze-dried diffusate and combinations of dialyzed and undialyzed extract did not restore lost activity. Even addition of the previously mentioned substances to an extract which had been dialyzed

TABLE 7. *Comparison of decarboxylation rates of pyruvic and α-ketoisocaproic acid by several enzyme preparations from Streptococcus lactis var. maltigenes*[a]

α-Keto acid	Enzyme source	Q_{CO_2}	Q_{O_2}	RQ
Isocaproic	Resting cells	8.75	—	—
	Disrupted mixture	7.15	—	—
	Cell-free extract	5.95	—	—
Pyruvic	Resting cells	9.30	6.10	1.53
	Disrupted mixture	3.50	2.04	1.70
	Cell-free extract	2.08	1.44	1.44
	Cell-free extract + lipoic acid, TPP, Mg^{++}[b]	2.18	1.31	1.67
	Cell-free extract + $K_3Fe(CN)_6$, TPP[c]	10.40	—	—

[a] Each flask contained 28.5 mg (dry weight) of cells or equivalent extract; *p*H of reaction mixture, 5.2; reaction time, 30 min. See Table 1 for other experimental details.

[b] Lipoic acid, 1.0 μmole; TPP, 1.0 μmole; $MgSO_4$, 10 μmoles.

[c] $K_3Fe(CN)_6$, 25 μmoles; TPP, 1.0 μmole.

TABLE 8. *Stability of cell-free extracts from Streptococcus lactis var. maltigenes*[a]

α-Keto acid	Storage temp	Storage time	$K_3Fe(CN)_6$[b]	Q_{CO_2} (N)	Percentage of original activity
	C	*days*			
Pyruvic	—	0	Present	77.8	100.0
	—	0	Absent	16.0	20.3
	4	1	Present	49.0	63.0
	21–25	1	Present	42.4	54.5
	−18	1	Present	73.0	93.9
Isocaproic	—	0	Absent	74.5	100.0
	4	1	Absent	35.8	48.2
	21–25	1	Absent	32.4	43.5
	−18	7	Absent	48.0	64.5

[a] Extracts equivalent to 2.5 mg of N were added to each flask; *p*H of reaction mixture, 5.2. See Table 1 for other experimental details.

[b] $K_3Fe(CN)_6$, 25 μmoles.

long enough to lose only one half of its activity resulted in α-ketoisocaproate decarboxylation at the rate expected for the one-half inactivated enzyme.

Decarboxylase activity for both pyruvate and α-ketoisocaproate was best preserved by freezing the extract. A summary of the effect of storage conditions is given in Table 8. The same extract was evaluated under all conditions. By handling the cell-free extract expeditiously, it was possible to obtain a preparation with about 30% greater activity for α-ketoisocaproate than that reported in Table 8.

DISCUSSION

The metabolism of α-keto acids in *S. lactis* var. *maltigenes* apparently is different from that reported for other microorganisms. Suomalainen and Oura (22) found that, in intact bakers' and brewer's yeast, low concentrations of straight-chain keto acids were decarboxylated at a rate which increased with the chain length of the acid, but that α-ketoisovalerate was less reactive than the straight-chain acids. Juni (9) found that cell-free extracts of bakers' yeast were most reactive for pyruvate, that activity decreased with increasing chain length, and that α-ketoisovalerate was less reactive than α-ketovalerate. Studies with purified yeast carboxylase (6) indicated comparable reaction rates for pyruvate and α-ketoisovalerate, but relative inactivity for α-ketoisocaproate. Oxidation did not occur during decarboxylation of any of the keto acids tested. King and Cheldelin (10) found that *Acetobacter suboxidans* possessed a yeast type of pyruvate decarboxylase and that the acetaldehyde formed was oxidized to acetate. Decarboxylation was not observed with α-ketoisovalerate and α-ketoisocaproate.

With *S. lactis* var. *maltigenes*, oxygen or a suitable electron acceptor is required for decarboxylation of pyruvate and α-ketobutyrate. Of the remaining keto acids tested, no oxygen was required and a preference was indicated for for the longer-chain acids branched at the penultimate carbon atom. Decarboxylation of α-ketoisocaproate and α-ketoisovalerate at the same rate, when present individually or simultaneously at saturation levels, would be expected if a common enzyme is involved. Simultaneous decarboxylation of α-ketoisocaproate and pyruvate occurred at nearly double the rate for the individual substrates. Although saturation levels for pyruvate were not attained at the concentrations employed, pyruvate at 10^{-2} N was required to give a Q_{CO_2} of 20.6 (Table 3). This concentration is twice that of combined pyruvate and α-ketoisocaproate, which resulted in a Q_{CO_2} of 19.0 (Table 5). On this basis, it must be concluded that the rate of the mixed substrate reaction was too rapid to be accounted for by a single enzyme. Inability to attain saturation levels of pyruvate could have been due to the activity of more than one CO_2-yielding reaction. Presence of acetoin and biacetyl in reaction mixtures from 0.02 N pyruvate suggests carboligase activity (18), and at this level of pyruvate some of the O_2 consumed was due to the conversion of acetoin to biacetyl.

Production of biacetyl agrees with an earlier report (5) that some isolates of malty and nonmalty cultures are Voges-Proskauer-positive. These reactions with pyruvate help clarify the previously unknown route of CO_2 formation in "homofermentative" streptococci (20).

Although acetaldehyde was not detected as one of the reaction products of pyruvate decarboxylation, its presence was indicated by a decrease in oxidation and an increase in CO_2 evolution in the presence of an aldehyde trapping agent. That oxidation did not increase in resting cells at pyruvate concentrations above 0.0025 N suggests the presence of an inhibitory intermediate which does not accumulate when ferricyanide serves as an electron acceptor with cell-free extracts. Arsenite inhibition indicates that lipoic acid may be involved in the reaction, but attempts to restore activity to cell-free extracts by addition of this cofactor were unsuccessful. Since arsenite is not a specific inhibitor for lipoic acid, but rather for compounds having adjacent sulfur atoms, a different cofactor may be involved, or the requirement may be for protein-bound lipoic acid (L. Reed and M. Koike, Federation Proc. **20**:238, 1961) which the extracts might not be able to synthesize. Failure of cyanide to inhibit oxidation indicates that a cytochrome system is not involved in the reaction.

Although the amount of 3-methylbutanal produced by nonmalty L-2 cells is considerable, the conditions employed were nearly ideal for decarboxylase activity and probably were much different from those prevailing in cultures during natural growth in milk. Detection of 3-methylbutanol as a product of α-ketoisocaproate metabolism is not surprising, as appreciable amounts of this alcohol were detected in milk cultures of the malty strain by Morgan et al. (16). Since the ultimate fate of α-ketoisocaproate is the corresponding alcohol as well as the aldehyde, a coupled reaction could occur when α-ketoisocaproate and pyruvate are decarboxylated simultaneously, thereby accounting for the decrease in O_2 uptake in the mixed substrate reaction. As observed in this and other laboratories, malty cultures generally grow faster, produce more acid, and attain higher cell numbers than nonmalty cultures. Since the malty aroma in milk cultures can usually be detected before any marked increase in lactic acid occurs, the greater vitality of such cultures may be due, in part, to the stimulation provided by early production of CO_2 (26) from the decarboxylation of α-keto acids or regeneration of nicotinamide adenine dinucleotide via the reduction of the resulting aldehydes to alcohols (21), or both.

Importance of the cell wall and membrane as thermal and ionic barriers was evidenced by enzyme activity of resting cells at temperatures which inactivate cell-free extracts and by activity over a broader *p*H range in resting cells. Disruption and removal of these structures were detrimental to pyruvate metabolism, which suggests that they may be the site of oxidative activity. Solubility differences at the lipid cell membrane (22) account for decarboxylation of α-ketoisocaproate at a more rapid rate than α-ketoisovalerate in resting cells. Failure to restore decarboxylase activity to dialyzed cell-free extracts indicates denaturation during dissociation of the conjugated enzyme. A similar effect has been noted on dialysis of yeast carboxylase (6).

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