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Metabolism of the Anaerobic Formation of Succinic Acid by *Saccharomyces cerevisiae*

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Abstract. 1. Succinic acid is formed in amounts of 0.2–1.7 g/l by fermenting yeasts of the genus *Saccharomyces* during the exponential growth phase. No differences were observed between the various species, respiratory deficient mutants and wild type strains.

2. At low glucose concentrations the formation of succinic acid depended on the amount of sugar fermented. However, the nitrogen source was found to be of greater importance than the carbon source.

3. Of all nitrogen sources, glutamate yielded the highest amounts of succinic acid. Glutamate led to an oxidative and aspartate to a reductive formation of succinic acid.

4. A reductive formation of succinic acid by the citric acid cycle enzymes was observed with malate. This was partially inhibited by malonate. No evidence was obtained that the glyoxylate cycle is involved in succinic acid formation by yeasts.

5. Anaerobically grown cells of *Saccharomyces cerevisiae* contained α-ketoglutarate dehydrogenase. Its activity was found in the 175 000 × g sediment after fractionated centrifugation. The specific activity increased 6-fold after growth on glutamate as compared with cells grown on ammonium sulfate.

6. The specific activities of malate dehydrogenase, fumarase, succinate dehydrogenase, succinyl-coenzyme A synthetase, α-ketoglutarate dehydrogenase and glutamate dehydrogenase (nicotinamide adenine dinucleotide dependent) were determined in yeast cells grown on glutamate or ammonium sulfate. Similar results were obtained with a wild type strain and a respiratory deficient mutant. The latter did not contain succinate dehydrogenase.

7. In fermenting yeasts succinic acid is mainly formed from glutamate by oxidation.

Key words: Succinic acid – Fermentation – *Saccharomyces cerevisiae* – α-ketoglutarate dehydrogenase.

Succinic acid is a regular by-product in the alcoholic fermentation of yeasts. It is formed in amounts of a few mg and up to 2 g/l in all products of fermentation (Whiting, 1976). It is the main acid produced by yeast.

Succinic acid is produced at the beginning of fermentation (Thoukis et al., 1965). The formation of succinic acid depends on the yeast strain (Castino, 1970) and varies with the composition of the nitrogen source as has been reported several times (Ribéreau-Gayon et al., 1959). Coote and Kirsop (1974) investigated the favourable action of glutamate, aspartate, asparagine and serine on the formation of succinic acid. Investigations of the influence of the metabolites of the citric acid cycle yielded different results. Mayer et al. (1964) found a formation of succinic acid from malic acid, whereas no influence of malic, fumaric and citric acids was reported by Wagener et al. (1971).

The exact mechanism of the formation of succinic acid during fermentation is not known. Several possibilities are being discussed. A reductive formation from glucose is assumed by Whiting (1976) and an oxidative formation by Oura (1976). Amino acids are regarded as precursors; they are assumed to be deaminated to either α-ketoglutaric acid (Wagener et al., 1971) or oxaloacetic acid (Sols et al., 1971; Oura, 1977) which are converted to succinic acid via enzymes of the citric acid cycle. Although no investigations of the different metabolic pathways have been reported, it can be assumed that succinic acid is formed by the same mechanism as α-ketoglutarate which is dependent on glutamate (Lewis and Rainbow, 1963; Lafon-Lafourcade and Peynaud, 1966).

Abbreviations. CoA = coenzyme A; EDTA = ethylenediaminetetraacetic acid; MTE = mannitol-Tris-SO_4-EDTA; TPP = thiamine pyrophosphate; YEP = yeast extract peptone

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Chapman and Bartley (1968) investigated the enzymes of the citric acid cycle of aerobically and anaerobically grown *Saccharomyces cerevisiae*. They found no α-ketoglutarate dehydrogenase in anaerobically cultivated cells. Therefore it was assumed that the citric acid cycle functions in a non cyclic manner under anaerobic conditions. As the oxidative pathway leads to α-ketoglutarate only, succinic acid should be formed by reduction.

In this paper the possible metabolic pathways leading to succinic acid by *Saccharomyces cerevisiae* were investigated during fermentation. As α-ketoglutarate dehydrogenase is apparently the key enzyme, it was attempted to demonstrate its presence in anaerobically cultivated cells.

Materials and Methods

Cultures. Various strains of the genus *Saccharomyces*, isolated from musts and wines, were used. The strains were from the collection of this institute.

Culture Conditions. The B-medium (glucose 100 g, $(NH_4)_2SO_4$ 1.5 g, KH_2PO_4 1.0 g, $MgSO_4 \times 7H_2O$ 1 g, $CaCl_2$ 0.5 g, inositol 40 mg, p-aminobenzoic acid 0.2 mg, biotin 0.02 mg, folic acid 0.02 g, niacin 1 mg, pantothenic acid 1 mg, pyridoxine hydrochloride 1 mg, riboflavin 0.5 mg, thiamine hydrochloride 0.5 mg, H_3BO_3 2 mg, $FeCl_3$ 2 mg, $ZnSO_4 \times 7H_2O$ 2 mg, $MnSO_4 \times 1H_2O$ 2 mg, KJ 1 mg, $CuSO_4 \times 5H_2O$ 1 mg, $MoO_4 \times 2H_2O$ 1 mg, $CoCl_2$ 1 mg, $LiSO_4 \times 2H_2O$ 1 mg, potassium hydrogen tartrate 20 mg, L-alanine 75 mg, L-arginine 350 mg, L-histidine 20 mg, L-methionine 40 mg, L-serine 50 mg, L-threonine 200 mg, L-tryptophane 40 mg, L-aspartic acid 50 mg, L-glutamic acid 300 mg, aqua dest ad 1 l, pH 3.3, total nitrogen: 500 mg/l) was used for growth and fermentation experiments. YEP-medium (yeast extract 3 g, peptone 10 g, KH_2PO_4 1 g, glucose 100 g, aqua dest ad 1 l pH 3.3) was used to cultivate cells for enzymatic investigations.

Fermentation Experiments. Yeast cells were cultivated in 200-ml-Erlenmeyer flasks with fermentation closures containing 150 ml medium at 25° C on a circular shaker (150 r.p.m.) until the CO_2-production ceased. For growth and enzymatic experiments 3-l-Erlenmeyer flasks with fermentation closures containing 2 l medium were used. The medium was sparged with O_2-free nitrogen gas for 15 min before and after inoculation. A 10% inoculum of cells grown anaerobically for 24 h was used.

Enzyme Preparation. The cells were collected by centrifugation at the end of the exponential growth phase. They were washed twice in potassium phosphate buffer pH 7.4, and suspended in MTE-buffer (mannitol 0.25 M, Tris-SO_4 20 mM pH 7.4, EDTA 1 mM). To prevent anaerobic induction of enzymes, cycloheximide (10 µg/ml) was added to all buffers immediately before harvesting the cells (Watson et al., 1970). The suspension was shaken in a refrigerated ball mill Braun MSK with glass beads (0.45–0.5 mm diameter) and prepared as described in "Results". After homogenisation all procedures were carried out at 4° C.

Analytical Determinations. Protein was determined using the Biuret method as described by La Rivière (1958). α-ketoglutaric and L-malic acids were determined by the enzymatic methods of Bergmeyer and Bernt (1970) and Hohorst (1970) respectively. The amino acids were determined by ion exchange chromatography (Weiller and Radler, 1976). Succinic, fumaric and malonic acids were determined by gaschromatography. The method was a modification of that used by Harmon and Doelle (1969). The 0.5 ml samples were adjusted to pH 1. They were lyophilized and esterified with 2 ml of 20% BF_3-methanol by shaking at 28° C over night. After hydrolysis of the BF_3-methanol complex, the esters were extracted twice with 1 ml of chloroform. The combined extracts were dried over anhydrous sodium sulfate. For analysis a gaschromatograph Varian type 1520B was used, stainless steel column 3 m length, 2 mm i.d., 5% DEGS on Chromosorb W/AW 60/80 mesh; injector 200° C, detector 220° C, oven 120° C; carrier gas: N_2 30 ml/min; H_2 30 ml/min, air 300 ml/min, sample 3 µl. Quantitative calculation with the computing integrator Autolab.

Enzyme Determinations. Specific activities are expressed in µmol substrate converted per mg protein and minute.

Determination of L-malate dehydrogenase (E.C. 1.1.1.37) according to Ochoa (1955), fumarase (E.C. 4.2.1.2) according to Hill and Bradshaw (1969) at 240 nm by spectrophotometric measurement, NAD-dependent glutamate dehydrogenase (E.C. 1.4.1.4) according to Doherty (1970), succinate dehydrogenase (E.C. 1.3.99.1) according to King (1963) with phenazine methosulfate and 2,6-dichlorophenolindophenol; GTP dependent succinyl-CoA synthetase (E.C. 6.2.1.4) according to Cha and Parks (1964), α-ketoglutarate dehydrogenase complex according to Holzer et al. (1963) using Sörensen phosphate buffer pH 7.5.

Chemicals. All enzymes, co-enzymes and oxaloacetic acid were purchased from Boehringer-Mannheim. DEGS and Chromosorb W/AW 60–80 mesh were obtained from Varian. All the other chemicals were purchased from Merck-Darmstadt.

Results

Formation of Succinic Acid by Growing Yeasts

Succinic Acid Production by Various Species of Saccharomyces. After using several media it was found that large amounts of succinic acid were produced in a synthetic medium with glutamate as the sole nitrogen source. Yeasts of the species *Saccharomyces cerevisiae*, *S. uvarum*, *S. rouxii*, *S. chevalieri*, *S. elegans*, *S. fructuum*, *S. ludwigii*, *S. rosei*, *S. oviformis* of which a total of 99 strains were investigated, formed 0.21–1.72 g/l succinic acid (Fig. 1). No significant differences were observed between *Saccharomyces cerevisiae* and the strains of other species. Similar amounts of succinic acid were produced by respiratory deficient strains and the corresponding wild type strains. *S. cerevisiae* strain 43 was used for the further investigations because it formed the largest amounts of succinic acid. Growth experiments showed that the formation of succinic acid occurs during the exponential growth phase only.

Influence of Various Nitrogen Compounds and Their Concentration on the Formation of Succinic Acid. Using non limiting amounts of 500 mg N/l, the influence of 21 amino acids and ammonium sulfate as sole nitrogen source on the formation of succinic acid by *Saccharomyces cerevisiae* strain 43 was investigated (Table 1). The amino acids promote growth of yeasts

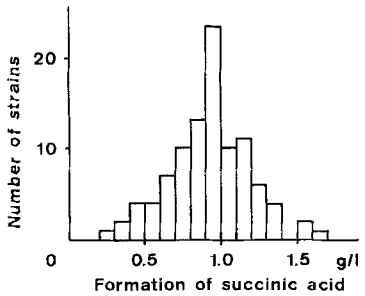

Fig. 1. Formation of succinic acid by yeasts of the genus *Saccharomyces* during fermentation. 150 ml B-medium containing 5.25 g glutamate per l, in Erlenmeyer flasks with fermentation closures, 25° C, on a circular shaker (150 r.p.m.). The fermentation was measured by determining the CO_2 production by weighing the fermentation flasks

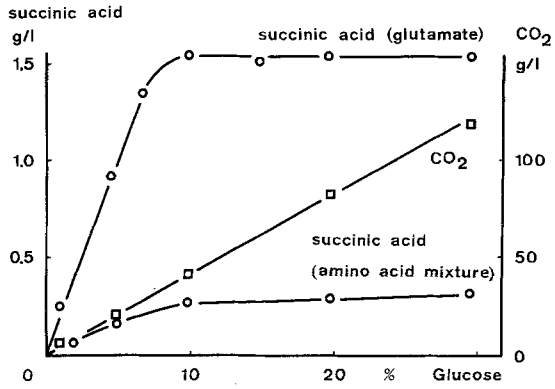

Fig. 2. The influence of glucose concentration on the formation of succinic acid by *Saccharomyces cerevisiae* strain 43. B-medium. Nitrogen source: glutamate or amino acid mixture. Experimental procedure as in Figure 1

Table 1. Formation of succinic acid by *Saccharomyces cerevisiae* strain 43 in B-medium with different nitrogen sources during fermentation. Experimental procedures as in Figure 1

Nitrogen source	Cells dry weight (g/l)	Succinic acid (g/l)
Glutamic acid	5.9	1.56
Proline	1.3	0.61
Glutamine	5.7	0.43
Threonine	2.8	0.33
Aspartic acid	6.1	0.27
Alanine	3.0	0.16
Serine	2.6	0.14
Tryptophane	3.3	0.14
Asparagine	5.5	0.13
Phenylalanine	2.8	0.13
$(NH_4)_2SO_4$	5.1	0.11
Valine	1.9	0.09
Arginine	5.0	0.08
Tyrosine	3.9	0.08
Methionine	3.1	0.07
Leucine	2.4	0.05
Isoleucine	2.6	0.04
Glycine	n.g.	–
Histidine	n.g.	–
Ornithine	n.g.	–
Lysine	n.g.	–
Cystine	n.g.	–

n.g. = no growth

differently as has been reviewed by Suomalainen and Oura (1971). No direct relation exists between the amount of cells and the amount of succinic acid formed. Ammonium sulfate was a good nitrogen source for cell growth but yielded little succinic acid. Similar amounts of cells were obtained with glutamic acid that led to formation of high amounts of succinic acid. Proline, glutamine, threonine and asparagine increased the succinic acid formation above the level of ammonium sulfate. Several amino acids did not support the growth of *S. cerevisiae* strain 43. The formation of succinic acid and the cell growth increased with the nitrogen concentration of the medium up to 500 mg nitrogen/l. Higher concentrations of nitrogen had no influence.

The influence of succinic acid formation from glutamate was investigated by adding varying concentrations of aspartate, proline, threonine and ammonium sulfate to the otherwise nitrogen free medium. Aspartate, ammonium sulfate, threonine and proline, when present in equimolar amounts to glutamic acid, decreased the succinic acid formation by 50%, 50%, 40% and 10% respectively. If the ratio was 1:9 the formation of succinic acid was not influenced by the minor compound. The increased formation of succinic acid was correlated with the proportional formation of α-ketoglutaric acid. It was found that generally 90% of the glutamate present were metabolized. These results indicate that different metabolic pathways are operative for the various amino acids. The assumption was confirmed by the investigation of the intracellular metabolites when yeast cells were grown on aspartic or glutamic acids. Cells grown on glutamate contained no fumarate and 0.160 mg of α-ketoglutarate per g fresh weight. Cells grown on aspartate contained small amounts of fumarate and 0.045 mg of α-ketoglutarate per g fresh weight.

Influence of the Concentration of Glucose on the Formation of Succinic Acid. The influence of glucose on the formation of succinic acid was investigated by using two different nitrogen sources. Up to a concentration of 8% glucose a linear relation was observed between the glucose concentration and the formation of succinic acid. This was independent of the nitrogen source. However, the total amount of succinic acid formed depended on the quality of the nitrogen source (Fig. 2). Similar results were obtained with *Sac-*

Table 2. The influence of α-ketoglutaric acid and malic acid on the formation of succinic acid by *Saccharomyces cerevisiae* strain 43. B-medium. Nitrogen source: ammonium sulfate. Experimental procedure as in Figure 1

Addition	Amount		Succinic acid formed (g/l)
	added (g/l)	decomposed (g/l)	
–	0	0	0.12
α-Ketoglutarate	0.5	n.d.	0.11
α-Ketoglutarate	5.0	1.3	0.76
α-Ketoglutarate	10.0	2.1	0.71
L-Malate	0.5	n.d.	0.11
L-Malate	5.0	1.3	0.25
L-Malate	10.0	2.4	0.42

n.d. = not determined

Table 3. The effect of malonic acid on the formation of succinic acid by *Saccharomyces cerevisiae* strain 43. B-medium, nitrogen source: glutamate or ammonium sulfate

Nitrogen source	Addition		Succinic acid formed (g/l)
	Malonate (M)	Malate (g/l)	
Glutamate	0	0	1.63
Glutamate	0.05	0	1.65
$(NH_4)_2SO_4$	0	10	0.38
$(NH_4)_2SO_4$	0.02	10	0.31
$(NH_4)_2SO_4$	0.05	10	0.25

charomyces rouxii strain 68, or when glucose was replaced by fructose or saccharose.

Influence of Metabolites of the Citric Acid Cycle and Glyoxylate on the Formation of Succinic Acid. Using ammonium sulfate as nitrogen source, the influence of L-malate, fumarate, α-ketoglutarate, isocitrate, citrate and glyoxylate on the formation of succinic acid was investigated with *Saccharomyces cerevisiae* strain 43. Only α-ketoglutarate and L-malate increased the formation of succinic acid (Table 2). As glyoxylic acid and isocitrate did not increase the formation of succinic acid, it is assumed that this acid is not formed by the glyoxylic acid cycle.

The Influence of Malonic Acid on the Formation of Succinic Acid. Malonic acid is known to inhibit succinate dehydrogenase and cytoplasmatic, soluble fumarate reductases (Hauber and Singer, 1967). Therefore it was attempted to distinguish between the reductive and oxidative formation of succinic acid of which the former should be inhibited by malonate. The results presented in Table 3 revealed that 0.05 M malonate partially inhibited the formation of succinic acid from L-malate but did not influence the formation of succinic acid from glutamate.

Investigation of the Enzymes Involved in the Formation of Succinic Acid in Anaerobically Grown Yeast

The results with fermenting cells of *Saccharomyces cerevisiae* had led to the assumption that the following enzymes may be involved in the formation of succinic acid. Glutamate dehydrogenase (NAD-dependent), α-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase and malate dehydrogenase. Therefore it was attempted to determine the activity of these enzymes in cell free extracts of anaerobically grown yeasts. As the oxidative formation of succinic acid from glutamate appeared most likely, special emphasis was placed on the investigation of α-ketoglutarate dehydrogenase.

Fractionation of Cell Free Extracts by Centrifugation. Anaerobically grown cells of *S. cerevisiae* were mazerated and fractionated according to the various methods of Chapman and Bartley (1968), Holzer et al. (1963) and Schatz and Racker (1966). None of the fractions showed the presence of α-ketoglutarate dehydrogenase. Therefore the method described in Figure 3 was employed which led to the detection of α-ketoglutarate dehydrogenase in anaerobically grown yeast cells.

Determination of the Enzyme Activities in the Various Fractions. After fractionation of yeast cells according to Figure 3 the various enzyme activities were determined in the different fractions. The results are presented in Table 4. The activity of α-ketoglutarate dehydrogenase was definitely detected in the fractions sed. 3 and sed. 2. The activity found in these sediments was higher than in the supernatant sup. 1. This is due to the presence of NADH oxidase in the supernatant, which interferes with the determination of α-ketoglutarate dehydrogenase. No NADH oxidase activity was found in the sediment sed. 3. Most of the activity (90 %) of succinate dehydrogenase was found in sed. 3. The other enzymes (glutamate dehydrogenase, fumarase, and malate dehydrogenase) are soluble and accordingly present in the supernatants.

In separate experiments the activity of succinyl-CoA synthetase was determined. Its specific activity in supernatant sup. 3 was 0.18, when the extract and the fractionation were prepared in 0.02 M potassium phosphate buffer, pH 7.4.

α-Ketoglutarate Dehydrogenase. By kinetic investigation of α-ketoglutarate dehydrogenase prepared from anaerobically grown cells of *S. cerevisiae* on glucose the following K_m-values were obtained: 3.9

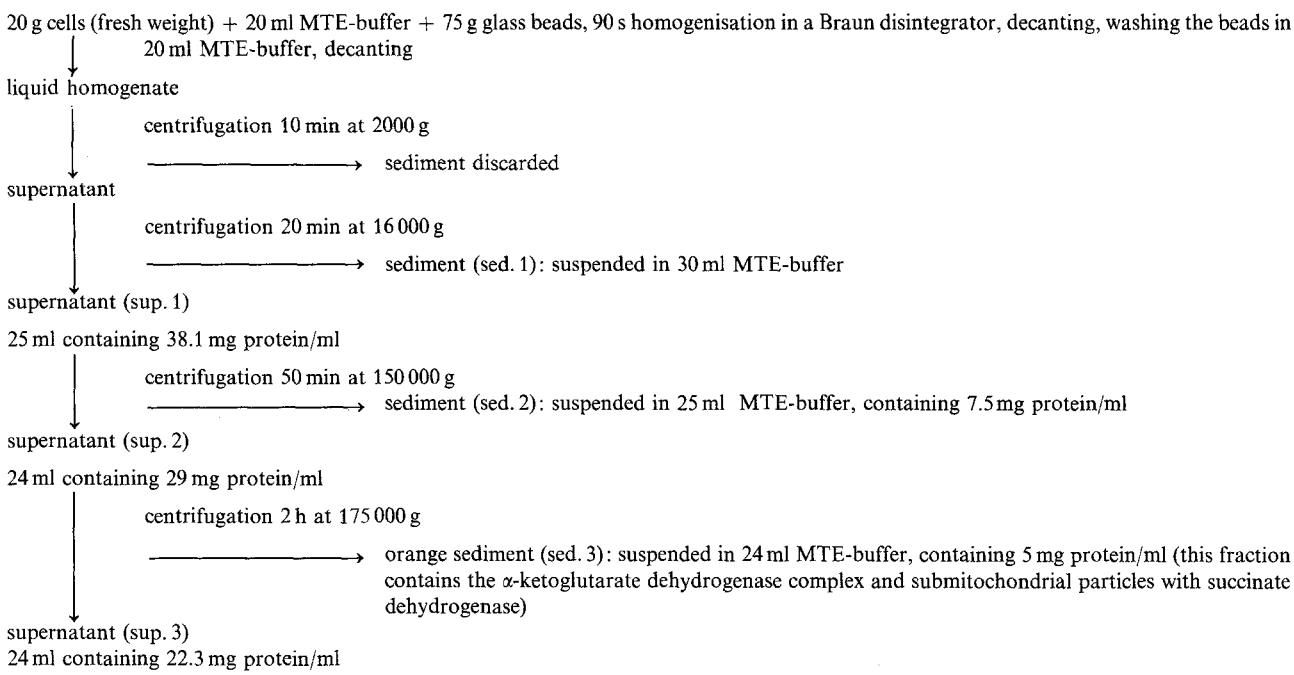

Fig. 3. Mazertion and fractionated centrifugation of cells of *Saccharomyces cerevisiae*

Table 4. Determination of the specific activities of the enzymes of succinic acid metabolism in the fractions obtained after centrifugation of cell free extracts of *Saccharomyces cerevisiae*. In supernatants (sup.) and sediments (sed.) of a crude extract of *Saccharomyces cerevisiae* the specific activities (μmol substrate converted/mg protein × min) of the following enzymes were determined: α-ketoglutarate dehydrogenase (KGDH), succinate dehydrogenase (SDH), L-malate dehydrogenase (MDH), NAD-dependent glutamate dehydrogenase (GluDH) and fumarase (FUM). Cultivation of cells in YEP-medium with an addition of 5 g/l L-glutamate

Time (min)	Centrifugation (g)	Fraction	Specific activities						Total protein (mg)
			KGDH	SDH	MDH	GluDH	FUM	Protein (mg/ml)	
10	1×10^3	sup.	n.m.	n.m.	n.d.	n.d.	n.d.	n.d.	n.d.
		sed.	0	0	n.d.	n.d.	n.d.	n.d.	n.d.
20	1.6×10^4	sup. 1	0.013[a]	0.02[a]	1.7	0.37	0.22	38.1	952
		sed. 1	0	0	n.d.	n.d.	n.d.	n.d.	n.d.
50	1.5×10^5	sup. 2	0.005	n.g.	2.2	n.d.	n.d.	29.5	708
		sed. 2	0.03	0.005	n.d.	n.d.	n.d.	7.5	187
120	1.75×10^5	sup. 3	0	0	2.64	0.35	0.33	22.3	535
		sed. 3	0.128	0.11	1.02	0.06	0.14	5.0	120

n.m. = not measurable; n.d. = not determined
[a] Values uncertain

$\times 10^{-4}$ for α-ketoglutaric acid, 1.2×10^{-6} for coenzyme A, and 9×10^{-5} for NAD. NADP did not replace NAD. The K_m-value for TPP was not determined because the coenzyme probably remains bound to the enzyme during mazeration and fractionation. The values obtained for *S. cerevisiae* strain 43 were found to be similar to those given by Holzer et al. (1963) for baker's yeast. This can be regarded as additional proof that *S. cerevisiae* contains α-ketoglutarate dehydrogenase even when cultivated on glucose in the absence of oxygen.

The Influence of L-Malate and Glutamate on the Enzymes of the Succinic Acid Metabolism. Under conditions that lead to the formation of different amounts of succinic acid in *Saccharomyces cerevisiae*, the specific activities of α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydro-

Table 5. Specific activities of α-ketoglutarate dehydrogenase (KGDH), succinate dehydrogenase (SDH), fumarase (FUM), malate dehydrogenase (MDH) and NAD-dependent glutamate dehydrogenase (GluDH) from *Saccharomyces cerevisiae* strain 43 were determined after growth in different media: (a) YEP + 500 mg N as glutamate or ammonium sulfate (YEP-Glu; YEP-NH_4) and YEP-NH_4 with an addition of 10 g/l L-malate (YEP-NH_4-mal); (b) nitrogen free B-medium with the same variations (B-Glu, B-NH_4, B-NH_4-mal). Succinic acid was determined in the medium after centrifugation of the cells after 15 h of growth. Specific activity: see Table 4

Medium	Specific activity					Succinic acid (g/l)	Cells fresh weight (g/l)
	KGDH	SDH	FUM	MDH	GluDH		
YEP-Glu	0.136	0.052	0.27	2.65	1.050	1.15	24
YEP-NH_4	0.023	0.096	0.23	2.15	0.072	0.12	21
YEP-NH_4-mal	0.025	0.105	0.28	2.44	0.079	0.25	21
B-Glu	0.078	0.035	0.33	2.12	1.400	1.34	29.5
B-NH_4	0.019	0.067	0.35	2.75	0.078	0.09	19
B-NH_4-mal	0.022	0.063	0.39	2.45	0.076	0.35	19.5

genase and glutamate dehydrogenase were determined. Using YEP and B-medium the effects of glutamate and L-malate were investigated. In previous experiments the former had resulted in oxidative succinic acid formation and the latter in reductive succinic acid formation.

When glutamate was used as the principal nitrogen source instead of ammonium sulfate, the specific activity of α-ketoglutarate dehydrogenase increased almost 6-fold (Table 5). The addition of 10 g/l of L-malate did not influence the activity of this enzyme. Cells grown in the presence of glutamate showed the lowest specific activity of succinate dehydrogenase when measured in oxidative direction. The reason for this variation is not abvious. Although succinate dehydrogenase is present in anaerobically grown yeast (Perlman and Mahler, 1974), its function in vivo is probably reductive only.

Malate dehydrogenase and fumarase were not influenced by the presence of L-malate or glutamate in the growth medium. Both enzymes are present in high activities and therefore not likely to be limiting in the citric acid cycle.

The specific activity of the NAD dependent glutamate dehydrogenase was increased 18-fold when glutamate was used as the main nitrogen source. This is in accordance with the results of Hierholzer and Holzer (1963).

The same investigations as presented in Table 5 were performed with a respiratory deficient mutant *S. cerevisiae* strain 311. Similar results were obtained for the enzymes α-ketoglutarate dehydrogenase, glutamate dehydrogenase, fumarase and malate dehydrogenase. Succinate dehydrogenase was not found in the mutant strain 311. This corresponds with the findings of Hauber and Singer (1967) that cytoplasmatic "petite"-mutants did not contain succinate dehydrogenase. The addition of malate led to an increased formation of succinic acid with *S. cerevisiae* strain 311. As this strain does not contain succinate dehydrogenase it is assumed that this reductive succinic acid formation is catalysed by cytoplasmatic fumarate reductases.

Discussion

Experiments with growing cultures of *Saccharomyces cerevisiae* had shown that the formation of succinic acid occurs during the exponential growth phase only. This is in accordance with Wagener et al. (1971), who had reported that succinic acid is excreted by yeasts at the beginning of fermentation.

Employing 99 different strains of several species of the genus *Saccharomyces* it was found that different amounts of succinic acid were formed by strains of the same species. Similar results were obtained by Coote and Kirsop (1974) with 8 strains of beer yeasts. However, Thoukis et al. (1965) did not observe an influence of the yeast strain on succinic acid formation.

Since Pasteur (1860) a direct relation between the amount of sugar fermented and the amount of succinic acid formed is known, see also Kleinzeller (1941). Sponholz and Dittrich (1977) regard succinic acid as a by-product of the sugar metabolism. However, our own investigations demonstrate that a direct and linear relation between the fermentation of glucose and the production of succinic acid exists up to a concentration of 8% glucose only. The total amount of succinic acid formed depended on the nitrogen source. This is an indication of a relation between amino acid metabolism and the biosynthesis of succinic acid.

When various metabolites were added to fermenting yeast cultures only α-ketoglutarate and glutamate increased the formation of succinic acid significantly. α-ketoglutarate which is partially metabolized increased the formation of succinic acid when ammonium sulfate

served as nitrogen source. This is due to the fact that α-ketoglutarate is used for transamination (Lewis and Rainbow, 1963) and assimilation of ammonium (Witt et al., 1964). In the presence of glutamate no decomposition of α-ketoglutarate was detected. Suomalainen et al. (1969) reported that yeasts neither absorb nor metabolize α-ketoglutaric acid.

Our experiments showed that glutamate led to the largest excretion of succinic acid which was not inhibited by malonate. Large amounts of succinic acid were also formed from proline, in spite of decreased yeast growth. This is probably due to the fact that proline is metabolized via glutamate (Duteurtre et al., 1971).

Chapman and Bartley (1968) had found that the synthesis of α-ketoglutarate dehydrogenase in yeast is inhibited by glucose. This enzyme was therefore regarded absent from yeast during anaerobic growth on 10% glucose. According to Whiting (1976) succinic acid is formed during fermentation by reduction only. Our investigations have shown that *Saccharomyces cerevisiae* strain 43 and the respiratory deficient mutant strain 311 both contain α-ketoglutarate dehydrogenase with a specific activity of up to 0.136, even when cultured under strictly anaerobic conditions. Chapman and Bartley (1968) had reported a specific activity of α-ketoglutarate dehydrogenase in aerobically grown cells of about 0.01 only. It appeares likely that these authors were unable to find α-ketoglutarate dehydrogenase in yeast cells grown under anaerobic conditions, for it can be assumed that the activity of this enzyme decreases by anaerobic growth as is the case with the other enzymes of the citric acid cycle. Probably due to the use of different buffers and methods of preparation, it has been possible to detect α-ketoglutarate dehydrogenase in the experiments described in this paper.

The specific acitity of α-ketoglutarate dehydrogenase is regulated by the nitrogen source, it is increased in cells grown on glutamate several fold when compared with cells grown on ammonium sulfate. These comparative enzymatic investigations are in accordance with the experiments with growing cells, that succinic acid is formed during fermentation by oxidation.

When L-malate or aspartate were added to fermenting yeast a smaller increase of succinic acid formation was detected than with glutamate or α-ketoglutarate. Cells grown with malate or aspartate contained fumarate and only little α-ketoglutarate. The biosynthesis of succinic acid from malate was partially inhibited by malonate. Therefore it is assumed that succinic acid is formed by reduction from malate and aspartate. An increased formation of succinic acid with malate was also observed with a respiratory deficient mutant. Investigations of Hauber and Singer (1967) have shown, that respiratory deficient mutants lack succinate dehydrogenase. However, when grown anaerobically, these mutants contain cytoplasmatic, soluble fumarate reductases, which are described by Tisdale et al. (1968) and reviewed by Thauer et al. (1977). Therefore it appears likely, that this minor reductive succinic acid formation may be catalysed by fumarate reductases. In our experiments only 10% of the malate metabolized were converted to succinic acid. The reductive formation of succinic acid from malate is probably a by-pass of the malate metabolism. In *Saccharomyces*, malate is partially decomposed to ethanol and CO_2 (Fuck and Radler, 1972). Using labelled malate Mayer et al. (1964) found that about half of the malate was converted to succinic acid. Wagener et al. (1971) had observed no influence of malate on succinic acid formation by fermenting yeasts.

Duntze et al. (1969) had reported that isocitrate lyase is not operative in anaerobic yeast growth. Our experiments had shown that isocitrate and glyoxylate did not influence the formation of succinic acid. Therefore it is assumed that the glyoxylic acid cycle is not part of the biosynthesis of succinic acid.

As only small amounts of succinic acid were formed when ammonium sulfate served as nitrogen source it is assumed that under these conditions the α-ketoglutarate formed is mainly used for the biosynthesis of amino acids (Witt and Holzer, 1964). The dependence of the succinic acid formation on the nitrogen source appears to be comparable with the formation of higher alcohols from the corresponding amino acids by the Ehrlich pathway which has been described by Vollbrecht and Radler (1973).

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