Distribution of ^{14}C -labelled carbon from glucose and glutamate during anaerobic growth of *Saccharomyces cerevisiae*

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The distribution of carbon from glucose and glutamate was studied using anaerobically grown *Saccharomyces cerevisiae*. The yeast was grown on glucose (20 g l⁻¹) as the carbon/energy source and glutamic acid (3.5 g l⁻¹) as additional carbon and sole nitrogen source. The products formed were identified using labelled [U- ^{14}C]glucose or [U- ^{14}C]glutamic acid. A seldom-reported metabolite in *S. cerevisiae*, 2-hydroxyglutarate, was found in significant amounts. It is suggested that 2-hydroxyglutarate is formed from the reduction of 2-oxoglutarate in a reaction catalysed by a dehydrogenase. Succinate, 2-oxoglutarate and 2-hydroxyglutarate were found to be derived exclusively from glutamate. Based on radioactivity measurements, 55%, 17% and 14% of the labelled glutamate was converted to 2-oxoglutarate, succinate and 2-hydroxyglutarate, respectively, and 55%, 9% and 3% of the labelled glucose was converted to ethanol, glycerol and pyruvate, respectively. No labelled glucose was converted to 2-oxoglutarate, succinate or 2-hydroxyglutarate. Furthermore, very little of the evolved CO₂ was derived from glutamate. Separation of the amino acids from biomass by paper chromatography revealed that the glutamate family of amino acids (glutamic acid, glutamine, proline, arginine and lysine) originated almost exclusively from the carbon skeleton of glutamic acid. It can be concluded that the carbon flow follows two separate paths, and that the only major reactions utilized in the tricarboxylic acid (TCA) cycle are those reactions involved in the conversion of 2-oxoglutarate to succinate.

Keywords: *Saccharomyces cerevisiae*, anaerobic growth, glutamate, redox balance, 2-hydroxyglutarate

INTRODUCTION

The nitrogen source strongly influences cellular metabolism and product formation in *Saccharomyces cerevisiae*, especially redox balancing under anaerobic conditions (Albers *et al.*, 1996). Glutamic acid plays a central role in nitrogen metabolism of *S. cerevisiae* and other organisms, regardless of whether it is used as the nitrogen source. Transamination reactions for amino acid biosynthesis mostly rely upon glutamic acid (Jones & Fink, 1982). The amino group from glutamic acid is transferred to the different amino acid precursors, yielding 2-oxoglutaric acid. Occasionally, other amino acids are used in the transamination reactions, such as

glutamine, alanine or aspartic acid. Conversely, the assimilation of ammonium results in the formation of glutamic acid in a reaction catalysed by glutamate dehydrogenase (Cooper, 1982). This enzyme exists as three isoenzymes, which may have different functions. The NAD⁺-dependent isoenzyme has a catabolic role while the NADP⁺-dependent isoenzymes are involved in anabolic reactions (Avendano *et al.*, 1997; Cooper, 1982; Miller & Magasanik, 1990; ter Schure *et al.*, 1995).

Uptake studies using radioactively labelled amino acids reveal that glutamic acid is, to a large extent, metabolized and excreted into the medium as deaminated derivatives (Woodward & Cirillo, 1977). Consequently, when glutamic acid is used as the sole nitrogen source, a large amount of extracellular 2-

Abbreviation: TCA, tricarboxylic acid.

oxoglutarate is produced (Lewis & Rainbow, 1963; Rankine, 1968). In a previous study on anaerobic growth in *S. cerevisiae*, increased formation of 2-oxoglutarate and succinate was found when glutamate rather than ammonium was used as a nitrogen source (Albers *et al.*, 1996). Succinate may be formed from 2-oxoglutarate via succinyl-CoA in the tricarboxylic acid (TCA) cycle, since this reaction takes place anaerobically in yeast (Heerde & Radler, 1978).

The aim of this study was to quantitatively determine how glutamic acid is catabolized under defined conditions using labelled substrates. Previous labelling studies on product formation have been made using only labelled glutamic acid (Woodward & Cirillo, 1977). However, by using either ^{14}C -labelled glucose or ^{14}C -labelled glutamic acid, it is possible to determine the source of carbon atoms in different excreted products and biomass. We identified two independent flows of carbon originating from either glucose or glutamate. These carbon flows result in a split of the TCA cycle. The implications that this has for the redox balance are discussed.

METHODS

Strain and media. An industrial strain of *Saccharomyces cerevisiae*, supplied by a baker's yeast manufacturer (Jästbolaget, Rotebro, Sweden) was used. The yeast was maintained on agar plates containing (l^{-1}) 20 g bacteriological agar, 10 g yeast extract, 20 g peptone, 20 g D-glucose. Inoculum and bioreactor cultures were grown in a defined medium (Albers *et al.*, 1996), with 20 g glucose l^{-1} as the carbon and energy source and 3.5 g glutamic acid l^{-1} as the nitrogen source. The inocula (50 ml) were grown aerobically for approximately 36 h, at 30 °C in 300 ml shake flasks. Ergosterol and Tween 80 were excluded from the inoculum cultures. The mineral components, including trace metals and antifoam, and glucose were autoclaved separately at 121 °C for 20 min. The ergosterol and Tween 80 solution was heated at 100 °C for 5–10 min in a water bath before addition to the medium (at final concentrations of 10 mg ergosterol l^{-1} and 0.42 g Tween 80 l^{-1}). The solutions of vitamins, glutamic acid and radioactive tracers were filter-sterilized. Radioactively labelled [$\text{U-}^{14}\text{C}$]glutamic acid (9.29 GBq mmol^{-1}) or [$\text{U-}^{14}\text{C}$]glucose (11.0 GBq mmol^{-1}) (Amersham) were used as tracers.

Bioreactor system and batch cultivation procedures. Cultures were grown in a Chemap CMF200 bioreactor system with a working volume of 700 ml. The bioreactor was equipped with a condenser, a sterilizable pH electrode and a temperature sensor. The agitation rate was controlled at 350 r.p.m., the temperature at 30 °C and the pH at 5.5 by addition of 2 M NaOH. To maintain anaerobic conditions, the fermenter was flushed with pure nitrogen (< 5 p.p.m. O_2 , Nitrogen Plus, AGA gas, Sweden) at a controlled flow rate of 0.2–0.3 vol. $\text{vol}^{-1} \text{min}^{-1}$ (at normal temperature and pressure) by a mass flow controller (Bronkhorst High-Tech). The mass flow controller was calibrated with a DryCal DC-1 flow calibrator (Bios International) to obtain the precise flow necessary for the determination of the CO_2 yield.

The bioreactor was autoclaved *in situ*, containing water. The bioreactor was emptied and filled with pre-sterilized medium. The fermenter was inoculated with 12 ml inoculum culture, giving an initial cell concentration of 0.09 g dry wt l^{-1} .

Analytical methods

Liquid components and biomass determination. Samples of the culture were centrifuged (15000 g) for 5 min and the cell-free supernatants were stored at –20 °C prior to analysis for liquid components.

Metabolites were measured with an HPLC (Waters) equipped with a UV detector (210 nm) and a refractive index detector. Glucose, glycerol, ethanol, pyruvic acid, succinic acid, 2-oxoglutaric acid and 2-hydroxyglutaric acid were separated on polymeric ion-exchange columns with 5 mM H_2SO_4 as the eluent. Two columns were used: Shodex SH 1011 (Showa Denko) at 55 °C with a flow rate of 1.0 ml min^{-1} , and Aminex HPX-87H (Bio-Rad) at 60 °C with a flow rate of 0.6 ml min^{-1} . Glutamic acid and acetic acid were analysed with enzymic kits (Boehringer Mannheim).

The biomass concentration was determined by OD_{610} measurements (Nova spec II, Pharmacia) which were recalculated to dry weight using a separately determined calibration curve. The dry weights used in the calibration curve were based on duplicate samples (10 ml, taken during growth up to the end of the cultivation), which were centrifuged (5000 g, 5–10 min), washed twice with distilled water and dried for at least 24 h at 110 °C. The maximum specific growth rate (μ_{max}) was calculated from biomass measurements during exponential growth by linear regression.

Gas analysis. The yield of CO_2 was determined in two separate cultivations. The exhaust gas was analysed continuously by an acoustic CO_2 and Oxygen Monitor, type 1308 (Brüel and Kjær) (Christensen *et al.*, 1995).

Radioactivity measurements. The radioactivity of compounds separated by the HPLC (Shodex column) was detected by a liquid scintillation detector (Beckman 171 Radioisotope detector), which was used in series with the UV detector. A scintillation cocktail for radiochromatography (Ready Flow III, Beckman) was used with a flow rate of 5.0 ml min^{-1} . For measurements of radioactivity in the biomass, 5 ml samples were prepared (as for dry weight analysis) and resuspended in 4 ml distilled water. CO_2 in the exhaust gas was absorbed in 5 M NaOH (100–300 ml). However, the absorption was not effective and could not be used for quantitative analysis. CO_2 is primarily formed from the decarboxylation of pyruvate under anaerobic conditions. Labelled CO_2 could therefore be estimated only from radioactively labelled ethanol (1 mol CO_2 per mol ethanol).

The radioactivity of the cell-free medium, biomass and absorbed CO_2 was measured with a liquid scintillator, Beckman LS 6000LL (Beckman), using a standard scintillation cocktail (Ready Safe, Beckman) (1 ml sample to 5 ml cocktail).

Analysis of radioactively labelled amino acids in biomass. The washed biomass suspension (2 ml) was centrifuged for 10 min (3300 g, 4 °C). The pellet was resuspended in 500 μl 20% HCl and 500 μl 90% formic acid and the tubes were sealed with stoppers. For protein hydrolysis, the tubes were kept at 100–110 °C for 24 h. The samples were dried with a flow of pure nitrogen and resolved in 100 μl distilled water. The insoluble debris was removed by centrifugation and the supernatant was transferred to another tube. Toluene (3 μl) was added to the sample before storage at 4 °C. The amino acids were separated by paper chromatography (Dörfel, 1959; Lewis & Rainbow, 1963) on Whatman paper no. 4. Two different eluents were used: the first (BAW) containing 60 ml 1-butanol, 15 ml acetic acid and 25 ml water, and the second (PIW) containing 37 ml pyridine, 32 ml isoamyl alcohol and 32 ml water. The chromatograms (length 35 cm) were run for 20 h and air-dried. The separated amino acids were stained

with ninhydrin (1.5 g ninhydrin, 10 g 2,4,6-trimethylpyridine per l acetone) and developed at 40 °C for 2–3 h. The amino acids were identified from standards of known composition. The different colours for different amino acids developed with the ninhydrin reagent also helped with identification. Radioactively labelled amino acids were detected by autoradiography using double-coated X-ray film (CEA reflex 25). The double coating was selected to detect as much as possible of the weak (0.2 MeV), energetically distributed β -particles from ^{14}C . The film was exposed for 14–19 d, developed with CEATank and fixed with CEAFix (CEA). Chemical darkening of the film was negligible.

Calculations. Calculations were made for carbon and degree of reduction balances. The degree of reduction for a compound with the C-molar formula $\text{CH}_a\text{O}_b\text{N}_c$ is defined with respect to molecular nitrogen according to equation 1 (Roels, 1983).

$$\gamma = 4 + a - 2b \quad (1)$$

The elemental composition of ash-free biomass used was $\text{CH}_{1.60}\text{O}_{0.45}\text{N}_{0.20}$ (Albers *et al.*, 1996).

Identification of 2-hydroxyglutarate. In the HPLC chromatograms a peak was observed with a retention time of approximately 8 min, which agreed with the retention time of a standard solution of DL-2-hydroxyglutaric acid, zinc salt (Sigma). The 2-hydroxyglutarate was further identified by mass spectrometry. The appropriate fraction was collected from the HPLC outlet. After reinjection into the HPLC system to check the purity, the unknown sample was analysed on a high-resolution mass spectrometer (Zab-Spec, VG Analytical, Fisons) operating in CI-mode with NH_3 as the ionizing gas. The mass spectrum was compared to a standard of the 2-hydroxyglutarate salt in HPLC eluent (5 mM H_2SO_4). Both samples showed characteristic peaks of masses 148 and 85, which indicated that the acid was present in the γ -lactone form.

RESULTS

Balances and yields

As expected, relatively high yields of 2-oxoglutaric and succinic acid were found (Table 1). However, the high yield of the metabolite 2-hydroxyglutaric acid, which was formed in similar amounts as succinate, was unexpected. Apparently, 2-hydroxyglutaric acid is a relatively important by-product for this strain when growing on glutamic acid. However, the formation of 2-hydroxyglutarate, previously unidentified in our early study (Albers *et al.*, 1996), contributes to only about 1% of the carbon recovery. Both the carbon and degree of reduction balances were in agreement for all experiments (Table 1). The μ_{max} was determined to be 0.33 h^{-1} , which agrees well with our earlier findings (Albers *et al.*, 1996).

Since all carbon atoms in the initial [^{14}C]glucose and [^{14}C]glutamic acid were labelled, the ratio between the radioactivity found in one compound and the radioactive source represents the yield, expressed as C-mol per C-mol glucose or glutamate, respectively. Therefore, if a compound is formed exclusively from a labelled substrate, the yields derived from radioactivity or concentration measurements should be identical. Both yields on glucose agreed well for ethanol and acetic acid

Table 1. Product yields on glucose, amount of assimilated glutamic acid used per mol glucose, and balances of carbon and degree of reduction during anaerobic growth of *S. cerevisiae*

Cultures were grown anaerobically with glucose as carbon/energy source and glutamic acid as both a carbon source and the sole nitrogen source. Means and standard deviations were calculated from six experiments unless otherwise stated.

Substance	Yield on glucose (mol mol ⁻¹)
Glutamic acid uptake*	0.13 ± 0.01*
CO ₂	1.61 ± 0.01†
Ethanol	1.62 ± 0.06
Glycerol	0.17 ± 0.005
Acetic acid	0.004 ± 0.000
Pyruvic acid	0.009 ± 0.002
Succinic acid	0.026 ± 0.003
2-Oxoglutaric acid	0.068 ± 0.004
2-Hydroxyglutaric acid	0.018 ± 0.002
Ash-free biomass‡	0.63 ± 0.03§
Type of balance	Recovery (%)
Carbon	98.4 ± 2.1
Degree of reduction	99.5 ± 2.9

* mol assimilated per mol glucose used.

† Mean and SD from two experiments.

‡ Corrected for the ash content, $96.7 \text{ mg (g dry wt)}^{-1}$ (Albers *et al.*, 1996).

§ Yield expressed in mol total carbon per mol glucose.

and to a lesser degree for glycerol and pyruvic acid (Table 2). The two types of yields on glutamate also correlated well for succinic acid, 2-oxoglutaric acid and 2-hydroxyglutaric acid (Table 2). The yield of CO₂ obtained from concentration measurements was very similar to the estimated radioactivity yield on glucose. Furthermore, when labelled glutamic acid was used as the tracer, almost no labelled CO₂ was detected.

Biomass was partly labelled in both cases. It seems plausible that glutamic acid and the amino acids belonging to the glutamate family were formed from the glutamic acid added and that all other amino acids were formed from glucose. This hypothesis was further examined using paper chromatography and autoradiography.

Distribution of radioactively labelled carbon

The distribution of labelled carbon showed a very different pattern depending on whether glucose or glutamate was initially labelled (Table 2). The carbon from glucose was mainly found in ethanol and glycerol, the two major products during anaerobic fermentation. Some labelled carbon was also found in the biomass, in

Table 2. Comparison of metabolite yields obtained from radioactivity and concentration measurements for anaerobically grown cultures of *S. cerevisiae* with glucose as the carbon/energy source and glutamic acid as the sole nitrogen source

Glucose or glutamic acid was initially uniformly labelled with ^{14}C . For concentration measurements, means and standard deviations were calculated from six experiments, except for CO_2 measurements, which were calculated from two experiments. For radioactivity measurements, means and standard deviations were calculated from two experiments.

Metabolite	Yield on glucose as measured by:		Yield on glutamic acid as measured by:	
	Concentration (C-mol C-mol ⁻¹)	Radioactivity [d.p.m. (d.p.m.) ⁻¹]	Concentration (C-mol C-mol ⁻¹)	Radioactivity [d.p.m. (d.p.m.) ⁻¹]
CO_2	0.27 ± 0.0011	$0.27 \pm 0.03^*$	2.53 ± 0.012	0.001 ± 0
Ethanol	0.54 ± 0.02	0.55 ± 0.06	5.10 ± 0.22	ND
Glycerol	0.083 ± 0.002	0.092 ± 0.011	0.78 ± 0.03	ND
2-Oxoglutaric acid	0.057 ± 0.003	ND	0.54 ± 0.03	0.55 ± 0.01
Succinic acid	0.017 ± 0.002	ND	0.16 ± 0.02	0.17 ± 0.01
2-Hydroxyglutaric acid	0.015 ± 0.001	ND	0.14 ± 0.01	0.14 ± 0.01
Pyruvic acid	0.005 ± 0.001	0.026 ± 0.007	0.05 ± 0.01	ND
Acetic acid	0.001 ± 0	Trace	0.01 ± 0	ND
Biomass	0.10 ± 0.005	0.064 ± 0.007	0.99 ± 0.06	0.16 ± 0.01
Sum of labelled compounds	—	1.01 ± 0.10	—	1.02 ± 0.06

* Calculated from the ethanol yield.

ND, Not detected.

pyruvic acid and in traces of acetic acid. The labelled glucose was recovered to 101 % (the standard deviation was 10 % from two experiments), using estimated CO_2 counts. The HPLC chromatograms showed clearly that neither 2-oxoglutarate, succinate nor 2-hydroxyglutarate were labelled (Fig. 1a) and consequently not formed from glucose.

Conversely, the carbon from glutamate was converted to 2-oxoglutarate and, through a further conversion via the TCA cycle, to succinate. 2-Hydroxyglutarate was also formed from glutamate. The recovery of labelled carbon from glutamate was 102 % (the standard deviation was 6 % from two experiments).

The paper chromatogram obtained with the butanol eluent (BAW) showed that amino acids in the glutamate family (glutamic acid, glutamine, proline, arginine and lysine) were all labelled when glutamate was labelled (Fig. 2a; Table 3). The results were, however, ambiguous since threonine overlaps with glutamate in spot no. VII and histidine overlaps arginine and lysine in spots nos IX and X, respectively. Only spots VII, IX and X were labelled with both tracers. To separate these amino acids additional chromatograms were made using a pyridine-based eluent (PIW) (Fig. 2b, Table 3). The spots containing threonine (V) and histidine (VI) were labelled only when glucose was used as the tracer and the amino acids in the glutamate family were labelled when glutamic acid was used as the tracer. Spot no. IX in the PIW chromatogram, containing only lysine and arginine, was labelled for both tracers, although very little was present with labelled glucose (Fig. 2b). Lysine

and arginine are not solely formed from glutamic acid (Jones & Fink, 1982). During lysine synthesis, CO_2 is fixed and incorporated into the carbon skeleton. Since CO_2 is mainly evolved during ethanol formation, carbon probably stems from glucose rather than from glutamate. The same is valid for the acetyl group from acetyl-CoA during arginine synthesis. The amount of carbon in amino acids from the glutamate family, corrected for the carbon from CO_2 and acetyl-CoA, was calculated to be 0.19 C-mol per C-mol glutamate used, using an amino acid composition previously reported (Albers *et al.*, 1996). This is in good agreement with the radioactivity yield of 0.16 (Table 2). The total recovery, including carbon in extracellular metabolites and the corrected yield of amino acid carbon, was determined to be 103 %. This finding supports the hypothesis that the amino acids from the glutamate family are synthesized from glutamate and not from glucose.

DISCUSSION

Distribution of carbon from glucose and glutamate

The results of the tracer experiments are described in Fig. 3 (for simplicity the formation of acetyl-CoA from acetaldehyde is not shown). Under experimental conditions with an excess of glucose and glutamate, there are two essentially non-overlapping flows of carbon originating from either glucose or glutamate. Therefore, only parts of the TCA cycle are in use when glutamate is the nitrogen source. In agreement with previous reports (Albers *et al.*, 1996; Coote & Kirsop, 1974; Heerde & Radler, 1978; Lewis & Rainbow, 1963; Rankine, 1968),

Fig. 1. HPLC chromatograms for ¹⁴C-containing compounds at the end of anaerobic cultivations of *S. cerevisiae* with glucose as carbon/energy source and glutamic acid as both a carbon source and the sole nitrogen source. The tracer used was glucose (a) or glutamic acid (b). The dotted lines show the retention time when the peaks of 2-oxoglutaric acid and succinic acid start.

formation of succinic acid and 2-oxoglutaric acid from glutamate was found in the present study also. The carbon used in the amino acid synthesis of the glutamate family of amino acid primarily originates from assimilated glutamate. In addition, a relatively large part of the carbon from glutamate is excreted in the form of 2-hydroxyglutarate.

2-Hydroxyglutaric acid and 2-hydroxyglutarate dehydrogenase

2-Hydroxyglutarate formation is well known for lactic acid bacteria, in which 2-oxoglutarate is reduced to form 2-hydroxyglutarate by 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2) (Radler, 1986). Using an NAD⁺-dependent enzyme from *Acidaminococcus fermentans*, Buckel & Miller (1987) determined that the equilibrium constant for 2-oxoglutarate formation is 1.47×10^{-12} and therefore formation of 2-hydroxyglutarate is greatly favoured. 2-Hydroxyglutarate dehydrogenase has also been found in *Alcaligenes* sp. (Suzuki *et al.*, 1985), *Peptococcus aerogenes* (Otawara *et al.*, 1984), rat and human liver cells (Jansen & Wanders, 1993) and *Escherichia coli* (Zhao & Winkler, 1996).

3-Phosphoglycerate dehydrogenase (EC 1.1.1.95) (the first enzyme in the synthesis of serine) in *E. coli* also catalyses both the reduction and oxidation of 2-oxoglutarate and 2-hydroxyglutarate, respectively, with NAD(H) as the cofactor (Zhao & Winkler, 1996). Furthermore, the enzyme has a higher affinity for both

the D-isomer of 2-hydroxyglutarate and 2-oxoglutarate than for 3-phosphoglycerate; hence 2-oxoglutarate reduction is favoured over 3-phosphoglycerate oxidation. The formation of 2-hydroxyglutarate in *S. cerevisiae* is less well studied, although Coote & Kirsop (1974) reported formation of the compound in both wort and synthetic medium by *Saccharomyces* sp. They found that 2-hydroxyglutarate is formed in relatively large amounts when glutamic acid is the nitrogen source. They also suggested that 2-hydroxyglutarate is formed from 2-oxoglutarate because of the chemical similarity between these compounds. The labelling technique used in the present study confirmed the hypothesis that 2-hydroxyglutarate was formed solely from glutamate.

Based on DNA sequence homology, the gene YER081W in *S. cerevisiae* encodes a putative 3-phosphoglycerate dehydrogenase (Achouri *et al.*, 1997). Furthermore, the gene YIL074C is highly homologous to YER081W (90.8% identity) and therefore probably also encodes a 3-phosphoglycerate dehydrogenase. YIL074C and YER081W are 46.9% and 47.4% identical, respectively, to 3-phosphoglycerate dehydrogenase from *E. coli* (encoded by *serA*). Although the yeast 3-phosphoglycerate dehydrogenases have not been fully characterized, NAD⁺ is the cofactor used and the enzyme is inhibited by the endproduct serine (Jones & Fink, 1982). As a preliminary test, batch cultivation of *S. cerevisiae* was done with addition of serine (400 mg l⁻¹) to the medium. The formation of 2-hydroxyglutarate was reduced by 50%, which suggests that 3-phosphoglycerate dehydrogenase also catalyses the formation of 2-hydroxyglutarate in *S. cerevisiae*. If the kinetics of the yeast 3-phosphoglycerate dehydrogenase are similar to the kinetics of the *E. coli* enzyme, there may be competition between oxidation of 3-phosphoglycerate to 3-phosphohydroxypyruvate and the reduction of 2-oxoglutarate to 2-hydroxyglutarate in *S. cerevisiae*. Under anaerobic growth on glutamate, the latter reaction may well be favoured, due to the high amounts of 2-oxoglutarate formed from glutamate and the surplus NADH formed during anaerobic growth.

3-Phosphoglycerate dehydrogenase has an interesting dual function. The enzyme functions in the anabolic pathway of serine synthesis and may also reduce the TCA cycle intermediate 2-oxoglutarate (Zhao & Winkler, 1996). Therefore, the enzyme may connect catabolic and anabolic carbon fluxes and may link regulation of central carbon catabolism to assimilative nitrogen metabolism in *S. cerevisiae*.

Redox balances with glutamate as the nitrogen source

The TCA cycle does not function during the experimental conditions, except for the reactions converting 2-oxoglutarate to succinate (Fig. 3). The non-functional part of the TCA cycle from citrate to 2-oxoglutarate and the reversed path from glutamate to 2-oxoglutarate affect the flow of reducing equivalents when compared to growth with ammonium as the nitrogen source.

Fig. 2. Paper chromatograms stained with ninhydrin reagent (left panel) and the corresponding autoradiograms (right panel) for separated amino acids from anaerobic cultivations with glucose as the carbon/energy source and glutamic acid as both a carbon source and the sole nitrogen source. The initial labelled carbon was in the form of glutamic acid (Glu*) or glucose (Glc*). The paper chromatograms were run for 20 h with (a) the butanol eluent (BAW) or (b) the pyridine eluent (PIW). Roman numerals correspond to the identified spots of amino acids, according to Table 3. The yellow spot of proline is not visible in the computer images of the paper chromatograms, but the placement of the spots is indicated with the corresponding number (BAW, V; PIW, IV).

When ammonium is used as the nitrogen source, mainly NADPH-dependent glutamate dehydrogenase catalyses the formation of glutamate. However, when glutamate is the nitrogen source, this enzyme is not required and the demand for NADPH is smaller (Avendano *et al.*, 1997).

Of the non-functional path of the oxidative TCA cycle, the only reaction which requires reducing equivalents is the formation of 2-oxoglutarate from isocitrate. This reaction is catalysed by three isoenzymes of isocitrate dehydrogenase, one using NAD⁺ and the other two using NADP⁺ as cofactors. During anaerobic growth on glucose and glutamate, only the constitutive NADP⁺-dependent isocitrate dehydrogenase is active (Nissen *et al.*, 1997; Zhao & McAlister-Henn, 1996). Under our

experimental conditions this important source of NADPH (Bruinenberg *et al.*, 1983) is nonexistent. However, in comparison with growth on ammonium as nitrogen source, the demand for NADPH is lower, since in the present case there is no demand for NADPH during glutamate formation.

The need for transamination in cultures grown on glutamate and hence the subsequent formation of 2-oxoglutarate has been reported (Albers *et al.*, 1996). The estimated amount of 2-oxoglutarate agrees well with the amount determined in both this and our earlier study. Consequently, the formation of 2-oxoglutarate may be seen as a measure of transamination and thus the uptake of glutamate appears to depend on the nitrogen requirement. The increased production of 2-oxoglutarate

Table 3. Identification of amino acids from different spots on the paper chromatograms, run with the butanol or pyridine eluent

The initially labelled carbon was in form of glutamic acid (Glu*) or glucose (Glc*). The amino acids from the glutamate family are in bold. Darkening of the X-ray film is marked with *. Spot numbers refer to the chromatograms in Fig. 2.

Butanol eluent				Pyridine eluent			
Spot no.	Amino acid	Spot on film		Spot no.	Amino acid	Spot on film	
		Glu*	Glc*			Glu*	Glc*
I	Ile, Leu		*	I	Ile, Leu, Phe		*
II	Phe		*	II	Met, Tyr		*
III	Met, Val		*	III	Val		*
IV	Tyr		*	IV	Pro	*	
V	Pro	*		V	Ala, Ser, Thr		*
VI	Ala		*	VI	His, Gly		*
VII	Glu, Thr	*	*	VII	Cys, Glu , Asp	*	*
VIII	Asp, Gly, Ser		*	VIII	Cys-Cys	*	*
IX	His, Lys	*	*	IX	Arg , Lys	*	*
X	Arg , His	*	*				
XI	Cys-Cys

Fig. 3. Carbon flows from glucose (open arrows) and glutamic acid (filled arrows) under anaerobic conditions in *S. cerevisiae*. The arrows for the synthesis of amino acids and the minor products are proportional.

does not contribute to the balance of reducing equivalents, since the transamination reactions are not coupled with NAD^+ or NADP^+ (Jones & Fink, 1982). Only the need for glutamine may contribute to NADH formation, either by the action of NAD^+ -dependent glutamate dehydrogenase or glutamate synthase (Miller & Magasanik, 1990).

The formation of 2-hydroxyglutarate in *S. cerevisiae* may serve as an additional sink for NADH. If previous theoretical calculations of anaerobic NADH formation (Albers *et al.*, 1996) are adjusted for the formation of 2-

hydroxyglutarate (using the yields reported in Table 1), a theoretical yield of glycerol formation of 0.14–0.16 mol per mol glucose is obtained. The theoretical and experimental (0.17 mol mol⁻¹; Table 1) yields are still in good agreement but now, unlike the earlier study, the upper limit of the theoretical value agrees with the experimental one.

The final redox reaction in the TCA cycle involves the conversion of 2-oxoglutarate to succinate (via succinyl-CoA), in a reaction catalysed by 2-oxoglutarate dehydrogenase. This mitochondrial enzyme uses NAD^+ as

a cofactor (Heerde & Radler, 1978; Wales *et al.*, 1980); succinate formation, therefore, gives rise to mitochondrial NADH formation. The substrate, 2-oxoglutarate, may be available both in the cytoplasm and mitochondrion, since the transport system for 2-oxoglutarate across the mitochondrial membrane is active both under aerobic and anaerobic conditions (Perkins *et al.*, 1973). The similar yields of 2-hydroxyglutarate and succinate (Table 1) may indicate that the formation of 2-hydroxyglutarate acts as a local sink for NADH, provided that the reduction takes place in the mitochondria.

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