Effect of Nitrogen Source on Lipid Accumulation in Oleaginous Yeasts

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The effect of various nitrogen sources on lipid accumulation by 17 species and strains of yeast was examined. Organic nitrogen sources resulted in considerably increased lipid contents only in strains of *Rhodospiridium toruloides*. Lipid accumulation in *Rs. toruloides* CBS 14 increased from 18% (w/w), with NH_4Cl as nitrogen source, to above 50% (w/w) when glutamate, urea or arginine was used. Stimulation of lipid production by glutamate was not observed when the yeast was grown in continuous culture with nitrogen-limiting medium. The increase in lipid content of glutamate-grown cells in batch culture was accompanied by a marked increase in the intracellular citrate concentration and its excretion from the cells. The pattern of citrate accumulation in glutamate-grown cells was mirrored by the accumulation of other metabolites, especially 2-oxoglutarate and NH_4^+ ions, which were produced as a result of the increased catabolism of glutamate. It is proposed that the products of glutamate metabolism in *Rs. toruloides* play a major role in regulating the flux of carbon to precursors of lipid biosynthesis, such as citrate.

INTRODUCTION

Work in our laboratory has been concerned with the biochemistry of various oleaginous yeasts which can accumulate large quantities of lipid (Ratledge, 1978, 1982; Rattray *et al.*, 1975). When the nitrogen source in the medium becomes depleted, protein and nucleic acid synthesis ceases but excess carbon continues to be metabolized to lipid. Under such conditions, non-oleaginous yeasts do not accumulate lipid. A hypothesis explaining lipid accumulation in yeasts has been proposed by Botham & Ratledge (1979). This states that intramitochondrial citrate accumulates primarily due to the decrease in intracellular AMP concentration, which leads to a decline in activity of the AMP-dependent NAD^+ :isocitrate dehydrogenase in the mitochondria. Citrate is then transported across the mitochondrial membrane in exchange for L-malate (Evans *et al.*, 1983) and is cleaved in the cytosol by ATP: citrate lyase to yield acetyl-CoA (and oxaloacetate), from which fatty acids are synthesized.

Certain yeasts, however, have been reported to produce higher concentrations of lipid when grown with an organic, rather than an inorganic, nitrogen source (Woodbine, 1959; Blinc & Hočevár, 1953; Witter *et al.*, 1974). No explanation for this has been forthcoming. In this paper, we have examined the effects of nitrogen sources on the lipid content of various yeasts. From such a study we hope to gain insight into the biochemical processes and control mechanisms operating in oleaginous yeasts and then be able to manipulate these to advantage.

METHODS

Yeasts. The yeasts used for this work were obtained from: the Central Bureau voor Schimmelcultures (CBS), Baarn, Holland; the National Collection of Yeast Cultures (NCYC), Food Research Institute, Norwich, UK; and the Institute for Fermentation (IFO), Osaka, Japan. *Candida curvata* D and *Trichosporon cutaneum* 40 were the kind gift of Professor E. G. Hammond, Iowa State University, USA.

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Abbreviation: GDH glutamate dehydrogenase.

Media. The glucose/salts medium for both batch and continuous culture experiments was that described by Evans *et al.* (1983). Glucose was used as sole carbon source in all experiments at 30 g l^{-1} . All nitrogen sources were added to give 0.13 g N l^{-1} . The pH was adjusted to 5.5 prior to sterilization. All amino acids were sterilized by filtration and then added to media prior to inoculation.

Culture conditions. Yeasts were grown at 30°C as batch cultures either in shake-flask (100 ml medium in 250 ml flask) or 1-litre vortex-aerated vessels without pH control. Continuous cultivation was carried out in a 1-litre chemostat (LH 500 series 11, LH Engineering, Stoke Poges, Bucks., UK) with the pH maintained at 5.5 by the automatic addition of NaOH. Addition of antifoam was maintained at preset intervals using an appropriate metering pump and timing clock. Incoming air was passed through two fibre-glass filters connected in series; the aeration rate was maintained at 1 vol. air per vol. medium per min. The volume within the fermenter was kept constant by using an overflow weir (5 mm diameter). Agitation, with flat-bladed impellers, was at 500 r.p.m. Steady-state conditions were maintained for at least six complete changes of medium in the vessel before alteration.

Biomass and other determinations. Samples (10 ml) were centrifuged at 5000 g for 5 min in preweighed, dried tubes and washed twice with 10 ml distilled water. The pellets were dried at 80°C over P_2O_5 in a vacuum oven to constant weight. Lipid was determined by a method based on that of Folch *et al.* (1957); ammonia was determined by the method of Chaney & Marbach (1962); citrate by the method of Dagley (1974); L-malate by the method of Gutmann & Wahlefeld (1974); DL-isocitrate by the method of Siebert (1974); 2-oxoglutarate by the method of Bergmeyer & Bernt (1974); L-glutamate by the method of Bernt & Bergmeyer (1974); glucose 6-phosphate (and fructose 6-phosphate) by the method of Lang & Michal (1974) and oxaloacetate by the method of Wahlefeld (1974).

Preparation of intracellular extracts (for isolation of metabolites). A HClO_4 digestion of washed cells was carried out according to the procedure described by Evans & Ratledge (1983b).

Preparation of cell extracts. Extracts were prepared by passage through a French pressure cell following the procedure described by Evans & Ratledge (1983b).

Enzyme assays. Protocols for the assay of ATP: citrate lyase (EC 4.1.3.8), NAD^+ and NADP^+ -dependent isocitrate dehydrogenase (EC 1.1.1.41), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), NAD^+ -malate dehydrogenase (EC 1.1.1.37), malic enzyme (EC 1.1.1.40), citrate synthase (EC 4.1.3.7), pyruvate carboxylase (EC 6.4.1.1), pyruvate dehydrogenase (EC 1.2.4.1), aconitase (EC 4.2.1.3) were as described by Evans & Ratledge (1983b). Phosphofructokinase (EC 2.7.1.11) was assayed by the method of Sols & Salas (1966); pyruvate kinase (EC 2.7.1.40) by the method of Bücher & Pfeleiderer (1955), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) by the method of Rutter *et al.* (1966), and NAD^+ - and NADP^+ -dependent glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4) by the method of Doherty (1970). Protein was determined by the method of Bradford (1976). NAD^+ : glutamate dehydrogenase and NADP^+ : glutamate dehydrogenase were partially purified from extracts of glutamate- and NH_4^+ -grown cells, respectively. Cell extracts were fractionated with $(\text{NH}_4)_2\text{SO}_4$ with the 40 to 70% saturation fraction being retained. The fractions were desalted by passage through a $30 \times 2.5\text{ cm}$ column of Sephadex G-50.

Studies with $[\text{U-}^{14}\text{C}]$ glutamate and $[\text{1,2-}^{14}\text{C}]$ acetate. $[\text{U-}^{14}\text{C}]$ Glutamate [$5\text{ }\mu\text{Ci}$; $52.6\text{ }\mu\text{Ci }\mu\text{mol}^{-1}$ ($1.95\text{ MBq }\mu\text{mol}^{-1}$)] or $[\text{1,2-}^{14}\text{C}]$ acetate [$5\text{ }\mu\text{Ci}$; $60\text{ }\mu\text{Ci }\mu\text{mol}^{-1}$ ($2.22\text{ MBq }\mu\text{mol}^{-1}$)] was added to cultures of *Rhodospiridium toruloides* CBS 14 growing in 1-litre vortex-aerated vessels at 30°C . Samples (1 ml) were filtered through Whatman grade A glass-fibre paper discs which had been pre-washed with distilled water. The cells were washed once with 0.9% (w/v) NaCl and once with a buffer containing 40 mM-Tris/HCl pH 7.0, 0.6 M-sorbitol and 0.9 mM-EDTA. The whole filter paper disc was transferred to a glass scintillation vial, dissolved in 1 ml Solute (Packard Instruments) and taken up in 10 ml of a toluene-based scintillation fluid. Lipid was extracted, as previously described, redissolved in chloroform and counted in a toluene-based scintillant. Residual radiolabel was estimated in culture filtrates using a dioxane-based scintillant containing: 4 g diphenyloxazole, 60 g naphthalene, 20 ml ethanediol, 100 ml methanol and 880 ml dioxane.

RESULTS

Effect of nitrogen sources on lipid accumulation in yeasts

The influence of three different nitrogen sources, namely NH_4Cl , asparagine and glutamate, on lipid and biomass production by 17 yeasts is shown in Table 1. Only a small number of the yeasts showed any variation in lipid content; the most significant of these were all strains of *Rhodospiridium toruloides*. Strain CBS 6016 was no exception to this finding, although previously it had been regarded as non-oleaginous because of a failure to detect ATP: citrate lyase in cell extracts (Boulton & Ratledge, 1981). However, this yeast has been re-examined in the light of the above findings and activity of ATP: citrate lyase has been detected (S. Gilbert,

Table 1. *Effect of different nitrogen sources on the production of biomass and lipid by various yeasts*

All yeasts were grown in batch culture in 1-litre vortex-aerated vessels at 30 °C. Nitrogen sources were added to give 0.13 g N l⁻¹. Cultures were harvested after 120 h growth.

Yeast	Nitrogen source . . . NH ₄ Cl		Asparagine		L-Glutamate	
	Biomass	Lipid	Biomass	Lipid	Biomass	Lipid
	(g l ⁻¹)	(%, w/w)	(g l ⁻¹)	(%, w/w)	(g l ⁻¹)	(%, w/w)
<i>Candida curvata</i> D	11.2	33	11.7	37	11.4	35
<i>Candida utilis</i> NCYC 359	8.9	6	8.0	8	9.2	8
<i>Hansenula saturnus</i> CBS 5761	10.4	22	10.4	29	10.4	30
<i>Lipomyces lipofer</i> CBS 944	10.5	38	10.6	35	9.8	38
<i>Lipomyces lipofer</i> CBS 5482	9.5	26	9.4	26	10.4	33
<i>Lipomyces starkeyi</i> CBS 1807	9.4	28	9.9	30	9.0	32
<i>Lipomyces starkeyi</i> CBS 1809	9.9	39	9.8	34	9.4	34
<i>Lipomyces starkeyi</i> CBS 6047	10.2	43	9.2	45	9.9	40
<i>Rhodospiridium toruloides</i> CBS 14	9.9	18	10.8	39	10.9	51
<i>Rhodospiridium toruloides</i> IFO 0559	10.2	33	10.9	42	10.6	49
<i>Rhodospiridium toruloides</i> CBS 5490	11.0	25	10.4	27	10.9	32
<i>Rhodospiridium toruloides</i> CBS 6016	10.9	15	11.2	34	11.0	44
<i>Rhodotorula glutinis</i> NCYC 59	6.9	23	7.2	23	6.6	23
<i>Rhodotorula graminis</i> CBS 5811	8.9	19	7.4	23	7.6	20
<i>Rhodotorula graminis</i> NCYC 502	8.0	24	9.6	29	8.0	27
<i>Saccharomyces cerevisiae</i> NCYC 33*	7.4	4	7.8	5	7.4	4
<i>Trichosporon cutaneum</i> 40	10.2	24	10.2	32	10.9	38

* Non-oleaginous; ATP :citrate lyase negative (cf. Boulton & Ratledge, 1981).

unpublished). The difference in results may be attributable to an unstable genetic make-up of this strain, which is a cross between two other strains of *Rs. toruloides*.

Growth on the different nitrogen sources had little effect on total biomass production and did not significantly decrease lipid production in any of the yeasts tested. In the cases where the lipid contents did vary, glutamate gave the highest increases. The largest increase in lipid content was found with *Rs. toruloides* CBS 14, in which it increased almost threefold, from 18% to 51% of the biomass. Figure 1 shows the striking change in cell morphology when the yeast was grown under the different conditions. This yeast was consequently chosen as a model organism for the investigation of this phenomenon.

The effect of a range of nitrogen sources on lipid accumulation by *Rs. toruloides* CBS 14 is shown in Table 2. All 20 nitrogenous compounds were able to support growth; the range in variation in final biomass concentration was only 1.7 g l⁻¹. The final lipid contents varied considerably, however. Inorganic nitrogenous compounds, including ammonium tartrate, produced less than 25% (w/w) lipid, whilst 12 of the organic sources increased lipid production to over 40% of the biomass.

Lipid accumulation by Rs. toruloides CBS 14 in batch culture

Figure 2 shows the patterns of lipid accumulation of *Rs. toruloides* CBS 14 grown with NH₄Cl or L-glutamate as nitrogen source. The time course of nitrogen exhaustion from the medium and biomass and lipid production were similar to those observed previously with *Rhodotorula* spp. (Enebo *et al.*, 1946; Kessell, 1968) and other oleaginous yeasts (Evans & Ratledge, 1983a). Up until 30 h growth, both culture profiles were similar but then changed abruptly as the glutamate culture began to accumulate substantially more lipid, which eventually reached 52% of the biomass.

The specific activities of 15 enzymes in cells from both NH₄⁺- and glutamate-grown cultures were determined to see if there were any obvious differences in the pathways of glucose catabolism and citrate metabolism which could relate to the different lipid contents of the cells

Fig. 1. *Rhodosporidium toruloides* CBS 14 grown on glucose with (a) NH_4^+ , lipid content 18%; (b) glutamate, lipid content 52%. Both photomicrographs taken without staining, negative phase, magnification = $\times 1250$. The low-lipid cells contain two small oil droplets, one at each end of the cell. The high-lipid cells show the two much larger oil droplets.

Table 2. *Effect of various nitrogen sources on lipid accumulation by Rs. toruloides* CBS 14

Cultures were grown in 250 ml shake flasks for 6 d at 30 °C with each nitrogen source at 0.13 g N l⁻¹.

Nitrogen source	Biomass (g l ⁻¹)	Lipid (%, w/w)	Nitrogen source	Biomass (g l ⁻¹)	Lipid (%, w/w)
NH_4Cl	9.9	18	Citrulline	9.2	44
$(\text{NH}_4)_2\text{SO}_4$	9.4	18	Threonine	10.4	46
Ammonium tartrate	9.8	20	Aspartate	9.5	46
NaNO_3	9.0	21	Asparagine	9.9	46
Leucine	8.7	29	Ornithine	9.2	48
Serine	9.2	30	Proline	9.8	49
Lysine	8.9	32	Allantoin	9.0	51
Glycine	9.6	38	Urea	10.3	52
Alanine	9.9	42	Glutamate	10.0	52
Glutamine	10.1	43	Arginine	9.8	54

(Table 3). However, no significant variation in the activity of any enzyme was found. The intracellular concentrations of numerous intermediary metabolites throughout the progress of the batch cultures are given in Fig. 3(a-h). The excretion of citrate into the medium was fivefold higher in the glutamate-grown culture than in the NH_4^+ -grown culture. Citrate excretion, as observed previously with *C. curvata* (Evans & Ratledge, 1983b) and with *Lipomyces starkeyi* (Boulton & Ratledge, 1983), did not begin until nitrogen had become exhausted from the

Fig. 2. Growth and lipid accumulation by *Rs. toruloides* CBS 14 in batch culture. The medium contained 30 g glucose l⁻¹ as carbon source and the nitrogen sources were : (a) NH₄Cl (0.5 g l⁻¹) and (b) L-glutamate (1.03 g l⁻¹). ○, Glucose; ●, biomass; □, lipid; ■, nitrogen source.

Table 3. Activities of cytoplasmic and mitochondrial enzymes in *Rs. toruloides* CBS 14

Cells were harvested after 40 h growth and separated into mitochondrial and cytosolic fractions as described by Evans *et al.* (1983). Specific activities are expressed in nmol substrate converted (mg protein)⁻¹ min⁻¹.

Enzyme	NH ₄ ⁺ -grown cells		Glutamate-grown cells	
	Cytosol	Mitochondria	Cytosol	Mitochondria
ATP:citrate lyase	74	0	82	0
NAD ⁺ :isocitrate dehydrogenase	0	124	3	109
NADP ⁺ :isocitrate dehydrogenase	98	0	102	0
NAD ⁺ :glutamate dehydrogenase	42	12	64	24
NADP ⁺ :glutamate dehydrogenase	423	78	546	59
Glucose-6-phosphate dehydrogenase	699	11	771	0
NAD ⁺ :malate dehydrogenase	1878	2673	2006	3015
Malic enzyme	79	0	106	0
Citrate synthase	41	5010	49	4973
Pyruvate carboxylase	4	89	0	12
Pyruvate dehydrogenase	0	112	0	119
Aconitase	0	83	0	79
Phosphofructokinase	142	0	199	0
Pyruvate kinase	897	6	1066	10
Aldolase	949	4	994	8

medium (25–30 h) and, by 40 h growth, eight times as much citrate had been excreted by the glutamate-grown cells as compared with the NH₄⁺-grown cells (see Fig. 3a). The intracellular concentrations of citrate also varied considerably between the two cultures (Fig. 3b). The concentration of citrate increased steadily over the first 40 h growth in the glutamate-grown cells, reaching a peak value which was 14 times that in the NH₄⁺-grown cells. After 40 h, the

Fig. 3. Concentrations of metabolites in cells of *Rs. toruloides* CBS 14 during batch culture. Closed circles, NH_4^+ -grown cells; open circles, glutamate-grown cells. (a) Extracellular citrate. Intracellular concentrations of metabolites: (b) citrate, (c) malate, (d) isocitrate. (e) NH_4^+ , (f) 2-oxoglutarate, (g) L-glutamate, (h) glucose 6-phosphate.

concentration of citrate in the intracellular pool decreased steadily until both cultures contained a basal concentration of citrate.

The intracellular concentration of malate was threefold higher in the glutamate-grown cells

than in the NH_4^+ -grown cells during the first 30 h growth but then decreased throughout the rest of growth, as it did in the NH_4^+ -grown cells (Fig. 3c). Interestingly, the concentration of malate throughout growth was always higher than the concentration of citrate, in both cultures. The intracellular concentration of isocitrate was low and almost the same in both cultures, with a peak occurring after 50 h growth (Fig. 3d). Oxaloacetate and fructose 6-phosphate (not shown in Fig. 3) were present only in trace amounts (<0.2 nmol per mg cell dry wt) in the cell extracts.

The most significant difference between the two cultures was in the contents of intracellular NH_4^+ (Fig. 3e). Cells from the NH_4Cl culture contained six times as much NH_4^+ during the first 15 h growth as did glutamate-grown cells, but this concentration then rapidly decreased to a basal level by 30 h growth. Conversely, the NH_4^+ content of glutamate-grown cells increased rapidly between 20 h and 30 h, after which it declined steadily to a basal level. This may be attributable to a rapid breakdown of the glutamate nitrogen source within the cells as the patterns of accumulation of NH_4^+ and 2-oxoglutarate in glutamate-grown cells were similar (Fig. 3e,f) and coincided with the disappearance of glutamate from the medium, which was readily accumulated by the glutamate-grown cells in the first 10 h of growth (see Fig. 3g). The concentration of 2-oxoglutarate in both NH_4^+ - and glutamate-grown cells increased steadily until 25 h growth when it was twofold higher in the glutamate-grown cells. The 2-oxoglutarate content of the NH_4^+ -grown cells then rapidly declined, so by 30 h that of the glutamate-grown cells was ninefold higher. The 2-oxoglutarate content of glutamate-grown cells remained twofold greater than that of NH_4^+ -grown cells throughout the rest of growth. Glutamate clearly underwent rapid catabolism in glutamate-grown cells, as only 10% of that originally accumulated remained by 30 h. The NH_4^+ -grown cells appeared to increase their glutamate pool during the early stages of growth but after 20 h this also declined to a third that of the glutamate culture (Fig. 3g).

The only other metabolite to show any significant variation throughout growth was glucose 6-phosphate (Fig. 3h). Between 20 h and 30 h growth, the cells from both cultures contained only trace amounts of this metabolite (which is what one could expect of a glycolytic intermediate during growth); however, the concentration of glucose 6-phosphate in NH_4^+ -grown cells rapidly increased between 30 and 60 h, whilst in glutamate-grown cells the concentration did not rise until 50 h growth. It must be stressed, however, that the concentrations of metabolites observed under these conditions have been determined in a dynamic system and so reflect flux patterns of intermediates during metabolism, where an increased flux and decreased metabolism would lead to a build-up of the metabolite in question.

L-Glutamate metabolism in Rs. toruloides CBS 14

It was considered possible that the high lipid accumulation in glutamate-grown *Rs. toruloides* CBS 14 was being directly caused by the nitrogen source itself, i.e. glutamate, or by one, or more, of the intermediates of its catabolism. If all the carbon from the added glutamate in a given culture was metabolized to lipid, it could only account for approximately 1 g of lipid (i.e. less than 20% of the total lipid). Addition of [$\text{U-}^{14}\text{C}$]glutamate to cultures of *Rs. toruloides* CBS 14 showed that glutamate carbon was indeed metabolized to lipid (Table 4). [$1,2\text{-}^{14}\text{C}$]Acetate was used as a control for the efficiency of metabolism and incorporation of a radiolabelled substrate into lipid. Of the glutamate carbon taken into the cell, 27% was recovered in the lipid fraction, indicating that not only was glutamate acting as a source of NH_4^+ (see Fig. 3e) but that the resultant 2-oxoglutarate was being actively metabolized by the cells. However, with only partial incorporation of the carbon atoms of glutamate into lipid, it was apparent that the increase in lipid concentration must be due to increased metabolic activity arising indirectly from glutamate being used as nitrogen source, rather than glutamate acting as a direct precursor of the lipid.

NAD^+ :glutamate dehydrogenase (NAD^+ :GDH) was probably responsible for the reciprocal changes in glutamate concentration with those of NH_4^+ and 2-oxoglutarate. Its specific activity was tenfold higher in glutamate-grown cells than in NH_4^+ -grown cells (Fig. 4a) in the early growth phase of the culture, i.e. between 20 h and 30 h. After this time, the activity

Table 4. Incorporation of [U - ^{14}C]glutamate and [$1,2$ - ^{14}C]acetate into lipid of *Rs. toruloides* CBS 14

Pulses (5 μ Ci) of radiolabelled glutamate and acetate were fed to batch cultures in vortex-aerated vessels after 35 h growth. Uptake and incorporation of radiolabel into cells and lipid was followed as described in Methods.

Culture	Time after addition of radiolabel (min)	Incorporation of radiolabel into cells (%)		Incorporation of radiolabel into lipid (%)	
		From glutamate	From acetate	From glutamate	From acetate
NH_4Cl -grown cells	10	59.7	70.4	8.2	37.9
	30	68.9	88.7	9.9	40.2
	45	85.5	97.5	10.9	40.9
L-Glutamate-grown cells	10	71.4	74.3	12.4	41.6
	30	80.8	89.1	21.8	42.8
	45	84.2	96.8	27.5	44.8

Fig. 4. Activities of the glutamate dehydrogenases of *Rs. toruloides* CBS 14 during batch culture with NH_4^+ and glutamate as nitrogen sources. (a) NAD^+ : glutamate dehydrogenase, (b) $NADP^+$: glutamate dehydrogenase. ●, NH_4^+ -grown cells; ○, glutamate-grown cells.

rapidly declined to a level similar to that found in NH_4^+ -grown cells. The peak in activity correlated with the depletion of the early intracellular pool of glutamate (Fig. 3g) and the concomitant production of NH_4^+ (Fig. 3e) and 2-oxoglutarate (Fig. 3f) in glutamate-grown cells. In NH_4^+ cultures, the activity of the anabolic $NADP^+$: glutamate dehydrogenase ($NADP^+$: GDH) increased markedly over the first 30 h growth and was approximately threefold higher than that in glutamate-grown cells (Fig. 4b). This activity in NH_4^+ -grown cells can therefore account for the low concentrations of intracellular NH_4^+ detected during the early stages of growth (20–30 h) in NH_4^+ -grown cells (Fig. 3e).

The kinetic properties of the NAD^+ - and $NADP^+$ -linked glutamate dehydrogenases are shown in Table 5. Although both reactions are reversible, the catabolic role of the NAD^+ : GDH

Table 5. Kinetic parameters of NAD^+ :glutamate and NADP^+ :glutamate dehydrogenases in *Rs. toruloides* CBS 14

Substrate	Apparent K_m (mM)	
	NAD^+ :GDH	NADP^+ :GDH
L-Glutamate	3.7	27.9
2-Oxoglutarate	9.2	1.4
NH_4^+	26.5	13.0
NAD^+	0.8	ND
pH optimum	8.2	8.6

ND, not determined.

Fig. 5. Growth and lipid production by *Rs. toruloides* CBS 14 in continuous culture on nitrogen-limited media with (a) NH_4Cl , (b) L-glutamate as nitrogen sources. ●, Biomass; ○, lipid.Table 6. Intracellular concentrations of metabolites in *Rs. toruloides* CBS 14 grown in continuous culture under nitrogen-limited conditions

Glucose (30 g l^{-1}) was used as carbon source with either NH_4Cl (0.5 g l^{-1}) or L-glutamate (1.05 g l^{-1}) as nitrogen source. D , dilution rate (h^{-1}). Concentrations are given as $\text{nmol (mg cell dry wt)}^{-1}$.

Metabolite	Concn in NH_4^+ -grown cells		Concn in glutamate-grown cells	
	$D = 0.02 \text{ h}^{-1}$	$D = 0.08 \text{ h}^{-1}$	$D = 0.02 \text{ h}^{-1}$	$D = 0.08 \text{ h}^{-1}$
Citrate	3.2	0	4.8	0
Malate	2.8	0	1.7	0
Isocitrate	1.0	2.4	2.0	3.7
2-Oxoglutarate	4.5	1.0	11.8	2.5
L-Glutamate	4.0	6.6	6.5	15.5
NH_4^+	1.5	7.5	2.5	5.5
Glucose 6-phosphate	0	1.0	0	2.0
Fructose 6-phosphate	0	0	0	1.5
Oxaloacetate	0	0	0.5	0
Extracellular citrate	2.0	0	2.0	0

and anabolic function of the $\text{NADP}^+:\text{GDH}$ in *Rs. toruloides* CBS 14 are clearly expressed by their very high apparent K_m values for NH_4^+ and glutamate, respectively. These results are not dissimilar to those reported for other yeasts (Brown, 1980; Brown *et al.*, 1974).

Growth and lipid production by Rs. toruloides CBS 14 in continuous culture

When *Rs. toruloides* CBS 14 was grown in a chemostat under nitrogen-limitation, the effect of L-glutamate on lipid production was lost (Fig. 5). Biomass and lipid accumulation were similar in both NH_4^+ - and glutamate-grown cultures over a range of dilution rates. The intracellular concentrations of key metabolites were monitored under steady-state conditions at two dilution rates in both cultures (see Table 6). The concentrations of most metabolites were close to the basal levels detected during batch cultures. Metabolites which accumulated to high concentrations in the glutamate-growing batch culture, i.e. NH_4^+ , L-glutamate, 2-oxoglutarate and citrate, were all found in low concentrations in the steady-state system of the chemostat.

DISCUSSION

A survey of the effect of various nitrogen sources on lipid accumulation in yeasts confirmed the early reports that certain yeasts produced much higher lipid contents when grown with an organic nitrogen source, rather than an NH_4^+ salt (Blinč & Hočevár, 1953; Woodbine, 1959). A comparison of the growth of *Rs. toruloides* CBS 14, as a yeast showing this effect to the greatest degree of those examined, revealed that metabolism in glutamate-grown cells was significantly different from that in NH_4^+ -grown cells. Accumulation and excretion of citric acid was far more pronounced in the glutamate-grown cultures, which indicated that the flux of carbon to citrate in glutamate-grown cells was somehow being stimulated. The much higher concentrations of intracellular NH_4^+ and 2-oxoglutarate in glutamate-grown cells showed that catabolism of the glutamate was occurring and that these products might be indirectly responsible for the increase in cellular citrate accumulation and excretion.

In the steady-state conditions of continuous culture, the intracellular concentrations of all these metabolites in glutamate-grown cells were very low and consequently the lipid content was as low as that in NH_4^+ -grown cells. This was presumably due to the fact that under nitrogen limitation, in the steady-state system, the amount of glutamate available per cell at the slow dilution rates was very low compared to the first 30 h of batch culture. The study of the chemostat culture thus revealed that the increase in lipid in batch culture cells was due to a transitory phenomenon – probably the build-up of NH_4^+ (or 2-oxoglutarate) in the early stages of growth. Such a transitory state cannot, of course, arise in a chemostat under normal conditions of operation.

The difference in concentrations of NH_4^+ , 2-oxoglutarate and glutamate in cells of the batch cultures of *Rs. toruloides* CBS 14 grown on NH_4Cl and on glutamate can be explained by the interplay in activity of the glutamate dehydrogenases observed during growth. Synthesis of the catabolic $\text{NAD}^+:\text{GDH}$ is derepressed (or induced) by growth on glutamate and its increased activity leads to the formation of intracellular NH_4^+ (Roon & Even, 1973; Pateman and Kinghorn, 1976). Conversely, the activity of the anabolic $\text{NADP}^+:\text{GDH}$ increased markedly in NH_4^+ -grown cells and would consequently prevent accumulation of NH_4^+ (Brown *et al.*, 1974; Brown 1980; Roon & Even, 1973). The decrease in activity of the $\text{NAD}^+:\text{GDH}$ in glutamate-grown cells after 30 h growth could be due either to its repression by NH_4^+ (Bernhardt *et al.*, 1965; Roon & Even, 1973) or to its rapid inactivation by reversible phosphorylation (Hemmings, 1978*a, b*, 1982). This would then prevent further catabolism of glutamate and build-up of NH_4^+ . The subsequent decrease in the NH_4^+ pool is then attributable to the increase in $\text{NADP}^+:\text{GDH}$ activity.

These results do not fully explain how such a large NH_4^+ pool accumulates in the first place. Possibly the various mechanisms of NH_4^+ assimilation in the cells are unaffected by growth on glutamate and hence operate only as efficiently as in NH_4^+ -grown cells. However, accumulation of glutamate from the medium and large increase in $\text{NAD}^+:\text{GDH}$ activity evidently release NH_4^+ faster than it can be assimilated, consequently increasing the pool size for a short period until the metabolism becomes adjusted.

It is possible that catabolism of the nitrogen source in the medium, to increase pool concentrations of metabolites such as NH_4^+ , 2-oxoglutarate or glutamate, can explain how numerous other organic nitrogen sources markedly stimulate lipid production (Table 2), and these aspects are described in detail in the following paper (Evans & Ratledge, 1984). The importance of these metabolites and the mechanisms by which they interact with the pathways of lipid biosynthesis in yeasts have been investigated, and these findings will be reported in due course.

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