Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*

James P. Wynn,[1] Adil A. Hamid,[2] Yonghua Li[1] and Colin Ratledge[1]

Author for correspondence: James P. Wynn. Tel: +44 1482 465507. Fax: +44 1482 465458.
e-mail: j.p.wynn@biosci.hull.ac.uk

[1] Centre for Advanced Lipid Research, Department of Biological Sciences, University of Hull, Hull HU6 7RX, UK

[2] Department of Microbiology, Universitii Kebangsaan, Malaysia, 43 600 Bangi, Salangor Darul Ehsan, Malaysia

The biochemical events associated with the onset of lipid accumulation in *Mucor circinelloides* and *Mortierella alpina*, under conditions of nitrogen-limited growth, have been elucidated; they differ in key aspects from those described in oleaginous yeasts. The NAD$^+$:isocitrate dehydrogenases of *Mc. circinelloides* and *Mort. alpina* were not absolutely dependent on AMP for activity. Furthermore, changes in the cellular adenine nucleotide pools and energy charge were different from those reported for oleaginous yeasts. In *Mc. circinelloides* ATP, ADP and AMP concentrations all decreased by 50% after nitrogen limitation, leading to a constant energy charge at the expense of the size of the total adenylate pool. Pyruvate carboxylase in *Mc. circinelloides* was cytosolic, having implications for the organization of lipid synthesis in filamentous fungi. As a result of the data obtained, a revised and more concerted mechanism for the initiation of storage lipid accumulation is put forward for filamentous fungi.

INTRODUCTION

As the nutritional benefits of certain long-chain polyunsaturated fatty acids are becoming appreciated so the demand for oils rich in these fatty acids is expanding. In particular, the roles of arachidonic acid [20:4n-6] (ARA) and docosahexaenoic acid [22:6n-3] (DHA) in neonate brain and nervous development have been reported and this has resulted in the inclusion of these fatty acids in baby formulae (Kyle, 1997). As amenable plant and animal sources for these fatty acids do not exist, oils rich in these fatty acids are being produced commercially using oleaginous micro-organisms. Arachidonic acid is being produced using the zygomycete fungus *Mortierella alpina* and DHA is being produced using the marine algae *Crypthecodinium cohnii* and *Schizochytrium* spp. (Ratledge, 1997; Wynn, 1998).

In attempts to optimize these processes and to realize new ones, a sound understanding of the regulation of cell lipid accumulation in the production organism is

required. Although the biochemical basis of microbial oleaginicity has been elucidated this work has been carried out almost exclusively in yeasts (Botham & Ratledge, 1979; Boulton & Ratledge, 1983; Evans & Ratledge, 1983, 1985a, b). A similar system has been tacitly assumed to apply to other groups of eukaryotic micro-organisms.

All the enzymes thought to be crucial for the accumulation of substantial amounts of storage lipid are present in both oleaginous yeasts and filamentous fungi. However, it has become apparent in the course of our more recent work that the regulation of lipogenesis may differ between filamentous fungi and yeasts. In the present study *Mucor circinelloides* was used as the principal model for oleaginous filamentous fungi. However, because of its commercial importance *Mort. alpina* was also included in order to verify the key results. This report indicates a number of significant differences in the biochemistry of lipid accumulation between oleaginous yeasts and oleaginous filamentous fungi. A revised and more concerted mechanism for the initiation of storage lipid synthesis when filamentous fungi experience N-limiting conditions is put forward. These findings have implications not only for the production of single-cell

oils but also for other commercial fermentations using filamentous fungi (e.g. those producing citric acid and gibberellic acid) in which lipid synthesis represents an undesirable by-product and therefore an unnecessary waste of substrate.

METHODS

Chemicals. Unless otherwise stated all fine chemicals were purchased from Sigma.

Cultivation of fungi. *Mucor circinelloides* CBS 108.16 and *Mortierella alpina* Peyron CBS 696.70 were initially cultivated in 1 litre magnetically stirred bottles containing 800 ml Kendrick medium (N-limiting) (Kendrick & Ratledge, 1992). These cultures were incubated for 16 h at 30 °C then used at 10% (v/v) to inoculate 4 litre (working volume) fermenters containing modified Kendrick medium containing ammonium tartrate at 2 g l^{-1} for both fungi, with glucose at 50 g l^{-1} for *Mc. circinelloides* and 30 g l^{-1} for *Mort. alpina*. Fermenters were incubated at 30 °C, stirred at 500 r.p.m. with aeration at 0·5 vol. vol.$^{-1}$ min^{-1} and pH maintained at 5·5 by automatic addition of KOH and HCl.

Production of cell extracts. Biomass was harvested by filtration (under reduced pressure) through a Whatman no. 1 filter and washed with cold distilled water. Cell extracts for the determination of enzyme activities were prepared by suspending mycelia in an extraction buffer (Wynn *et al.*, 1998) and disrupted either by passage twice through a French pressure cell at 35 MPa or by a single pass through a 'One Shot' cell disrupter (Constant Systems) at 640 MPa. Disrupted cell suspensions were centrifuged at 10 000 g for 10 min and the supernatant retained for enzyme analysis. Protein concentrations were determined using the method of Bradford (1976) with BSA as a standard.

Preparation of spheroplasts and isolation of mitochondria. *Mc. circinelloides* was grown in 1 litre stirred bottles, as described above. Harvested biomass was washed twice with spheroplasting buffer (50 mM Tris/HCl, pH 6·5, containing 1·2 M sorbitol) then suspended in the same buffer containing chitinase/chitosanase preparation (Vanheeswijck, 1984) at 2 mg ml^{-1} and incubated for 3 h at 30 °C. The treated biomass was collected by centrifugation (10 000 g for 10 min at 4 °C) and the spheroplasts formed were disrupted by resuspension in 50 mM Tris/HCl (pH 7·5) and incubated at 20 °C for 1 h. The disrupted cells were fractionated by centrifugation at 5000 g for 10 min and then the supernatant centrifuged at 16 000 g for 20 min. The cell preparations were maintained at 4 °C throughout. The supernatant and the pellet formed at 16 000 g were retained as the 'cytosolic' and 'mitochondrial' fractions respectively.

Estimation of enzyme activities. NAD^{+}:isocitrate dehydrogenase (NAD:ICDH) was assayed using the method of Kornberg (1955). AMP deaminase activity was assayed using a reaction volume containing 100 mM KH$_2$PO$_4$/KOH (pH 7·1), 2 mM MgCl$_2$, 0·5 mg BSA ml^{-1}, 2 mM NaH$_2$PO$_4$, 2 mM ATP (pH 7·1), 5 mM AMP. The assay volume was incubated for 10 to 30 min at 30 °C and the NH$_4^+$ liberated was quantified using the indophenol method (Chaney & Marbach, 1962). Adenylate kinase was determined using a method based on that of Sottocasa *et al.* (1967). The reaction volume contained 67 mM Tris/HCl (pH 7·5), 5 mM ADP, 0·5 units hexokinase/glucose-6-phosphate dehydrogenase mix (from baker's yeast), 5 mM MgSO$_4$, 0·2 mM NADP$^+$ and 10 mM glucose. The increase in A_{340} was measured. Phosphofructokinase (PFK) was assayed as described by Sols & Salas (1966), citrate

synthase as described by Parvin (1969), succinate dehydrogenase as described by Schwitzguebel *et al.* (1981), pyruvate kinase as described by Worthington Enzymes (1979) and pyruvate carboxylase as described by Seubert & Weicker (1969). The assay reactions for citrate synthase, succinate dehydrogenase, pyruvate kinase and pyruvate carboxylase were supplemented with 0·1% (w/v) Triton X-100.

Determination of nucleotide concentrations. The concentrations of adenine nucleotides (ATP, ADP and AMP) were determined using the luciferin/luciferase system to quantify ATP (Speilmann *et al.*, 1982). Samples (20 ml) from the fermenter were rapidly quenched (< 0·5 s) by collection in sterile Universal tubes containing 4 ml conc. H$_2$SO$_4$. Quenched samples were diluted 1:10 with 5 mM glycylglycine/KOH buffer (pH 7·8) and, if necessary, the pH adjusted to 7·8. Cell debris was removed by centrifugation at 10 000 g for 10 min and then the supernatant was diluted 1:10 with glycylglycine buffer. The diluted supernatant was either analysed immediately or stored at −20 °C for a maximum of 2 weeks prior to analysis. ATP was quantified by adding 50 μl of the sample prepared above to 50 μl 100 mM glycylglycine buffer (pH 7·8) containing 5 mM MgSO$_4$, 0·5 mM phospho*enol*pyruvate and 100 μl ATP assay mix (Sigma). ADP was quantified by conversion to ATP in the same reaction mixture as described above but with the addition of 1 unit pyruvate kinase (type 1: crude preparation from rabbit muscle) and incubated at 37 °C for 10 min prior to the addition of the ATP assay mix. ADP was calculated by subtraction of the amount of ATP detected previously. AMP was likewise quantified by its conversion to ATP and then subtraction of the amount of ATP + ADP detected in the sample. AMP was converted to ATP by addition of 1 unit myokinase (from porcine muscle) and 1 unit pyruvate kinase followed by a 10 min incubation at 37 °C prior to the addition of the ATP assay mix.

Determination of culture dry weight. A 20 ml sample of the culture was harvested on to a washed, dried and pre-weighed filter (Whatman no. 1). The filtrate was retained and analysed for culture glucose and ammonium concentrations (see below). Harvested biomass was washed with distilled water and then dried at 110 °C to constant weight. The weight of the biomass was determined gravimetrically.

Analysis of the culture supernatant. The glucose concentration in the culture medium was determined using a GOD-Perid test kit (Boehringer Mannheim) according to the manufacturer's instructions. The ammonium concentration in the culture filtrate was determined using the indophenol test (Chaney & Marbach, 1962).

Analysis of intracellular metabolites. Samples were collected into tubes containing 4 ml 5·8 M HClO$_4$. Samples were centrifuged at 10 000 g for 10 min and the supernatant adjusted to pH 7·4 with NaOH. The isocitrate and citrate concentrations in the samples were determined as described by Siebert (1974) and Dagley (1974) respectively. Samples of culture medium were also analysed for isocitrate and citrate to allow corrections to be made for extracellular metabolites.

Determination of culture dO$_2$ and CO$_2$ evolution. The dissolved oxygen concentration (dO$_2$) of the culture medium in the fermenters was continuously recorded using galvanic O$_2$ electrodes (Mettler-Toledo, Woburn, MA, USA) as a percentage of the O$_2$ concentration in the equilibrated culture medium immediately prior to inoculation. The CO$_2$ evolution by the fungal cultures was determined by measuring the CO$_2$ in the fermenter exhaust using a CO$_2$ analyser (Analytical Development Co., Hoddesdon, UK).

2858

RESULTS

The dO_2 in *Mc. circinelloides* cultures decreased during the period of active cell growth in the presence of NH_4^+ in the culture medium (Fig. 1). After N-exhaustion the dO_2 recovered to an equilibrium point between 70% and 80%. The CO_2 content of the fermenter exhaust gas exhibited similar but opposite trends to the dO_2 data, with CO_2 concentration in the exhaust gas increasing during active growth (i.e. prior to N-exhaustion) then decreasing as the culture experienced N-limitation. Changes in CO_2 evolution were rapid, occurring within minutes of the exhaustion of the N-source.

The NAD:ICDH activities of two oleaginous fungi, *Mc. circinelloides* and *Mort. alpina*, were examined at a range of isocitrate and AMP concentrations *in vitro* (see Fig. 2a, b). The fungal enzymes were not dependent on AMP for activity in the presence of saturating levels of isocitrate. At low isocitrate concentrations AMP increased the affinity of the NAD:ICDH for isocitrate. The K_m of NAD:ICDH for isocitrate in the presence of 1 mM AMP was tenfold lower in *Mc. circinelloides* (from 3·2 mM to 0·3 mM) and twofold lower in *Mort. alpina* (from 4·6 mM to 2·7 mM) than when determined in the absence of AMP. As a result of the increased affinities for isocitrate of the NAD:ICDHs from the fungi in the presence of AMP, activity was detectable and maximal at lower concentrations of isocitrate when AMP was present in the system (see Fig. 2a, b).

Attempts to determine the intracellular concentration of isocitrate in *Mc. circinelloides* failed to detect this metabolite at any stage of growth, the limit of detection being $< 1·5$ nmol (mg dry wt)$^{-1}$. This corresponded to an intracellular concentration of less than 1 mM (see
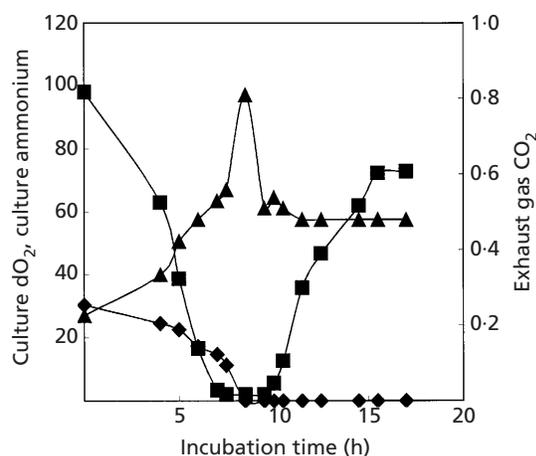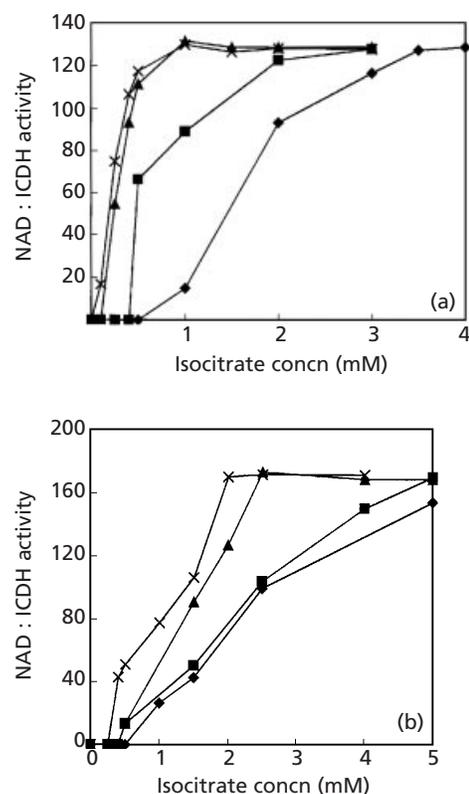

Fig. 2. Effect of isocitrate and AMP concentration on the activity of NAD$^+$:ICDH from (a) *Mc. circinelloides* and (b) *Mort. alpina*. The activity of NAD$^+$:ICDH [nmol min^{-1} (mg protein)$^{-1}$] in a dialysed cell extract was measured, *in vitro*, in the presence and absence of AMP. This experiment was repeated three times and representative data are presented. AMP concentrations: ◆, 0 mM; ■, 0·1 mM; ▲, 0·5 mM; ×, 1·0 mM.

Fig. 1. Effect of N-exhaustion on O_2 consumption and CO_2 evolution by *Mc. circinelloides*. The dO_2 in the culture medium and CO_2 in the exhaust gas were determined during growth of *Mc. circinelloides* in a 4 litre fermenter under batch conditions on a medium with a high C:N ratio. This experiment was repeated three times and representative data are presented. ◆, Culture ammonium tartrate concentration (g l^{-1}); ■, dO_2 (% original saturation); ▲, exhaust gas CO_2 (%, v/v).

Discussion). In contrast, intracellular citrate was detectable both before and after N-exhaustion. A fourfold increase in the citrate concentration, from $3·7 \pm 6$ nmol (mg dry wt)$^{-1}$ (mean $\pm$ SEM; $n = 3$) to $16·1 \pm 4·7$ nmol (mg dry wt)$^{-1}$ ($n = 5$), occurred after N-exhaustion, corresponding to an intracellular citrate concentration of 1–2 mM prior to N-exhaustion and 4–8 mM after N-exhaustion.

When the activity of AMP deaminase was determined, in triplicate batch cultures of *Mc. circinelloides*, an increase in activity was reproducibly observed at the point when the cultures became N-limited and was coincident with the onset of lipid accumulation (see Fig. 3a). The increase in activity of AMP deaminase was, however, transitory and activity returned to basal levels within 5 h of N-exhaustion (see Fig. 3a). Similar data were obtained with *Mort. alpina* (Fig. 3b).

Throughout the cultivation period, the activity of adenylate kinase was high in comparison to the activity of AMP deaminase and it increased over threefold after the depletion of the culture N-source. The specific activity of adenylate kinase in *Mc. circinelloides* was 518 ± 154 nmol min^{-1} (mg protein)$^{-1}$ ($n = 5$) prior to N-
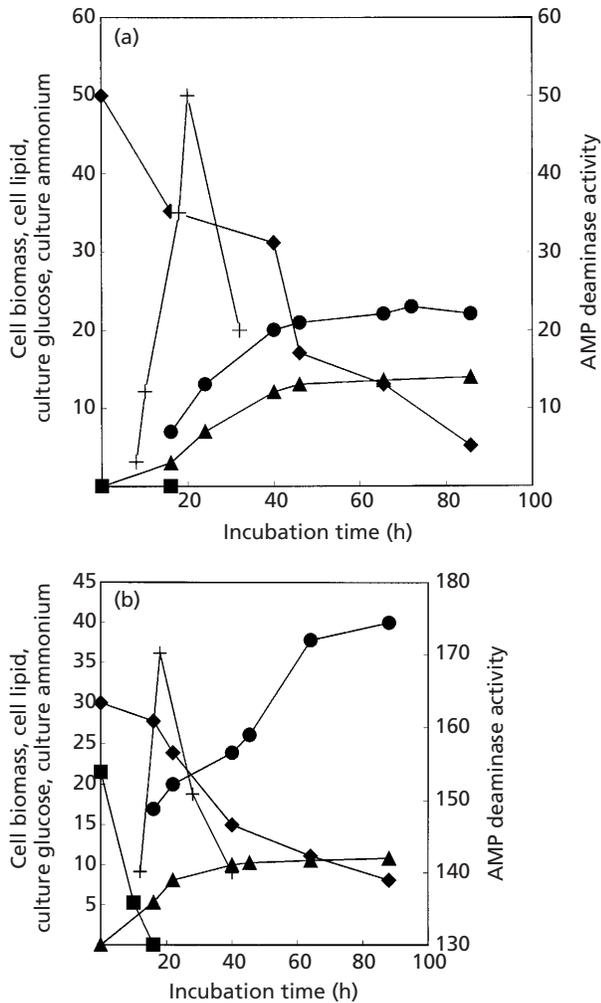
Fig. 3. Changes in cell growth and AMP deaminase activity in response to depletion of the N-source in (a) *Mc. circinelloides* and (b) *Mort. alpina* in a 4 litre fermenter. The AMP deaminase activity [nmol min^{-1} (mg protein)$^{-1}$] was determined at intervals during growth in a 4 litre fermenter under batch conditions on a medium with a high C:N ratio. This experiment was repeated three times and representative data are presented. ▲, Cell biomass (g l^{-1}); ●, cell lipid (%, w/v, dry wt); ◆, culture glucose concentration (g l^{-1}); +, AMP deaminase activity [nmol min^{-1} (mg protein)$^{-1}$]; ■, culture ammonium concentration (g l^{-1}).

exhaustion and 1739 ± 226 nmol min^{-1} (mg protein)$^{-1}$ ($n = 4$) after N-exhaustion.

The stimulation of AMP deaminase and adenylate kinase activities, at the time of N-exhaustion, led to an almost immediate decrease in the concentrations of ATP, ADP and AMP. The concentrations of all three adenine nucleotides decreased by 50% during the 2–3 h period immediately after N-exhaustion (see Fig. 4). ADP was the most abundant nucleotide [concentration varying from 3·7 to 1·1 nmol (mg dry wt)$^{-1}$], followed by ATP [between 1·6 and 0·5 nmol (mg cell dry wt)$^{-1}$]. The AMP concentration was consistently the lowest [0·9–0·4 nmol (mg dry wt)$^{-1}$]. Although the cellular total

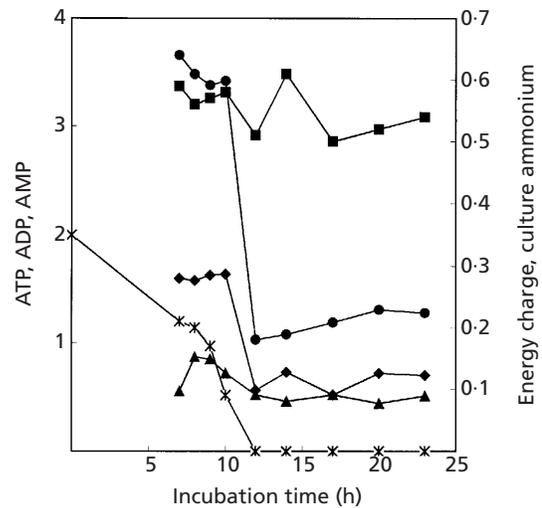nucleotide concentration decreased by 50% at the point when the cells became growth-limited by the depletion of the N-source, the cellular energy charge remained relatively constant at between 0·5 and 0·6 as there were concomitant changes in all three adenine nucleotides.

Both citrate and NH$_4^+$ affected PFK activity in *Mc. circinelloides*, though in an antagonistic fashion (Fig. 5). PFK activity, *in vitro*, was increased 50% by 5 mM NH$_4^+$. However, higher concentrations of NH$_4^+$ reversed the stimulation so that at 15 mM NH$_4^+$ the activity of PFK was equivalent to the activity in the absence of NH$_4^+$. Citrate, in contrast, inhibited the activity of PFK *in vitro*, causing a 50% decrease in activity at 5 mM and an abolition of activity at 15 mM. The effect of NH$_4^+$, in the presence of citrate, on the activity of PFK confirmed the antagonistic affect of these two compounds. In the presence of 1 mM citrate, the stimulation of PFK by 4 mM NH$_4^+$ was maximal and only half that observed in the absence of citrate.

When a cell-free extract from *Mc. circinelloides* was subjected to subcellular fractionation, the activity of pyruvate carboxylase [21 nmol min^{-1} (mg protein)$^{-1}$] was recovered in the soluble fraction along with that of pyruvate kinase [5 mg min^{-1} (mg protein)$^{-1}$] and did not appear in the cytosolic fraction. Pyruvate carboxylase activity was clearly separated from the activity of the mitochondrial marker enzymes citrate synthase [37 nmol min^{-1} (mg protein)$^{-1}$] and succinate dehydro-
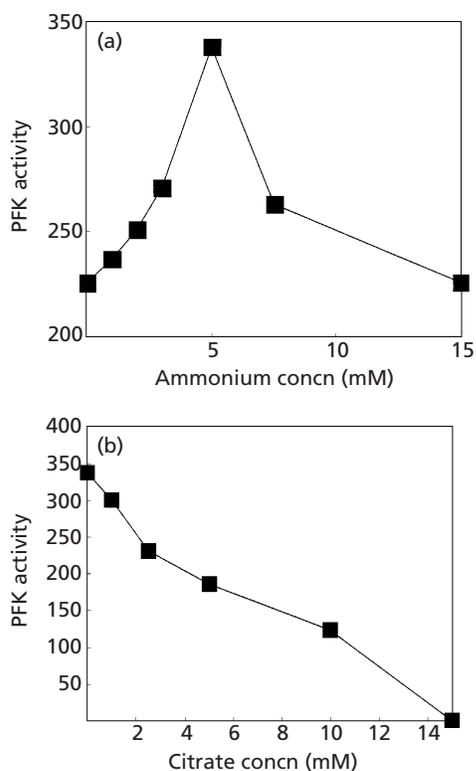
Fig. 5. Effect of (a) NH_4^+ and (b) citrate on the activity of PFK of *Mc. circinelloides in vitro*. Citrate and NH_4^+ were included in the *in vitro* assay of PFK to establish the impact of these effectors on the activity [nmol min^{-1} (mg protein)$^{-1}$] of this enzyme. This experiment was repeated three times and representative data are presented.

genase [32 nmol min^{-1} (mg protein)$^{-1}$], which were recovered exclusively in the mitochondrial pellet.

DISCUSSION

Changes in O_2 consumption and CO_2 evolution in response to N-limiting conditions and implications for metabolic switching

The decrease in O_2 consumption and CO_2 evolution at the time of N-exhaustion from the cultures is direct evidence of the down-regulation of cell metabolism in general and the citric acid cycle in particular. As the dO_2 of the culture did not return to 100 % saturation the cells evidently remained metabolically active after N-exhaustion, with glucose assimilation continuing and an increase in both cell dry weight and storage lipid content of the cells.

To correlate this switch in metabolic flux to the overall down-regulation of metabolic activity is a large and complex task. However, with our focus being on the mechanism of lipid accumulation we have aimed to elucidate the biochemical events underlying this rapid cellular response to N-limitation and hence the transition from the trophophase to the idiophase when lipid

and other secondary products are accumulated. Only by a sound understanding of this biochemistry can rational attempts at increasing lipid/secondary product accumulation using molecular techniques be made. Previous attempts to increase the production of secondary products by gene cloning without a sound biochemical background have highlighted the 'hit and miss' (mainly miss) nature of this approach (Rangasamy & Ratledge, 2000; Ratledge, 2000; Roehr *et al.*, 1996; Ruijter *et al.*, 2000).

The enzymes thought to be important in the initiation of lipid accumulation (ATP:citrate lyase, AMP deaminase and NAD^+:isocitrate lyase) in yeasts (Evans & Ratledge, 1985a, b) have all been reported in filamentous fungi (Botham & Ratledge, 1979; Wynn *et al.*, 1998) and their presence in *Mc. circinelloides* has been confirmed in this and previous studies (Wynn *et al.*, 1997).

NAD^+:ICDH in oleaginous fungi

The characteristics of the NAD:ICDHs of the two fungi examined were similar (though the enzyme from *Mucor* had a higher affinity for isocitrate than that from *Mortierella*) but were clearly distinct from those described for the NAD:ICDH from oleaginous yeasts (Botham & Ratledge, 1979; Evans *et al.*, 1983; Evans & Ratledge, 1985b). Both fungi are oleaginous – with *Mc. circinelloides* capable of accumulating 25 % (w/w dry wt) lipid and *Mort. alpina* capable of accumulating 40 % lipid (Wynn *et al.*, 1999) – however, the NAD:ICDH activities in these fungi were not absolutely dependent on AMP and, therefore, more closely resembled the enzyme from non-oleaginous micro-organisms (Evans *et al.*, 1983; Atkinson *et al.*, 1965). It is important to note, however, that NAD:ICDH was only active in the absence of AMP at non-physiological concentrations of isocitrate; i.e. 3 mM and 5 mM for *Mc. circinelloides* and *Mort. alpina* respectively. Although we could not detect isocitrate within the cells it is likely that the concentration of isocitrate will be approximately 5 % of the citrate concentration (Siebert, 1974), which was measured maximally at 15–20 nmol (mg dry wt)$^{-1}$. This would give a concentration of isocitrate of less than 1 nmol (mg dry wt)$^{-1}$, i.e. less than 0·5 mM assuming 2–4 μl H_2O (mg dry wt)$^{-1}$ (Knowles, 1977). At this concentration of isocitrate, NAD:ICDH would be down-regulated by the decrease in AMP concentration observed in the cells as a result of the increase in AMP deaminase activity triggered by N-exhaustion from the culture medium: see below.

AMP deaminase activity

Activation of AMP deaminase plays a key role in decreasing the concentration of AMP in oleaginous yeasts entering N-limited growth (Evans & Ratledge, 1985a). A similar situation occurred in the filamentous fungi studied, with a peak of AMP deaminase activity coincident with N-exhaustion causing a decrease in the intracellular AMP concentration (see below). The de-

crease in AMP concentration was coincident with the decrease in CO_2 evolution (see Fig. 1), which was indicative of a major decrease in the activity of the citric acid cycle. That the increase in activity of AMP deaminase was directly responsible for the decline in activity of NAD:ICDH, thereby affecting the overall activity of the citric acid cycle, is entirely consistent with these observations. The situation is more complex than in oleaginous yeasts in that AMP deaminase subsequently returned to its original activity, but this did not lead to any concomitant increase in AMP concentration or in the rate of CO_2 evolution. Therefore, the effect of the increase in AMP deaminase, though transitory, nevertheless may have initiated a cascade of connected biochemical events that rapidly led to the initiation of lipid accumulation.

Changes in adenylate pool size and cellular energy charge

The increased activity of AMP deaminase led to a rapid decrease in the cellular AMP, from 0·8 nmol (mg dry wt)$^{-1}$ (0·2–0·4 mM) prior to N-exhaustion to 0·5 nmol (mg dry wt)$^{-1}$ (0·13–0·25 mM) after N-exhaustion. AMP remained at this concentration even after AMP deaminase returned to its initial activity (Fig. 4). This approximately 50% decrease in AMP concentration, although not as dramatic as the 90% decrease reported for some yeasts (Boulton & Ratledge, 1983; Mitsushima et al., 1978), would nevertheless still lead to a significant down-regulation of NAD:ICDH activity, and thereby rapidly diminish the carbon flux through the citric acid cycle. Although the AMP concentration measured represented the total intracellular, rather than intra-mitochondrial, concentration this is thought unimportant as the mitochondrial membrane in eukaryotic micro-organisms is permeable to AMP (Matsushima et al., 1978; Bartels & Jensen, 1979). Therefore, the intra-cellular and intra-mitochondrial concentrations of AMP are likely to be equivalent.

Although a decrease in the AMP concentration associated with the depletion of the N-source in Mc. circinelloides resembles the situation reported for oleaginous yeasts (Boulton & Ratledge, 1983; Mitsushima et al., 1978), changes in the adenylate pool and the cellular energy charge differed from those previously reported. Whereas in yeasts the decrease in AMP was accompanied by an increase in the intra-cellular ATP/AMP ratio (Solodovnikova et al., 1998) and, therefore, energy charge, in Mc. circinelloides this did not happen. During the transition from N-replete to N-limited growth, the cellular concentrations of all three adenine nucleotides decreased by approximately 50% and the energy charge remained constant. This is similar to the situation reported in bacteria and fish, where during metabolic stress the cellular energy charge was maintained within closely defined limits at the expense of the total adenylate pool size, by the combined action of AMP deaminase and adenylate kinase (Atkinson, 1977; Woo & Chiu, 1997).

Regulatory properties of PFK

A preliminary study of the PFK of Mc. circinelloides demonstrated regulatory properties similar to those reported for the enzyme from other fungi (Roehr et al., 1996). Activity was stimulated by the presence of NH_4^+ and inhibited by citrate, with these effectors acting antagonistically. Therefore under N-limiting conditions a decrease in the intracellular NH_4^+ and an increase in citrate concentration would down-regulate the carbon flux through the glycolytic pathway, via inhibition of PFK activity. It is possible that the build-up of citrate due to the down-regulation of the citric acid cycle (see above) plays a role in regulating the rate of glycolysis (due to the inhibition of PFK) and therefore its own synthesis.

Subcellular localization of pyruvate carboxylase

A cytosolic pyruvate carboxylase activity in Mc. circinelloides is in accord with the observations in other fungi (Osmani & Scrutton, 1985; Klitsch et al., 1991). In this regard filamentous fungi are distinct from yeasts, in which both mitochondrial and cytosolic pyruvate carboxylases have been reported (Evans et al., 1983; Rohde et al., 1991; Van Urk et al., 1989), and animals, where pyruvate carboxylase is mitochondrial (Böttger et al., 1969; Taylor et al., 1978). A cytosolic pyruvate carboxylase would allow the cytosolic generation of NADPH for lipid biosynthesis via the co-operation of pyruvate carboxylase, malate dehydrogenase and malic enzyme to produce a 'transhydrogenase cycle' generating NADPH at the expense of NADH and ATP.

Conclusions

The biochemical mechanism responsible for the initiation of lipid accumulation in oleaginous fungi differs in several fundamental ways from that observed in oleaginous yeasts. As a result, the hypothesis explaining the onset of lipid accumulation in oleaginous yeasts requires modification before it can be applied to filamentous fungi. Although many of the enzymes involved in both systems are the same, the mechanism that operates in filamentous fungi is a somewhat more complicated and concerted process than that reported for yeasts.

The following hypothesis is put forward to explain the switching from active growth to the stationary phase in filamentous fungi.

1. As the N-source in the medium reaches a concentration below the K_m value of the uptake mechanisms the intracellular NH_4^+ concentration decreases. Because NH_4^+ is an activator of PFK the decrease in its concentration will lead to a decrease in the carbon flux through the glycolytic pathway (Fig. 6).

2. A reduced flux of carbon through the glycolytic pathway (and therefore ultimately the citric acid cycle) will limit ATP generation, so the energy charge within
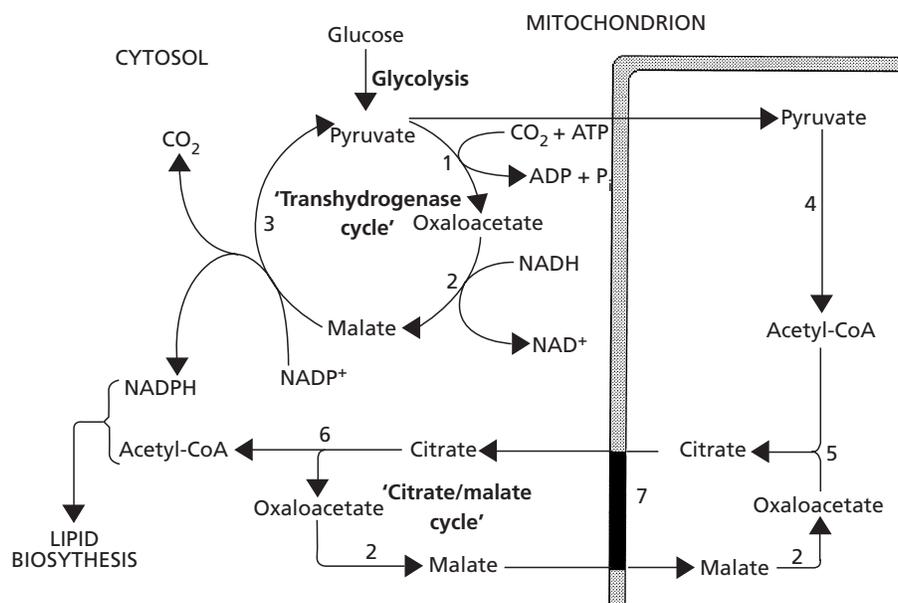
Fig. 6. Scheme showing how the proposed citrate/malate cycle and the cytosolic 'transhydrogenase' cycle could provide sufficient precursors (acetyl-CoA and NADPH) for lipogenesis in filamentous fungi. Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate lyase; 7, citrate/malate transport.

the cell will begin to decrease and this will activate AMP deaminase in order to maintain the energy charge within strictly controlled limits.

3. As a result of the activation of AMP deaminase, the AMP concentration will decrease and further limit the activity of the citric acid cycle (via NAD:ICDH activity), further restricting ATP generation; this will lead to a feed-forward process so that the energy charge can only be maintained by decreasing the total adenylate pool to a minimum level (approx. 50% of its trophophase level). Flux through the citric acid cycle will be further limited to a basal level, thereby restricting the supply of intermediates for anabolic processes, which will be further down-regulated by the decreased cellular ATP concentration, bringing them into line with the growth-limited environment.

4. When NAD:ICDH activity falls, citrate accumulates, which could further limit PFK activity. Citrate is translocated into the cytosol for cleavage by ATP:citrate lyase to provide cytosolic acetyl-CoA for lipogenesis.

5. The cytosolic pyruvate carboxylase together with ATP:citrate lyase and malate dehydrogenase allow a 'transhydrogenase cycle' to generate cytosolic NADPH independently from the 'citrate/malate cycle' (Evans *et al.*, 1983), thereby providing sufficient reducing power for lipogenesis.

That *Mc. circinelloides* and *Mort. alpina* have such an involved mechanism for the sensing of oncoming N-limited growth should not be a surprise. These fungi are commonly isolated from soil, which is a N-limited habitat, so that intricate mechanisms to deal with N-

depletion from the environment would be an expected environmental adaptation.

REFERENCES

Atkinson, D. A. (1977). *Cellular Energy Metabolism and its Regulation.* New York: Academic Press.

Atkinson, D. E., Hathaway, J. A. & Smith, E. C. (1965). Kinetics of regulatory enzymes: kinetic order of the yeast diphosphopyridine nucleotide isocitrate dehydrogenase reaction and a model for the reaction. *J Biol Chem* **240**, 2682–2690.

Bartels, P. D. & Jensen, P. J. (1979). Role of AMP in regulation of the citric acid cycle in mitochondria from bakers yeast. *Biochim Biophys Acta* **582**, 246–259.

Botham, P. A. & Ratledge, C. (1979). A biochemical explanation for lipid accumulation in *Candida* 107 and other oleaginous micro-organisms. *J Gen Microbiol* **114**, 361–375.

Böttger, I., Wieland, O., Brdiczka, D. & Pette, D. (1969). Intracellular localisation of pyruvate carboxylase and phospho-enolpyruvate carboxylase in rat liver. *Eur J Biochem* **8**, 113–119.

Boulton, C. A. & Ratledge, C. (1983). Use of transition studies in continuous cultures of *Lipomyces starkeyi*, an oleaginous yeast, to investigate the physiology of lipid accumulation. *J Gen Microbiol* **129**, 2871–2876.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.

Chaney, A. L. & Marbach, E. P. (1962). Modified reagents for determination of urea and ammonium. *Clin Chem* **8**, 130–132.

Dagley, S. (1974). Citrate UV spectrophotometric determination. *Methods Enzymatic Anal* **3**, 1562–1565.

Evans, C. T. & Ratledge, C. (1983). Biochemical activities during lipid accumulation in *Candida curvata*. *Lipids* **18**, 630–635.

Evans, C. T. & Ratledge, C. (1985a). The role of NAD^+:isocitrate dehydrogenase in lipid accumulation by the oleaginous yeast *Rhodosporidium toruloides* CBS 14. *Can J Microbiol* **31**, 845–850.

Evans, C. T. & Ratledge, C. (1985b). Possible regulatory roles of ATP:citrate lyase, malic enzyme, and AMP deaminase in lipid accumulation by *Rhodosporidium toruloides* CBS 14. *Can J Microbiol* **31**, 1000–1005.

Evans, C. T., Scragg, A. H. & Ratledge, C. (1983). A comparative study of citrate efflux from mitochondria of oleaginous and non-oleaginous yeasts. *Eur J Biochem* **130**, 195–204.

Kendrick, A. & Ratledge, C. (1992). Desaturation of poly-unsaturated fatty acids in *Mucor circinelloides* and the involvement of a novel membrane-bound malic enzyme. *Eur J Microbiol* **209**, 667–673.

Klitsch, W. M., Kubicek, C. P. & Scrutton, M. C. (1991). Intracellular location of enzymes involved in citrate production by *Aspergillus niger*. *Can J Microbiol* **37**, 823–827.

Knowles, C. J. (1977). Microbial metabolic regulation by adenine pools. *Symp Soc Gen Microbiol* **27**, 241–283.

Kornberg, A. (1955). Isocitric dehydrogenase of yeast (TPN). *Methods Enzymol* **1**, 705–709.

Kyle, D. J. (1997). Production and use of a single cell oil highly enriched in arachidonic acid. *Lipid Technol* **9**, 116–121.

Mitsushima, K., Shinmyo, A. & Enatsu, T. (1978). Control of citrate and 2-oxoglutarate formation in *Candida lipolytica* mitochondria by adenine nucleotides. *Biochim Biophys Acta* **538**, 481–492.

Osmani, S. A. & Scrutton, M. C. (1985). The subcellular localisation and regulatory properties of pyruvate carboxylase from *Rhizopus arrhizus*. *Eur J Biochem* **147**, 119–128.

Parvin, R. (1969). Citrate synthase from yeast. *Methods Enzymol* **13**, 16–19.

Rangasamy, D. & Ratledge, C. (2000). Genetic enhancement of fatty acid synthesis by targeting rat liver ATP:citrate lyase into plastids of tobacco. *Plant Physiol* **122**, 1231–1238.

Ratledge, C. (1997). Microbial lipids. In *Biotechnology*, 2nd edn, vol. 7, pp. 133–197. Edited by H. J. Rehm & G. Reed. Weinheim: VCH.

Ratledge, C. (2000). Look before you clone – A comment on 'Properties of *Aspergillus niger* citrate synthase and effects of *citA* overexpression on citric acid production' by G. H. G. Ruijter, H. Panneman, D.-B. Xu & J. Visser *FEMS Microbiol Lett* **184** (2000), 35–40. *FEMS Microbiol Lett* **189**, 317–318.

Roehr, M., Kubicek, C. P. & Komínek, J. (1996). Citric acid. In *Biotechnology*, vol. 6, *Products of Primary Metabolism*, 2nd edn, pp. 311–145. Edited by H. J. Rehm, G. Reed, A. Pühler & P. Stadler. Weinheim: VCH.

Rohde, M., Lim, F. & Wallace, J. C. (1991). Electron microscopic localisation of pyruvate carboxylase in rat liver and *Saccharo-myces cerevisiae* by immunogold procedures. *Arch Biochem Biophys* **290**, 197–201.

Ruijter, G. H. G., Panneman, H., Xu, D.-B. & Visser, J. (2000). Properties of *Aspergillus niger* citrate synthase and effects of *citA* overexpression on citric acid production. *FEMS Microbiol Lett* **184**, 35–40.

Schwitzguebel, J. P., Moller, I. M. & Palmer, J. M. (1981). Changes in density of mitochondria and glyoxysomes from *Neurospora crassa*: a re-evaluation utilizing silica sol gradient centrifugation. *J Gen Microbiol* **126**, 289–295.

Seubert, W. & Weicker, H. (1969). Pyruvate carboxylase from *Pseudomonas*. *Methods Enzymol* **13**, 258–260.

Siebert, G. (1974). Isocitrate UV spectrophotometric determination. *Methods Enzymatic Anal* **3**, 1570–1576.

Solodovnikova, N. Y., Sharyshev, A. A., Medentsev, A. G., Voloshin, A. N., Morgunov, I. G. & Finogenova, T. V. (1998). Dynamics of the adenine nucleotide pool in *Yarrowia lipolytica* cells overproducing organic acids. *Microbiology* **67**, 28–32.

Sols, A. & Salas, M. (1966). Phosphofructokinase III. Yeast. *Methods Enzymol* **9**, 436–442.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967). Separation and some enzymatic properties of the inner and outer membranes of rat liver mitochondria. *Methods Enzymol* **10**, 448–463.

Speilmann, H., Jacob-Müller, U. & Schulz, P. (1982). Simple assay of 0·1–1·0 pmol of ATP, ADP and AMP in single somatic cells using purified luciferin luciferase. *Anal Biochem* **113**, 172–178.

Taylor, D. J., Crabtree, B. & Smith, G. H. (1978). The intracellular location of pyruvate carboxylase, citrate synthase and 3-hydroxyacyl-CoA dehydrogenase in lactating rat mammary gland. *Biochem J* **171**, 273–275.

Van Urk, H., Schipper, D., Breedveld, G. J., Mak, P. R., Scheffers, W. A. & van Dijken, J. P. (1989). Localisation and kinetics of pyruvate-metabolizing enzymes in relation to aerobic alcoholic fermentation in *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621. *Biochim Biophys Acta* **992**, 78–86.

Vanheeswijck, R. (1984). The formation of protoplasts from *Mucor* species. *Carlsberg Res Commun* **49**, 597–609.

Woo, N. Y. S. & Chiu, S. F. (1997). Metabolic and osmoregulatory responses of the sea bass *Lates calcarifer* to nitrate exposure. *Environ Toxicol Water Quality* **12**, 257–264.

Worthington Enzymes (1979). Pyruvate kinase. In *Enzymes and Related Biochemicals*, pp 179–180. Bedford, MA: Millipore Corporation.

Wynn, J. P. (1998). Microorganisms as sources of nutritionally important polyunsaturated fatty acids. *Proceedings of the 12th Forum on Applied Biotechnology*, part 1, pp. 1215–1222. ISSN 0368-9697.

Wynn, J. P., Kendrick, A. & Ratledge, C. (1997). Sesamol as an inhibitor of growth and lipid metabolism in *Mucor circinelloides via* its action on malic enzyme. *Lipids* **32**, 605–610.

Wynn, J. P., Hamid, A. A., Midgley, M. & Ratledge, C. (1998). Widespread occurrence of ATP:citrate lyase and carnitine acetyltransferase in filamentous fungi. *World J Microbiol Biotechnol* **14**, 145–147.

Wynn, J. P., Hamid, A. A. & Ratledge, C. (1999). The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. *Microbiology* **145**, 1911–1917.