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The Physiological Significance of Citric Acid in the Control of Metabolism in Lipid-Accumulating Yeasts

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Introduction

Citric acid occupies a key position in the intermediary metabolism of all living cells where it has a multifunctional role in regulating both anabolic and catabolic pathways in these cells (Srere, 1972). Besides the well-documented oxidation of citrate in the tricarboxylic acid cycle, it also serves to supply the acetyl-CoA and oxaloacetate required for numerous biosynthetic pathways (Goodwin, 1968; Lowenstein, 1968) and acts as a positive and negative modulator of several key enzymes during primary metabolism (Srere, 1965). It is, therefore, hardly surprising that the concentration of such an important metabolite is under stringent control within the cell. However, in several micro-organisms the synthesis and metabolism of citrate appears totally to escape regulation (Shennan and Levi, 1974; Marchal, Vandecasteele and Metche, 1977; Röhr and Kubicek, 1981). As a result of this lack of control, either citric acid itself, or products derived principally from the acetyl-CoA moiety of the citrate molecule, are accumulated or excreted in large quantities.

Investigation of the physiology of citric acid production in the most commonly used industrial micro-organism, *Aspergillus niger*, (Perlman and Sih, 1960; Mattey, 1977; Röhr and Kubicek, 1981) has led to the belief that excessive citrate accumulation may be attributable to a stricture in the operation in the tricarboxylic acid cycle, the enzymes affected probably being either aconitase, isocitrate dehydrogenase or α-ketoglutarate dehydrogenase. Synthesis of citrate may be increased still further by certain nutrient deficiencies, such as manganese, which cause impairment of protein synthesis leading to accumulation of other important regulatory metabolites, for example NH_4^+

Abbreviations: FACEs, fatty acyl CoA esters; ICDH, isocitrate dehydrogenase; PFK, phosphofructokinase.

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ions, which can counteract any feedback inhibition by the accumulating citrate (Röhr and Kubicek, 1981). Overproduction of citric acid by yeasts has also been well documented (Mitsushima, Shinmyo and Enatsu, 1978; Bartels and Jensen, 1979). The synthesis of itaconic acid may also depend on the overproduction of citric acid, the major precursor to this commercially important compound (Winskill, 1983).

Under various nutrient limitations, certain yeasts and moulds can accumulate, and excrete, large quantities of the disaccharide, trehalose, or of polysaccharides such as glycogen and pullulan (Manners, 1971; Phaff, 1971). The overproduction of the precursor to these polysaccharides may also be regulated by feedback of an elevated cytosolic pool of citrate (Sols, Gancedo and Delafuente, 1971). Finally, the synthesis and metabolism of citrate has now been shown to be the key regulatory event responsible for the accumulation of large quantities of intracellular lipid by various yeasts and moulds (Ratledge, 1982) and may also be essential for extracellular lipid production by micro-organisms (Stodola, Deinema and Spencer, 1967). This review considers the role of citric acid in the regulation of lipid accumulation by oleaginous yeasts and will endeavour to show the central position occupied by citrate in the co-ordination of metabolic pathways in these yeasts and to develop a metabolic model of lipid accumulation to explain some of the less well-understood aspects of microbial citric acid production. It will be emphasized that the regulation of yeast lipid biosynthesis is a multi-site, highly integrated process and that no single regulatory mechanism can explain all the observations described.

The distribution of citrate during lipid accumulation by yeasts

Yeast cells can provide a natural system for accumulation of large quantities of lipid (*Figure 1*) in that the cellular lipid content can vary from as little as 0·5% (w/w) to as much as 70% (w/w) of the cell dry weight (Rattray, Schibeci and Kidby, 1975; Ratledge, 1978, 1982).

The oleaginous yeasts are regarded as ones which can accumulate 20% or more of their biomass as lipid (Ratledge, 1978) though the precise lower limit for inclusion is not defined. A possible biochemical determinant for classification of a yeast as being oleaginous may be the possession of the enzyme ATP: citrate lyase (ATP citrate (*pro*-3*S*) lyase (*see* page 351)). A list of yeasts currently classed as oleaginous is given in *Table 1*. The accumulating lipid is principally triacylglycerol, i.e. the same lipid that is the major constituent of commercial plant oils and fats. This lipid is stored within the yeast cell as discrete droplets (*see Figure 1*). Considerations for the exploitation of yeasts, and indeed moulds, as potential sources of oil have been advanced elsewhere and readers are referred to these reviews for greater information concerning the types of oils and fats which micro-organisms can produce, and of the various substrates which might be used in their production (Ratledge, 1978, 1982, 1984, 1985; Ratledge and Boulton, 1985).

The fundamental requirement for lipid accumulation is the deficiency of a particular nutrient, usually nitrogen, and an excess of carbon. In batch

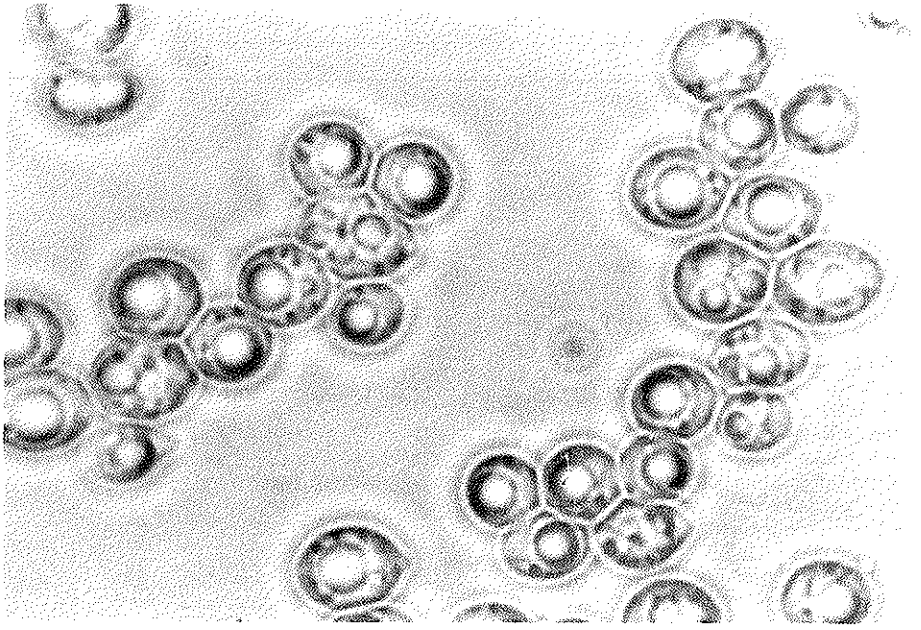


Figure 1. *Candida curvata* D with lipid content of 33% grown on glucose/NH_4^+ medium. Negative phase microscopy; magnification approx. 1600. (Cells usually contain one droplet of variable size.)

culture, when the nitrogen is exhausted, synthesis of nitrogenous cellular components (protein, DNA, RNA etc.) ceases while the carbon continues to be channelled into lipid, *see Figure 2* (Kessell, 1968; Gill, Hall and Ratledge, 1977; Evans and Ratledge, 1983a, b). Lipid biosynthesis in all yeasts is a cytosolic process (Schwitzer, 1984) with acetyl-CoA as the basic precursor unit (Lynen, 1980). In non-oleaginous yeasts, such as *Saccharomyces cerevisiae* and *Candida utilis*, acetyl-CoA is believed to originate from the mitochondria via the carnitine acetyltransferase (EC 2.3.1.7) reaction (Kohlhaw and Tan-Wilson, 1977) as the mitochondrial membrane is relatively impermeable to acetyl-CoA; *see also* pages 362–364. In oleaginous yeasts, as in mammalian-cell systems (Srere, 1972), citrate is believed to be the principal acetyl donor for fatty acid biosynthesis (Botham and Ratledge, 1979). Mitochondrial citrate is transported to the cytosol where it is cleaved by ATP: citrate lyase (ATP citrate (*pro*-3*S*) lyase (EC 4.1.3.8) to yield acetyl-CoA and oxaloacetate (I).

$$\text{citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + P_i \quad \text{(I)}$$

This is the only known mechanism of cytosolic citrate metabolism in eukaryotic cells and has not been detected in non-oleaginous micro-organisms (Boulton and Ratledge, 1981a).

Table 1. Oleaginous yeasts

Organism	Lipid content (%)
Candida curvata (also classified as *Apiotrichum curvatum*)	58
Candida diddensiae (= *diddensii*)	37
Candida lipolytica, see *Yarrowia lipolytica*	
Candida paralipolytica, see *Yarrowia lipolytica*	
Candida sp. no. 107 (NCYC 911)	42
Cryptococcus albidus var. *aerius*	63
Cryptococcus albidus var. *albidus*	65
Endomyces vernalis (= *Endomycopsis vernalis*) see *Trichosporon pullulans*	
Geotrichum candidum	50
Hansenula saturnus	28
Lipomyces lipofer (= *lipoferus*)	64
Lipomyces starkeyi	63
Lipomyces tetrasporus	66
Oidium lactis, see *Geotrichum candidum*	
Rhodosporidium toruloides	66
Rhodotorula glutinis	71
Rhodotorulum gracilis, see *Rhodosporidium toruloides*	
Rhodotorula graminis	41
Rhodotorula mucilaginosa	28
Rhodotorula suganii, see *Rhodosporidium toruloides*	
Trichosporon cutaneum	45
Trichosporon pullulans	65
Trigonopsis variabilis	40
Yarrowia lipolytica	36
Zygolipomyces lactosus, see *Lipomyces tetrasporus*	

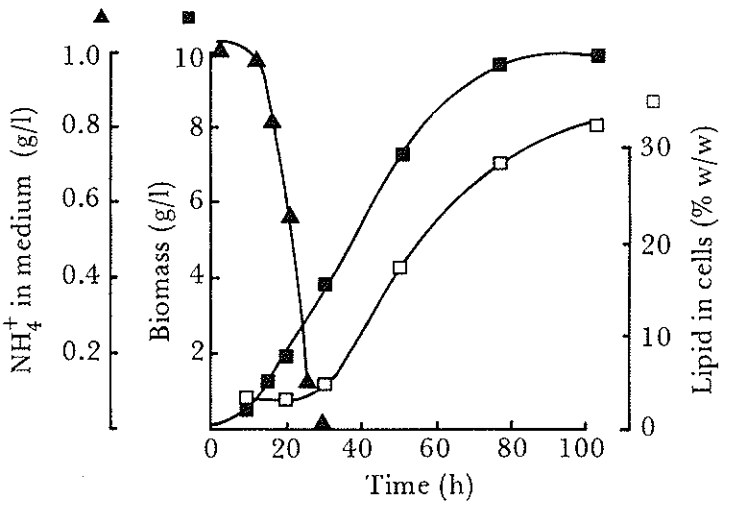


Figure 2. Lipid production in a typical oleaginous yeast, *Candida curvata* D, growing in batch culture. Biomass (■); lipid % dry wt (□) and NH_4^+ in culture medium (▲); (from Evans and Ratledge, 1983a).

In order to understand fully the importance of citrate in the metabolism of oleaginous yeasts, the intracellular and extracellular concentrations of this metabolite have been extensively monitored in batch, transition and continuous cultures (Boulton and Ratledge, 1983b; Evans and Ratledge, 1983b, 1984a). Using *Candida curvata* as a model oleaginous yeast, it was found that citrate was rapidly transported out of the cells and into the culture medium soon after exhaustion of the medium nitrogen source (*Figure 3a*). Citrate also accumulated intracellularly for the first 40 h growth, then decreased steadily as the cellular lipid content increased. Similar fluctuations in citrate concentration were also seen in chemostat transition experiments* using *Lipomyces starkeyi* (*see Figure 3b*). In these studies it was found that, as the culture medium became progressively nitrogen-limited, citrate was again excreted from the cells and accompanied by a marked increase in the intracellular citrate content. Under the steady-state conditions of continuous culture, citrate was detected only at the lower dilution (growth) rates (*see Figure 3c*). No citrate could be detected in cell or medium when the growth rate was increased above $0{\cdot}1\ h^{-1}$.

The above observations can be interpreted in terms of the difference in metabolic activity in the relevant cellular compartments. During the early growth stages an unimpaired tricarboxylic acid cycle ensures that all mitochondrial citrate is rapidly oxidized. However, the onset of nitrogen limitation appears to provide the metabolic signal necessary to promote the accumulation of intracellular citrate. The citrate must then leave the mitochondria and accumulate in the cytosol prior to its excretion from the cell (*Figure 4*). This indicates that cytosolic citrate metabolism (reaction 2, *Figure 4*), at the time of excretion from the cell, is considerably lower than the rate of citrate efflux from the mitochondria (reaction 3, *Figure 4*) (Evans, Scragg and Ratledge, 1983a). It was found that after 40 h growth, only a small amount of citrate was now expelled from the cells and was accompanied by a steady decrease in the intracellular citrate pool. This interplay could be explained by events prior to reaction 5 given in *Figure 4*; that is by either (1) decreased *de novo* citrate synthesis (reaction 1, *Figure 4*); (2) increased metabolism of citrate through the tricarboxylic acid cycle (reaction 2, *Figure 4*); (3) restricted supply to the cytosol (reaction 2, *Figure 4*); (4) increased cytosolic citrate metabolism (reaction 4, *Figure 4*), or a combination of these events (Evans, Scragg and Ratledge, 1983a,b). The importance of these metabolic activities will now be discussed in relation to lipogenesis in yeasts.

Nitrogen limitation and its effect on mitochondrial citrate synthesis and metabolism

The depletion of cellular nitrogen modifies yeast primary metabolism (protein, nucleic acid synthesis etc.) to a conditon that efficiently accommodates

* Chemostat transition experiments are the observations made between the disturbance of one steady state and the creation of a second, different set of steady-state conditions. In the experiments described, steady-state conditions with carbon (glucose) as limiting nutrient are established then perturbed by switching to nitrogen (NH_4^+)-limiting medium. The events during the transition of cells from one steady state to the next are then monitored (*see Figure 3b*).

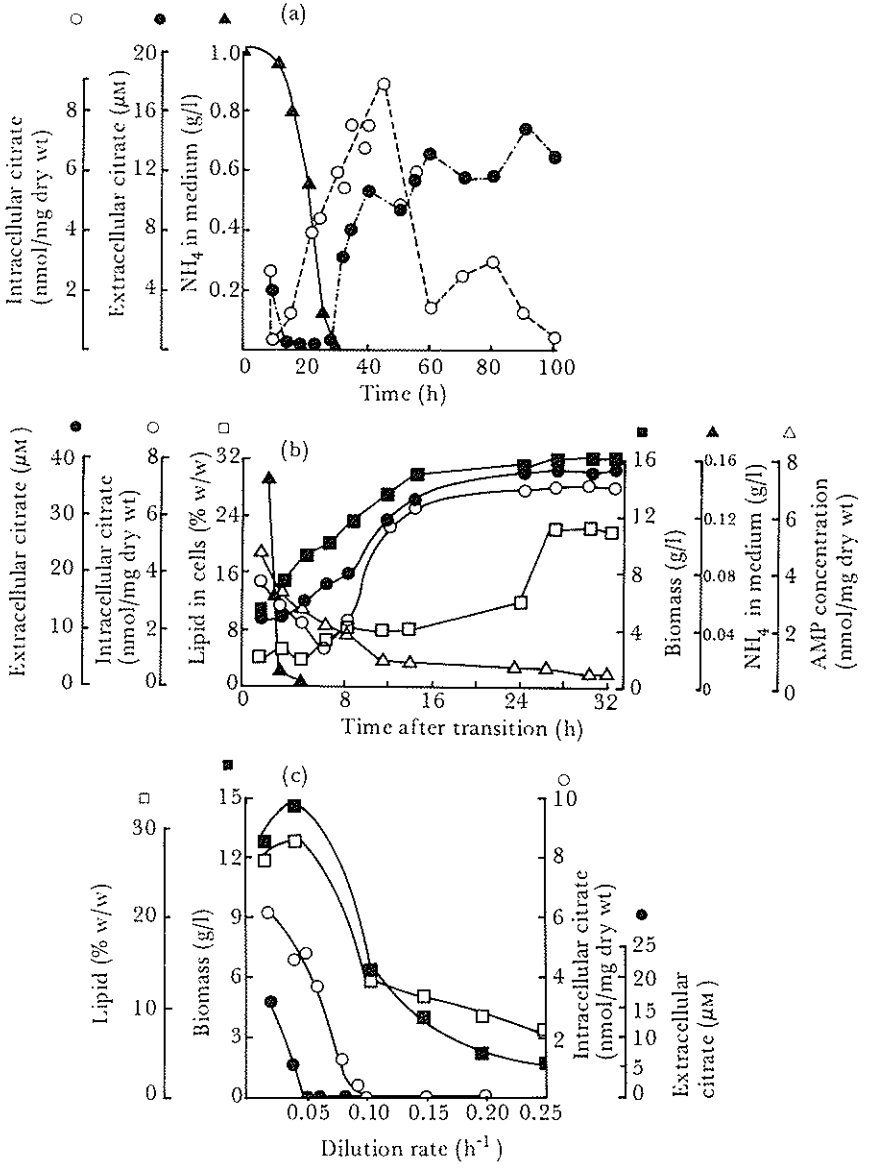


Figure 3. Accumulation of citric acid and other metabolites during lipid accumulation. (a) In *Candida curvata* D during batch growth (cf. *Figure 2*) (from Evans and Ratledge, 1983b). (b) In *Lipomyces starkeyi* during transition from carbon-limiting (low lipid) growth to nitrogen-limiting (high lipid) growth in continuous culture (*see* footnote on p. 353) (from Boulton and Ratledge, 1983b). (c) In *Candida curvata* D in continuous culture (from Evans and Ratledge, 1983b). Symbols: Biomass (■); lipid (□); extracellular citrate (●); intracellular citrate (○); NH_4^+ in culture medium (▲); intracellular AMP (△).

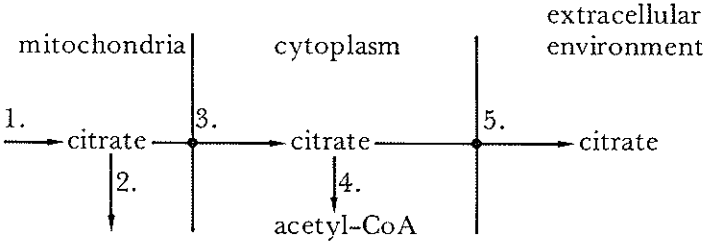


Figure 4. Flow of citrate between various compartments: mitochondrial, cytoplasmic and extracellular. Enzyme systems involved: 1: citrate synthesis (*see* reaction IV); 2: citrate metabolism via tricarboxylic acid cycle (*see* reactions III and VI); 3: citrate translocase (mitochondrial) (*see* reaction V); 4: ATP: citrate lyase (*see* reaction I); 5: citrate translocase (cytoplasmic).

the excess carbon available to the cells. This has been shown to be effected by a decrease in the intracellular concentration of AMP, which rapidly followed depletion of the nitrogen source (*see Figure 3b*) (Boulton and Ratledge, 1983b). Although the decrease in the absolute concentration of nitrogen is the main contributory factor for diminishing cellular AMP content, it has also been shown that AMP deaminase (EC 3.5.4.6), which catalyses reaction II,

$$\text{adenosine monophosphate} + H_2O \rightarrow \text{inosine monophosphate} + NH_3 \quad \text{(II)}$$

and which is active during nitrogen limitation, also serves to deplete the AMP pool (Evans, 1983; Yoshino, Miyajima and Tsushima, 1976; Yoshino and Murakami, 1981, 1982). The exhaustion of the cellular nitrogen source and AMP content must therefore somehow influence the mechanisms regulating the citrate concentration of the cell, as *Figures 3a–c* show an increased distribution of this metabolite throughout growth under nitrogen limitation.

ORIGIN AND REGULATION OF CYTOSOLIC CITRATE CONCENTRATION

The mitochondrial citrate synthesized during the yeast-cell cycle is normally oxidized in the tricarboxylic acid cycle: thus, if citrate is to enter the cytosolic compartment, its oxidation must be prevented to permit binding to the citrate translocase in the mitochondrial membrane (Evans, Scragg and Ratledge, 1983a). It was proposed by Botham and Ratledge (1979) that the normal oxidative role of the tricarboxylic acid cycle could be interrupted by inactivation of the NAD^+: isocitrate dehydrogenase (EC 1.1.1.41) (III).

$$\text{isocitrate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + NADH + CO_2 \quad \text{(III)}$$

This enzyme in oleaginous yeasts has been shown to have a specific requirement for AMP and when the medium nitrogen source is depleted the intracellular AMP concentration falls, so inactivating this enzyme. Indeed, an

11-fold decrease in cellular AMP content was observed during transition to nitrogen-limited conditions (*Figure 3b*) (Boulton and Ratledge, 1983b).

The activation of NAD^+:isocitrate dehydrogenase (NAD^+:ICDH) by AMP has now been shown to be a characteristic of the enzyme in numerous yeasts but absolute dependency on AMP has been observed only in oleaginous yeasts (Botham and Ratledge, 1979; Evans, Scragg and Ratledge, 1983b; Boulton and Ratledge, 1984). The latter workers also showed that ATP inhibited NAD^+:ICDH activity, especially in oleaginous yeasts, which emphasized that the ATP:AMP ratio is probably the major regulatory parameter controlling the oxidation of citrate in the TCA cycle (Atkinson, 1977; Mitsushima, Shinmyo and Enatsu, 1978). The enzyme from the oleaginous yeast *Rhodosporidium toruloides* has also been shown to be regulated by a number of metabolites including inhibition by citrate, which, by feeding forward, would help regulate its own accumulation (Evans, 1983; Evans and Ratledge, 1985b).

The second enzyme which was considered as a potential control of intracellular citrate accumulation is the mitochondrial citrate (*si*)-synthase (EC 4.1.3.7) (IV) (Hathaway and Atkinson, 1965; Srere, 1972; Weitzman and Dawson, 1976; Atkinson, 1977):

$$\text{acetyl-CoA} + \text{oxaloacetate} \rightarrow \text{citrate} + \text{CoA} \qquad \text{(IV)}$$

Although the enzyme in *S. cerevisiae* is reportedly modulated by several effectors, especially adenine nucleotides (Weitzman and Dawson, 1976; Atkinson, 1977), that from the oleaginous yeast *Candida* sp. 107 (NCYC 947) was only weakly regulated by ATP and ADP and is now considered of little physiological significance in controlling the supply of citrate for lipid biosynthesis (Boulton and Ratledge, 1980).

Regulation of mitochondrial citrate transport

Although the mechanisms for preventing mitochondrial citrate oxidation in yeasts have now been established, the means of transporting this accumulated citrate to the cytosol have been relatively neglected. Because of the separate compartmentation of citrate synthesis and fatty acid synthesis in yeasts, it is conceivable that the supply of acetyl-CoA, the substrate for lipid biosynthesis, is limited by the rate of efflux of citrate from the mitochondria to the cytosolic compartment (Lowenstein, 1968; Evans, Scragg and Ratledge, 1983a,b; Zammit, 1981). Mitochondria isolated from numerous oleaginous and non-oleaginous yeasts have been shown to possess an active carrier system for tricarboxylic anions, exhibiting specificity for citrate, isocitrate and *cis*-aconitate as well as L-malate (Evans, Scragg and Ratledge, 1983a). The malate–citrate exchange

$$\text{citrate}_{\text{IN}} + \text{malate}_{\text{OUT}} \rightleftharpoons \text{citrate}_{\text{OUT}} + \text{malate}_{\text{IN}} \qquad \text{(V)}$$

was shown to be stimulated by phosphate and pyruvate in both oleaginous and non-oleaginous yeasts. This stimulation is attributed to the operation of the citrate translocase as a proton-compensated electroneutral carrier, which transports a proton in the same direction as the citrate during exchange (McGivan and Klingenberg, 1971; Robinson *et al.*, 1971; Evans, Scragg and Ratledge, 1983a), i.e. the exchange is malate^{2-} for citrate^{3-} + H^+.

It was shown (Evans, Scragg and Ratledge, 1983a) that rates of citrate efflux were significantly greater from mitochondria of oleaginous yeasts than from mitochondria of non-oleaginous yeasts, *C. utilis* and *S. cerevisiae* (*Figure 5*). However, when mitochondria from non-oleaginous yeasts were exposed to fluorocitrate (which is a potent inhibitor of aconitase (VI)) there was a marked increase in citrate efflux so that it was now similar to the rate observed with untreated mitochondria from oleaginous yeasts. As fluorocitrate specifically inhibits aconitase activity, this indicated that the transport of citrate and its intramitochondrial metabolism were in direct competition (*see Figure 4*). The first enzyme of mitochondrial citrate catabolism is aconitase (aconitate hydratase; EC 4.2.1.3) (VI):

$$\text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \text{isocitrate} \qquad \text{(VI)}$$

This enzyme in yeasts, as far as we are aware, is not subject to any modulation by metabolic effectors. The equilibrium of the aconitase reaction is strongly in favour of citrate formation so that very little isocitrate can be detected in metabolically active mitochondria from either type of yeast (Evans, Scragg and Ratledge, 1983a,b). Furthermore, it was also shown that addition of ATP to mitochondria increased the rates of citrate efflux, whereas AMP markedly decreased citrate efflux. Both these effects were more pronounced in oleaginous yeasts and were shown to be attributable to the effects on the mitochondrial NAD^+:ICDH (III) (Evans, Scragg and Ratledge, 1983b). The degree of regulation by adenine nucleotides, in each yeast, thus determines the flux of carbon through the latter enzyme and is ultimately reflected in the rate at which citrate is effluxed from mitochondria (*see Figure 5b*). The sensitivity of the NAD^+:ICDH to the change in concentration of several key metabolites during nitrogen limitation is therefore an important control in regulating the amount of lipid accumulated by yeasts (Evans, Scragg and Ratledge, 1983b; Evans and Ratledge, 1985b).

It has been demonstrated in oleaginous yeasts that both radiolabelled [U-^{14}C] malate and [U-^{14}C] pyruvate, on entering the mitochondria, are quickly metabolized to citrate which is then effluxed (Evans, Scragg and Ratledge, 1983a). These observations showed that it was only the acetyl-CoA moiety of the effluxed citrate which was derived from pyruvate and not the oxaloacetate moiety; the latter intermediate was derived by oxidation of the *L*-malate, the counter anion used in the exchange for citrate. Thus, the continual efflux of citrate is dependent on the activity of the mitochondrial pyruvate dehydrogenase system and malate dehydrogenase (EC 1.1.1.37) but not on that of pyruvate carboxylase (EC 6.4.1.1) (*see Figure 6*). No significant

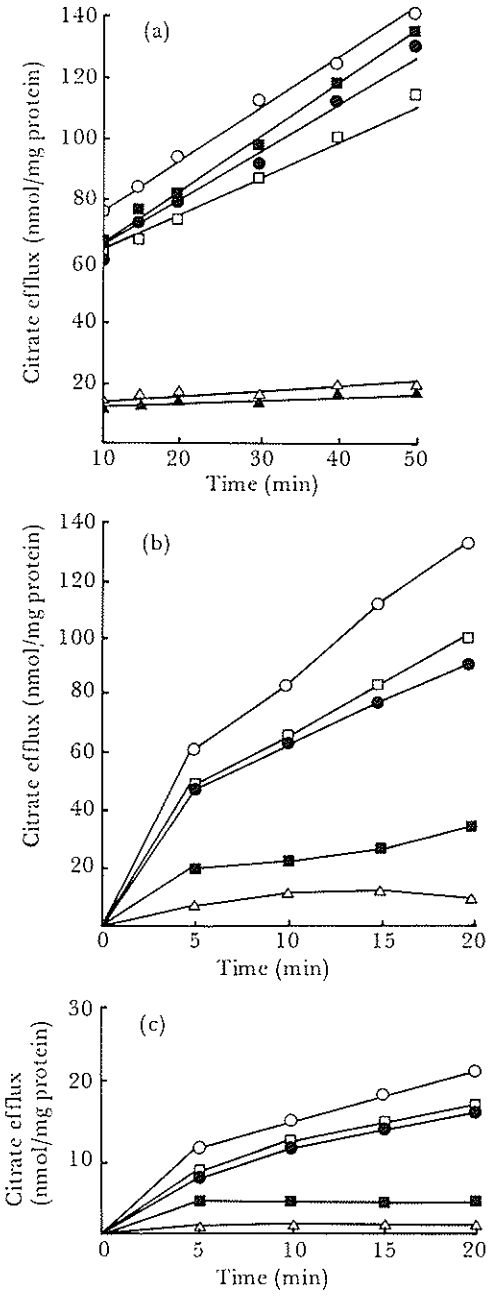


Figure 5. Efflux of citrate from yeast mitochondria (from Evans, Scragg and Ratledge, 1983a, b). (a) Using mitochondria from oleaginous yeasts: *Candida* S_{12} (○); *C. curvata* D (●); *Rs. toruloides* (□) and *L. starkeyi* (■); and from non-oleaginous yeast: *Candida utilis* NCYC 359 (△) and *C. utilis* NCYC 707 (▲). (b) and (c) Effect of ATP and AMP on citrate efflux from mitochondria of *C. curvata* (b) and *C. utilis* NCYC 359 (c): control (no additions) ●: AMP at 0·5 mM (■) and 5 mM (△); ATP at 0·5 mM (□) and 5 mM (○).

role for the latter enzyme in lipid production by yeasts has been found, to date.

These results indicate the existence of a malate–citrate shuttle which can efficiently transfer reducing equivalents (in the form of malate) from cytosol to mitochondria, and acetyl groups (in the form of citrate) to the cytosol (*see Figure 6*). As is apparent from *Figure 6* the operation of this shuttle during lipogenesis in yeasts would depend on the supply of cytosolic L-malate. The concentration of malate would be controlled by the activity of ATP:citrate lyase and not the cytosolic malate dehydrogenase, which is always very active (Evans, Scragg and Ratledge, 1983a). Thus, the rate of citrate efflux could well be controlled by the rate at which the cleavage enzyme can operate on the transported citrate. The operation of such a shuttle in mammalian systems has already been proposed (Wit-Peeters, Scholte and Elenbaas, 1970; Robinson and Oei, 1975), whereas in non-oleaginous yeasts it is unlikely that under lipogenic conditions such a shuttle system is active, because of the absence of ATP:citrate lyase and the low activity of the citrate translocase itself (Evans, Scragg and Ratledge, 1983a). However, in the citrate-accumulating yeast *Yarrowia* (= *Candida* = *Saccharomycopsis*) *lipolytica*, although ATP:citrate lyase is absent, nevertheless there is an active citrate–malate translocase system which serves the same purpose — to remove the citrate which is accumulating within the mitochondria (Evans, Scragg and Ratledge, 1983a).

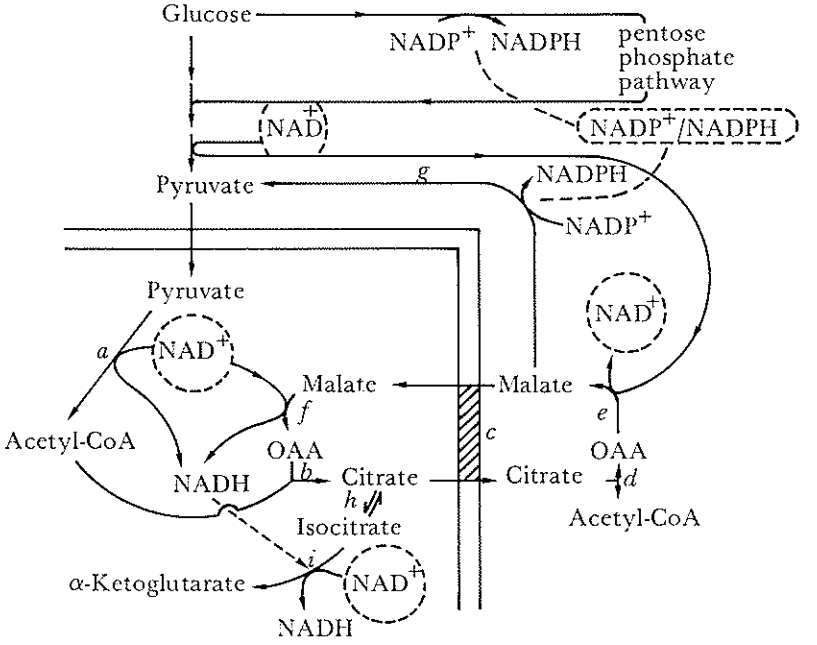


Figure 6. Generation of reducing equivalents during production of acetyl-CoA in oleaginous yeasts. Reactions: *a*, pyruvate dehydrogenase; *b*, citrate synthase; *c*, citrate/malate translocase; *d*, ATP: citrate lyase; *e* and *f*, malate dehydrogenases; *g*, malic enzyme; *h*, aconitase; *i*, isocitrate dehydrogenase (NAD$^+$-linked). (*See also* note added in proof, page 371).

Figure 6 also shows the existence of malic enzyme (malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$); EC 1.1.1.40) (VII)

$$\text{malate} + NADP^+ \rightarrow \text{pyruvate} + CO_2 + NADPH \qquad \text{(VII)}$$

in these yeasts. This enzyme could be advantageous during lipogenesis by supplying pyruvate and NADPH, but possibly detrimental by inhibiting citrate efflux by removing cytosolic L-malate. Malic enzyme, together with ATP:citrate lyase, has been implicated in the 'pyruvate cycle' in adipose tissue, where NADPH is produced from L-malate, derived in turn from oxaloacetate, by being recycled as pyruvate (Leveille and Hanson, 1966; Rognstad, 1969, 1980). However, this scheme makes no provision for the exit of citrate from the mitochondrion in the first place. Thus, in oleaginous yeasts, where a relatively large cytosolic pool of citrate is generated and maintained, an active malate–citrate shuttle must be the favoured mechanism of citrate transport. Although no definite role for malic enzyme has been determined in oleaginous yeasts, it is possible that it serves to regulate the malate–citrate shuttle, in order to maintain a balance between the rate of citrate efflux, NADPH generation, and fatty acid synthesis. The mechanism by which malate is provided extramitochondrially in the first instance is as yet uncertain in these yeasts.

It has also been demonstrated that the efficiency of the mitochondrial citrate translocase itself is directly affected by very low concentrations of long-chain fatty acyl-CoA esters (FACEs) (Evans, Scragg and Ratledge, 1983c). It was shown that citrate efflux, from a wide range of yeast mitochondria, was severely inhibited by FACEs; those with chain lengths of between 14 and 18 carbon atoms were the most potent inhibitors (*see Figure 7*). Over 50% inhibition of citrate transport was observed using palmitoyl-CoA and oleoyl-CoA, at approximately 4–5 μM, when the citrate translocase was saturated with L-malate as counter anion. This inhibition was shown to be competitive with L-malate. The possibility that the FACEs were exerting their effect by acting as detergent was eliminated and, furthermore, the inhibition could be reversed by adding bovine serum albumin.

As indicated by *Figure 7b*, the control of the citrate transport system in yeasts would therefore depend upon the availability of cytosolic L-malate and the build-up of long-chain FACEs as negative modulators. Although the concentration of FACEs in yeast cells during lipogenesis remains to be determined, in mammalian and avian liver, values for these molecules of 15–130 μM have been recorded (Volpe and Vagelos, 1976). This inhibition by FACEs could provide a stringent regulatory mechanism, either for controlling the amount of lipid accumulated by a yeast or for ensuring that lipid biosynthesis was readily inhibited if degration of the stored triacylglycerol was initiated for any reason (Evans, Scragg and Ratledge, 1983c). More recent work from the Hull laboratories indicates that the latter explanation is the preferred one, in that when yeasts are starved, initiation of lipid degradation is very rapid (within about 30 min) and lipid biosynthesis can no longer be detected (C. Ratledge and J.E. Holdsworth, unpublished results). This would

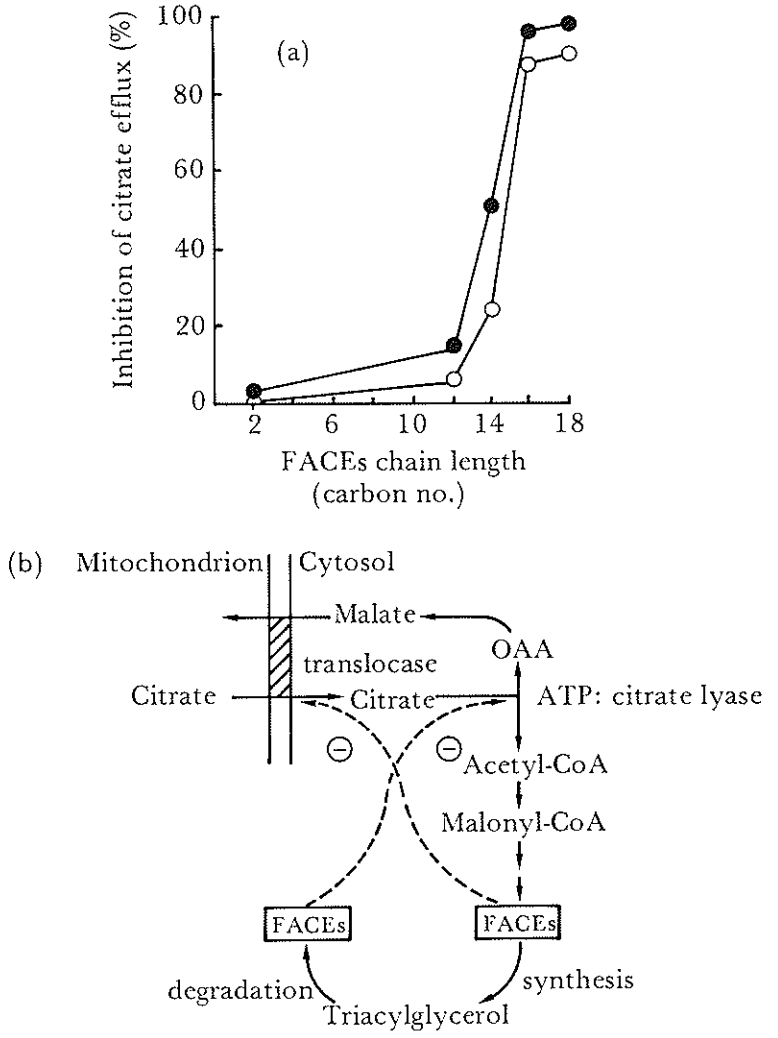


Figure 7. Fatty acyl-CoA esters (FACEs) and role in regulation of fatty acid biosynthesis. (a) Relationship between chain length of FACEs (at 35 μM) and inhibition of mitochondrial citrate efflux in *C. curvata* (○) and *C. utilis* (●); (from Evans, Scragg and Ratledge, 1983c). (b) Sites of inhibitory action of FACEs. (FACEs may arise as intermediates of fatty acid biosynthesis and thus regulate the extent of lipid accumulation or, more probably, as intermediates of lipid degradation and thus prevent biosynthesis occuring simultaneously with degradation.)

imply that fatty acid biosynthesis is being inhibited, rather than that the appropriate enzymes are being repressed. This inhibition is probably by the action of the FACEs, released from triacylglycerols during degradation, acting on both the citrate–malate translocase and also on ATP:citrate lyase (Boulton and Ratledge, 1981b, 1983a).

In the case of citric acid-excreting yeasts and moulds (e.g. *Yarrowia lipolytica* and *Aspergillus niger*), it would be interesting to know if the

characteristics of mitochondrial citrate efflux are similar to those demonstrated in oleaginous yeasts. It has been shown that L-malate does act as counter anion for efflux in the citric acid-excreting yeast *Yarrowia lipolytica* (Mitsushima, Shinmyo and Enatsu, 1978; Evans, Scragg and Ratledge, 1983a). It is possible, however, that in this yeast the oxaloacetate-regenerating reaction is pyruvate carboxylase rather than malate dehydrogenase, as malate is not regenerated. Thus, considerable citric acid synthesis could be maintained by a constant supply of pyruvate, but this does not make any provision for the exit of citrate from the mitochondria. The nature of the counter anion, and its metabolic fate on entering the mitochondria, could reveal some important regulatory aspects of microbial citrate metabolism.

The role of ATP: citrate lyase

The importance of the citrate cleavage enzyme, ATP:citrate lyase (I), has already been highlighted in this review because of its influence over the citrate transport system. Although the possession of this enzyme is essential in yeasts that rely on citrate as acetyl donor, its mere presence is insufficient to cause lipid accumulation (Boulton and Ratledge, 1981a; Evans and Ratledge, 1984a). The enzyme constitutes the only means of cytosolic citrate metabolism in eukaryotic cells and is found only in yeasts capable of accumulating substantial amounts of lipid (Botham and Ratledge, 1979; Boulton and Ratledge, 1981a). In mammalian systems ATP:citrate lyase has been shown to occupy a key role in lipogenesis, where its activity has been shown to decrease during starvation and greatly increase on refeeding (Kornacker and Lowenstein, 1965) as a direct consequence of changes in the actual amount of the enzyme protein (Yen and Mack, 1980). As mentioned previously, provision of acetyl-CoA in the cytosol of non-oleaginous yeasts is via the action of carnitine acetyltransferase (EC 2.3.1.7) (VIII) (Kohlhaw and Tan Wilson, 1977):

$$\text{carnitine} + \text{acetyl-CoA} \rightarrow \text{acetylcarnitine} + \text{CoA} \qquad \text{(VIII)}$$

This enzyme has also been found to occur in oleaginous yeasts (Ratledge and Gilbert, 1985) although at a substantially lower activity than in non-oleaginous micro-organisms. Its purpose, therefore, may be to supplement the combined activities of citrate translocase (V) and ATP:citrate lyase (I): however, as much higher activities, i.e. derepressed levels, are found in yeasts grown on triacylglycerols as carbon source, the enzyme's principal function may be to transport acetate into, rather than out of, the mitochondria when conditions are appropriate.

There is no correlation between the final cellular lipid content of an oleaginous yeast and the maximum specific activity of the ATP:citrate lyase (as measured in crude extracts) in yeast cells (Boulton and Ratledge, 1981a; Ratledge and Gilbert, 1985). In batch culture of the oleaginous yeast *Candida curvata* the activity of ATP:citrate lyase was shown to increase fivefold as the

culture progressed from a nitrogen-sufficient to a nitrogen-limited condition (*see Figure 8a*). The increase in ATP:citrate lyase activity presumably explains the cessation of citrate excretion and the decrease in the intracellular citrate pool observed (*Figure 3*). This is then reflected in the concomitant increase in cellular lipid content. Similar, but more dramatic, variations in ATP:citrate lyase activity were observed in continuous culture at the lower dilution rates ($D = 0{\cdot}02$–$0{\cdot}05\ h^{-1}$) where the dilution rate $D = F/V$ (F = total medium flow rate and V = fermenter working volume) (*Figure 8b*). These results support the conclusion reached with another oleaginous yeast, *Lipomyces starkeyi*, that ATP:citrate lyase is the rate-limiting step in lipid biosynthesis, although, in transition experiments with this yeast (*see Figure 8c*), no significant variations in activity of ATP:citrate lyase were observed (Boulton and Ratledge, 1983b). From a dilution rate of $0{\cdot}05\ h^{-1}$ to washout ($D = 0{\cdot}25\ h^{-1}$), citrate was undetectable, either intra- or extracellularly, even though ATP:citrate lyase activity was undiminished and the specific rate of lipid biosynthesis continued to increase. These observations would indicate that the rate of supply of citrate to the cleavage enzyme is now the limiting reaction during lipogenesis (Evans and Ratledge, 1983b). The restriction in citrate supply is probably at the level of the mitochondrial NAD^+:ICDH (III) which, at the higher growth rates, would metabolize all the synthesized citrate and prevent efflux (Evans, Scragg and Ratledge, 1983c).

ATP:citrate lyase from *L. starkeyi* has been partially purified and its regulatory properties determined (Boulton and Ratledge, 1983a). The most significant findings showed that the enzyme was inhibited by ADP, activated by increasing energy charge, and severely inhibited by low concentrations of long-chain FACEs (Boulton and Ratledge, 1981b). Like the inhibition of citrate efflux by FACEs, discussed above, that of ATP:citrate lyase was shown to be reversible and not attributable to the detergent properties of these molecules. This type of regulation provides further evidence for a physiological role of ATP:citrate lyase in the control of lipogenesis, and contests the generally accepted premise that acetyl-CoA carboxylase (EC 6.4.1.2) (IX),

$$\text{acetyl-CoA} + CO_2 + \text{ATP} \rightarrow \text{malonyl-CoA} + \text{ADP} + P_i \qquad \text{(IX)}$$

is the 'first' enzyme of lipid biosynthesis and the controlling reaction of this pathway (Volpe and Vagelos, 1976). Furthermore, the inhibitory effects of FACEs on ATP:citrate lyase observed in *L. starkeyi* are an order of magnitude higher than those reported for acetyl-CoA carboxylase from *S. cerevisiae* (White and Klein, 1965; Lynen, 1967) and also from the oleaginous yeast, *Candida* 107 (NCYC 947) (Gill and Ratledge, 1973) — *see* pages 361–362. This regulatory property of ATP:citrate lyase, coupled with that of the mitochondrial citrate translocase, must therefore constitute an effective means of restricting further flow of carbon through the lipid-synthesizing pathway when sufficient lipid reserves have been laid down or are being mobilized (*see Figure 7b*).

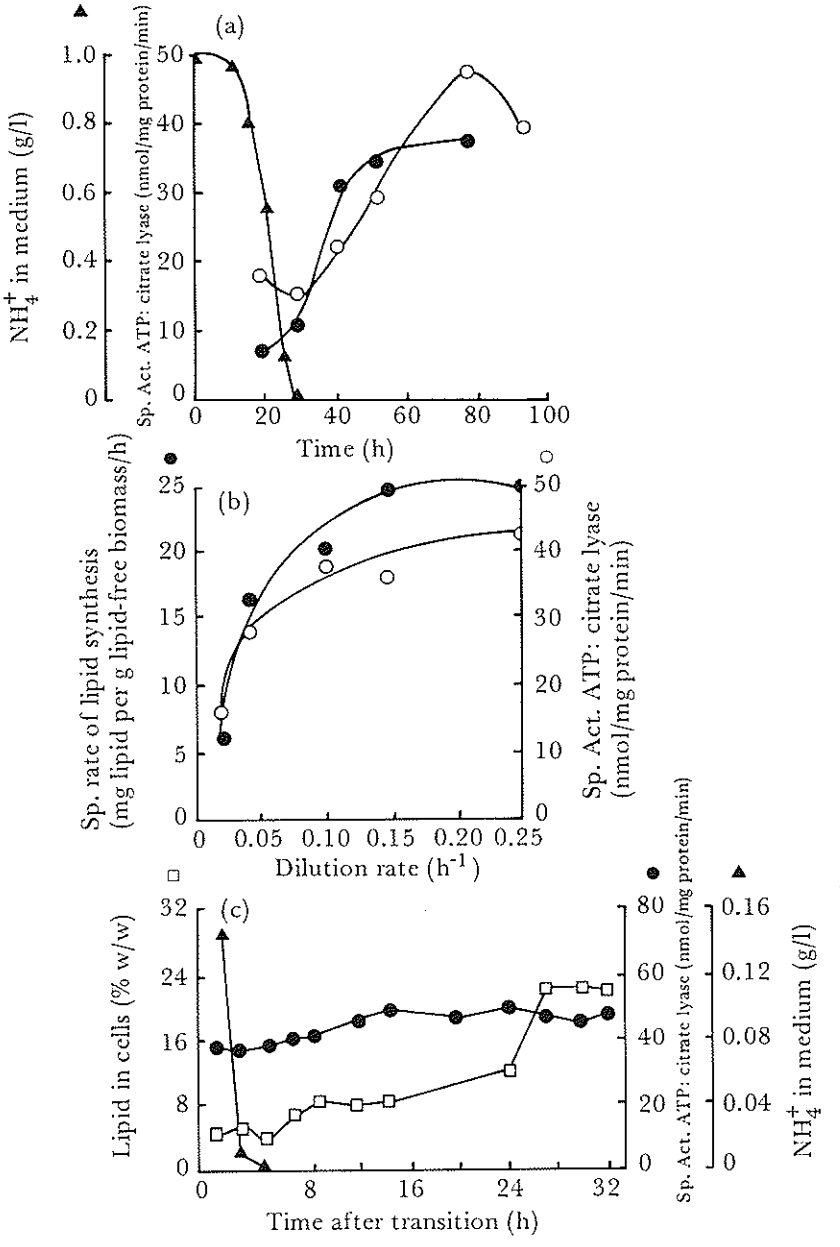


Figure 8. ATP: citrate lyase activity during growth of oleaginous yeasts. (a) In batch culture of *C. curvata* (●) and *Rs. toruloides* (○) related to NH_4^+ utilization: ▲ = NH_4^+ in medium (from Evans and Ratledge, 1983b; Evans, 1983). (b) In continuous culture of *C. curvata* (●) related to specific rate of lipid synthesis (mg lipid synthesized per g lipid-free biomass per hour), (○); (from Evans and Ratledge, 1983a). (c) In transition culture (*see Figure 3b*) of *L. starkeyi* (●) related to NH_4^+ in medium (▲) and lipid content of cells (□) (from Boulton and Ratledge, 1983b).

Compartmentation and concentration of citrate in yeast cells

Before describing the metabolic sites at which citrate can function as both a positive and negative modulator in yeasts, it is important to stress the extent to which this metabolite is distributed and accumulated in the various cellular compartments. During growth of an oleaginous yeast under nitrogen-sufficient conditions, the concentration of intracellular citrate has been found to be very low, namely 0·2 mM in *Rhodosporidium toruloides* (Evans and Ratledge, 1984a), 0·5 mM in *C. curvata* (Evans and Ratledge, 1983b) and 0·95 mM in *L. starkeyi* (Boulton and Ratledge, 1983b). Under these conditions very little, if any, citric acid is excreted into the medium. As shown in *Figures 3* and *9*, the onset of nitrogen limitation results in approximately a tenfold increase in the intracellular citrate content. Thus, after 30 h growth, the total cellular citrate content increased to 2·2 mM with *Rs. toruloides* and to

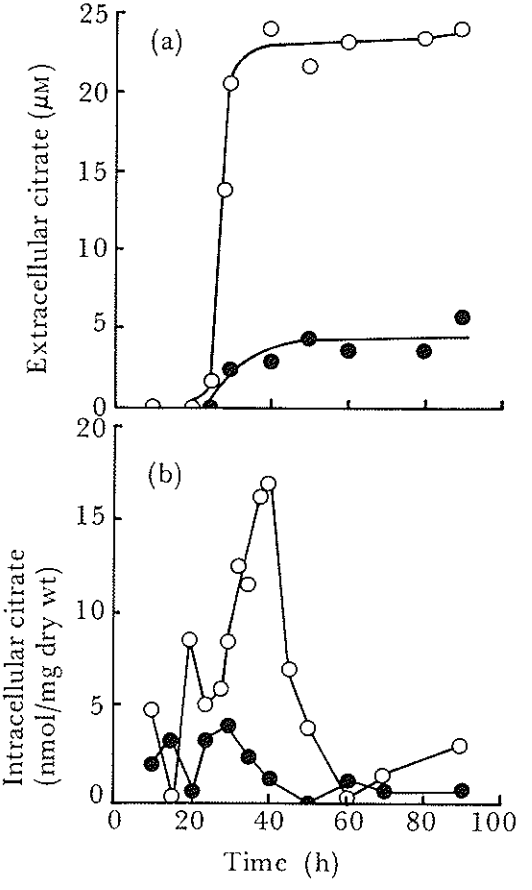


Figure 9. Concentration of citrate in *Rs. toruloides* CBS 14 during batch culture with NH_4^+ as N source (closed circles) or with glutamate as N source (open circles). (a) Extracellular citrate; (b) intracellular citrate (from Evans and Ratledge, 1984b).

4·6 mM with *C. curvata*. In *L. starkeyi*, however, the transition from nitrogen excess to nitrogen limitation resulted in only a threefold increase in cellular citrate content. The depletion of the medium nitrogen source also resulted in concomitant excretion of citrate into the medium to levels of between 15 μM and 40 μM, as measured with the latter three oleaginous yeasts.

Under the steady-state conditions of continuous culture, citrate (both intra- and extracellular) is detected only at the lower dilution rates ($D < 0{\cdot}05\ h^{-1}$; *see Figure 3c*). At these dilution rates the intra- and extracellular concentrations are approximately 3 mM and 15 μM respectively in all oleaginous yeasts examined to date. All the figures cited so far can be regarded as typical of oleaginous yeasts cultivated under the usually employed lipid-accumulating conditions (high glucose and low NH_4^+) where the final lipid content varies from 20% to 35% w/w. However, marked differences in citrate accumulation have been observed with the oleaginous yeast *Rs. toruloides* CBS 14 cultivated under a variety of conditions. The use of L-glutamate, or urea, as nitrogen source in batch culture resulted in intra- and extracellular citrate concentrations of approximately 8 mM and 250 μM respectively, after 40 h growth (Evans and Ratledge, 1984a) *see Figure 9a,b*). The intracellular citrate content then steadily decreased during lipogenesis to about 0·2 mM after 70 h growth.

Use of metabolic inhibitors with *Rs. toruloides* appeared to result in very high intracellular citrate contents (Evans, 1983). Addition of the fatty acid synthesis inhibitor, cerulenin, resulted in an elevated citrate pool to approximately 12 mM. Fluoroacetate, which is metabolized via citrate synthase to yield fluorocitrate, the specific inhibitor of aconitase (VI), was found to increase the intracellular citrate concentration to between 12 and 15 mM for the remainder of growth (*Figure 10*). More dramatic was the excretion of citric acid induced by addition of this inhibitor. This gave a concentration of citrate in the medium of greater than 1 mM (Evans, 1983). It must be stressed, however, that all the concentrations of citrate observed during growth of these oleaginous yeasts have been determined in a dynamic system and so reflect the result of flux patterns during metabolism, where an increased flux and decreased metabolism would lead to a detectable build-up of this metabolite. Not surprisingly, increases in the concentrations of intracellular citrate led to substantial increases in the amount of lipid accumulated (*Figure 10b*). Isolated yeast mitochondria contain relatively high quantities of endogenous citrate (Evans, Scragg and Ratledge, 1983a); those from non-oleaginous yeast were shown to contain between 11 and 15 mM citrate, whereas in oleaginous yeast mitochondria the endogenous citrate concentrations were between 32 and 50 mM. Mitochondria of the citric-acid-excreting yeast, *Yarrowia lipolytica*, contained approximately 24 mM citrate and thus were similar to the mitochondria of oleaginous yeasts. There are, therefore, striking metabolic similarities between oleaginous yeast and citric-acid-accumulating micro-organisms, except, of course, that the latter cells do not contain ATP:citrate lyase (Boulton and Ratledge, 1981a). These figures are comparable to those relating to isolated mammalian mitochondria from perfused liver, which have been shown to contain between 11 and 30 mM citrate (Rolleston, 1972). The fact that yeast mitochondria with such high

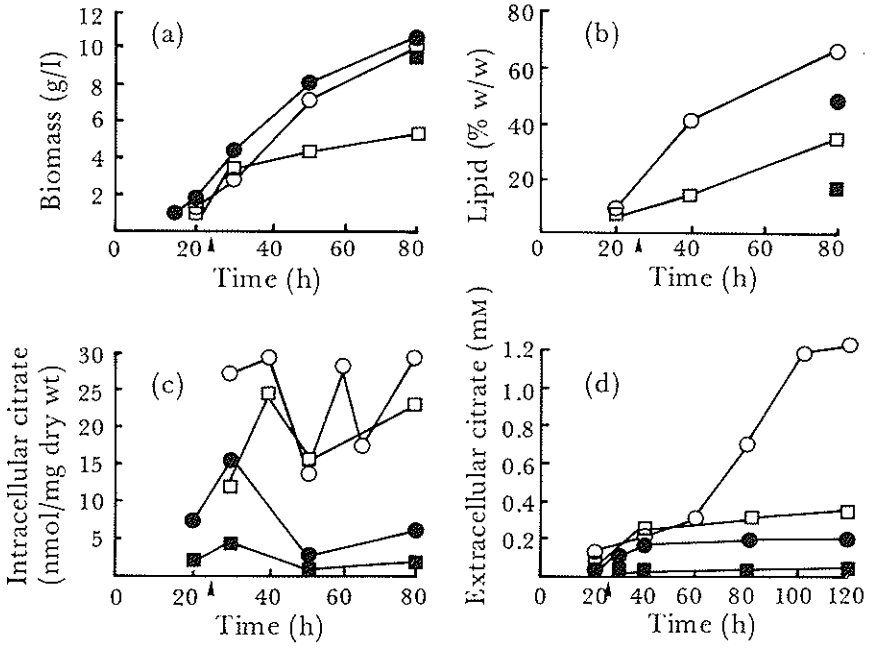


Figure 10. Effect of fluoroacetate, added at 25 h (arrowhead), on the growth (a), lipid content (b) and intra- and extracellular citrate (c) and (d) concentrations of *Rs. toruloides* CBS 14 grown in batch culture on glucose with NH_4^+ (squares) or glutamate (circles) as nitrogen sources. Open symbols, controls; closed symbols with fluoroacetate added.

endogenous citrate contents were isolated at all was surprising as the method of preparation was relatively slow. However, it is possible that, prior to extraction of mitochondria from sphaeroplasts, the concentration of exchangeable anions in the cytosol decreased significantly to prevent any citrate effluxing from mitochondria.

To the authors' knowledge, no data are available for concentrations of citrate in the cytosolic compartment in yeasts, probably because of the lack of a rapid and reliable method of separating mitochondrial and cytosolic fractions in yeast cells. Although the values for intra- and extracellular citrate concentration measured in oleaginous yeasts, and cited in this review, are modest in comparison with those measured in citric-acid-accumulating yeasts and moulds (*see* Röhr and Kubicek, 1981), these figures may well be an underestimate. For example, the cytosolic citrate concentration may be much larger than could be predicted from the values quoted if one considers that the intracellular aqueous volume will decrease dramatically with the ever-expanding size of the lipid globules (*see Figure 1*). These globules can account for over 75% of the cell volume in certain cases (Evans, 1983).

Citrate as a positive and negative modulator of key enzymes in lipid-accumulating yeasts

The effects of citrate on various aspects of yeast metabolism have been well documented (Goodwin, 1968; Srere, 1972). In *S. cerevisiae*, and mammalian

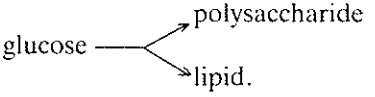
tissues, citrate has been shown to activate the cytosolic acetyl-CoA carboxylase (IX) (White and Klein, 1965; Rasmussen and Klein, 1968; Bloch and Vance, 1977; Wakil, Stoops and Joshi, 1983) and to inhibit glycolysis at the levels of 6-phosphofructokinase (EC 2.7.1.11) (X) (Uyeda, 1979; Sols, Gancedo and Delafuente, 1971) and pyruvate kinase (EC 2.7.1.40) (XI) (Hess, 1973; Barbalace, Chambliss and Brady, 1971; Barwell, Woodward and Brunt, 1971):

$$\text{fructose 6-phosphate} + \text{ATP} \rightarrow \text{fructose 1,6-bisphosphate} + \text{ADP} \quad \text{(X)}$$

$$\text{phospho}\textit{enol}\text{pyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP} \quad \text{(XI)}$$

Acetyl-CoA carboxylase from the oleaginous yeast *Candida* sp. 107 (NCYC 947) has also been shown to be activated by citrate, but that in the non-oleaginous *Candida utilis* was not (Gill and Ratledge, 1973; Botham, 1978; Botham and Ratledge, 1979).

The most important regulatory role attributed to citrate in oleaginous yeasts is its inhibitory effect on phosphofructokinase (PFK) (Evans and Ratledge, 1984c). This enzyme is a well-established control point regulating the flux of carbon through the glycolytic pathway in almost all organisms (Uyeda, 1979) and the enzyme in oleaginous yeasts is no exception. PFK from *Rs. toruloides* has been purified (approximately 92-fold) and shown to have a molecular weight of 700 000 (Evans and Ratledge, 1984c). At physiological concentrations of its substrates (fructose 6-phosphate and ATP at 1 mM), activity of PFK was reduced to zero in the presence of 5 mM citrate. Even in the presence of saturating amounts of fructose 6-phosphate, citrate at concentrations of 5 mM and 10 mM still resulted in strong inhibition: 45% and 85% inhibition respectively. The inhibition was shown to be competitive with the concentration of fructose 6-phosphate rather than ATP. Only NH_4^+ ions could significantly alleviate this inhibition by citrate: thus, 10 mM NH_4^+ raised the K_i for citrate inhibition from 0·9 mM to 7·2 mM. Such high intracellular concentrations of NH_4^+ occur when this yeast is grown on glutamate or other organic nitrogen sources rather than NH_4^+ itself, which appears to be transported at a rate closely governed by the rate at which it is used. In the *Rhodosporidium* yeasts, therefore, lipid accumulation is seen to any appreciable extent only when organic nitrogen sources are used, as it is only under these circumstances that the inhibition of PFK by citrate can be relieved by NH_4^+. It is possible that, in other species of oleaginous yeasts (cf. *Table 1*), NH_4^+ is more freely taken up by the cells, thus readily reversing any inhibition by citrate of PFK activity. If PFK is inhibited, this has the effect of diverting the incoming substrate, i.e. glucose, into storage polysaccharides and carbohydrates (glycogen, trehalose etc.):



In *Rs. toruloides*, there is an inverse correlation in the amounts of polysaccharide and lipid accumulating in the cell (Evans and Ratledge, 1984c).

Besides PFK, pyruvate kinase (XI) from the same yeast has also been found to be sensitive to inhibition by physiological concentrations of citrate (Evans and Ratledge, 1985a). This inhibition was competitive with phospho-*enol*pyruvate and shown to have a K_i of 340 μM. NH_4^+, L-glutamate and fructose 1,6-bisphosphate were shown to be activators. Similar results for the enzyme from the citrate-accumulating mould, *Aspergillus niger*, have also been reported (Meixner-Monori, Kubicek and Röhr, 1984).

The mitochondrial NAD^+:isocitrate dehydrogenase (III) from this yeast was also shown to be significantly inhibited by citrate (Evans and Ratledge, 1985b). Relatively high concentrations of citrate (> 20 mM) were required for complete inhibition *in vitro*, although it is conceivable that intramitochondrial citrate concentrations may be considerably greater than 20 mM during lipogenesis, as outlined on pages 365–367. Malic enzyme (VII) from the oleaginous yeasts *C. curvata* and *Rs. toruloides* was also inhibited by citrate (C.T. Evans and R. Mapleston, unpublished results) but the physiological importance of this inhibition during lipid accumulation is still not clear, as the exact role of malic enzyme in yeasts has yet to be fully elucidated (*see* page 360).

In vivo modulation by citrate of the activity of the enzymes mentioned above is thus a major factor in regulating the flux of carbon into lipid by oleaginous yeast (*see Figure 11*). The degree to which the various metabolic controls will be exerted will depend not only on the absolute concentration of citrate in the micro-environment of the enzyme, but also on the concentration of other modulators of enzyme activity: for example, fructose 1,6-bisphosphate, ATP, ADP, AMP, glutamate, α-ketoglutarate and NH_4^+ ions. This type of complex control is similar in broad outline to that proposed for the regulation of citric acid accumulation by *Aspergillus niger* (Ma, Kubicek and Röhr, 1981; Röhr and Kubicek, 1981; Habison, Kubicek and Röhr, 1983; Meixner-Monori, Kubicek and Röhr, 1984), although there are differences in the exact control mechanisms of the key enzymes.

Summary and conclusions

The fact that citric acid is excreted in considerable quantities by oleaginous yeasts under various conditions is indicative of a large cytosolic pool which will saturate its means of metabolism, ATP:citrate lyase. Thus, it is this enzyme which can be regarded as the rate-limiting factor in lipid biosynthesis because it controls the flux of acetyl-CoA to the fatty-acid-synthesizing systems, and is also responsible for the accumulation of the cytosolic citrate pool. This build-up of citrate may then initiate 'secondary controls' of lipid accumulation (*see Figure 11*). Activation of acetyl-CoA carboxylase by citrate ensures that cytosolic acetyl-CoA generated in the ATP:citrate lyase reaction is efficiently utilized for lipid synthesis. Inhibition of glycolysis by citrate restricts the flow of carbon to pyruvate and hence to citrate itself, and so prevents further build-up of this metabolite in the cytosolic compartment. This then enables ATP:citrate lyase once more to deplete the existing citrate pool, thereby restoring the balance between lipid synthesis and restrictive flux through glycolysis.

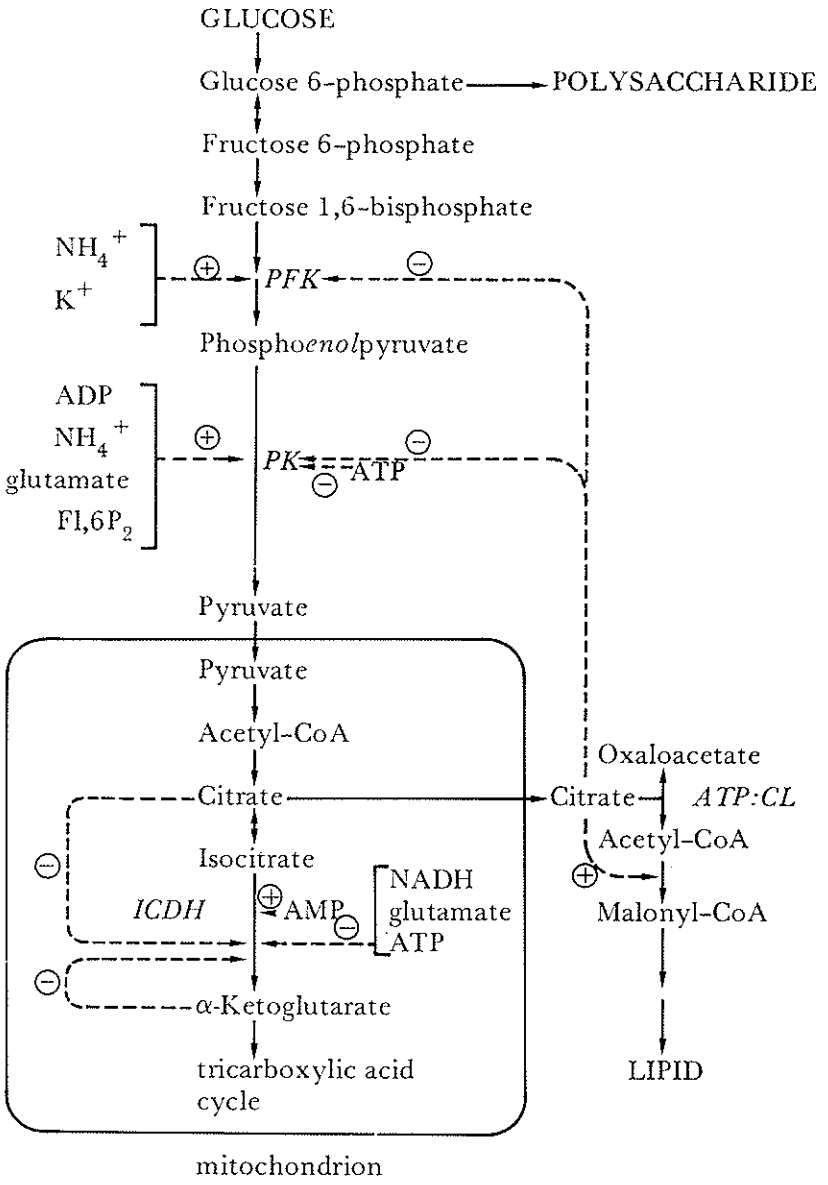


Figure 11. Synopsis of regulatory controls of citrate and other metabolites on the flux of carbon from glucose to lipid. *PFK* = phosphofructokinase; *PK* = pyruvate kinase; *ATP:CL* = ATP:citrate lyase; *ICDH* = isocitrate dehydrogenase; $Fl,6P_2$ = fructose 1,6-bisphosphate.

In conclusion, therefore, yeast cells possess only two mechanisms of citrate metabolism — one by mitochondrial oxidation and the other by cytosolic cleavage. Thus, it is hardly surprising that citrate has such an influence on the key enzymes of cytosolic carbon metabolism for, in cells lacking ATP:citrate lyase or possessing only very low *in vivo* activity, the transport of citrate to the

cytosol would result in a build-up of a non-utilizable metabolite. In non-oleaginous yeasts, such as *Saccharomyces*, the occurrence of cytosolic citrate signals an overflow of carbon from mitochondria which, if continued, would lead to loss of carbon from the yeast's cellular economy. Feedback inhibition of glycolysis by citrate thus reduces the flux of carbon entering mitochondria and so restores the balance between synthesis and oxidation of citric acid.

Oleaginous yeasts, on the other hand, could be regarded as more advanced species in that they possess a system of citrate metabolism in the most appropriate cellular compartment — the cytosol. This activity, coupled with a citrate-activated acetyl-CoA carboxylase, ensures that these yeasts can rapidly adjust to a lipid-synthesizing mode once citrate is allowed to accumulate. The flow of citrate to the cytosol in oleaginous yeasts is promoted by the high sensitivity of the NAD^+:isocitrate dehydrogenase to regulation by adenine nucleotides in these yeasts. Further inhibition of this enzyme by α-ketoglutarate, L-glutamate, NADH and citrate also increases the build-up of citrate in certain oleaginous yeasts (Evans and Ratledge, 1985b). A further specialization acquired by some oleaginous yeasts is the ability to stimulate or protect glycolysis, from citrate inhibition, by accumulation of regulatory metabolites such as NH_4^+ ions, thereby ensuring an unrestricted flow of carbon to the lipid-synthesizing system. A summary of the pathway leading to lipid accumulation, and the appropriate controls, is depicted in *Figure 11*.

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Note added in proof

Since this review was prepared, we have developed our consideration of the role of malic enzyme (page 360) and as a result a small but significant change has to be made to the flow of metabolites given in *Figure 6*. We now consider (Evans and Ratledge, 1985c) that malate will exit from the mitochondrion in exchange for the incoming pyruvate but will then return in exchange for citrate. The malate will be formed intramitochondrially from oxaloacetate which therefore fulfils the dual role of acting as precursor of citrate as well as malate. The cytosolic pool of malate again fulfils two roles: to provide NADPH via malic enzyme (VII) and to exchange for citrate (V).

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