Glycerol is not an inhibitor of mitochondrial citrate oxidation by *Aspergillus niger*

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We have re-assessed the hypothesis that an accumulation of intracellular glycerol triggers the accumulation of citric acid by *Aspergillus niger* by inhibiting the activity of the mitochondrial NADP-dependent isocitrate dehydrogenase isoenzyme. To this end, we have incubated mycelia of *A. niger* with 0.5 M glycerol, which resulted in a maximal intracellular glycerol pool level of 0.92 M, comparable to that determined during the early phase of citrate accumulation. This addition affected neither the uptake of [1,5-¹⁴C]citrate from the medium nor the rate of the subsequent release of ¹⁴CO₂ by the mycelia, indicating no effect on citrate oxidation. Mitochondria isolated from mycelia previously loaded with 0.5 M glycerol contained 8% of the total mycelial glycerol. They released ¹⁴CO₂ from exogenously added [1,5-¹⁴C]citrate at the same rate as mitochondria isolated from mycelia not loaded with 0.5 M glycerol. The addition of glycerol had no effect on the activity of the purified NADP-specific isocitrate dehydrogenase, but appeared to inhibit the activity in crude cell-free extracts of *A. niger*. We conclude that the intracellular accumulation of glycerol does not affect the rate of mitochondrial citrate oxidation and is therefore, in contrast to previous claims, not a trigger of citrate accumulation.

Keywords: *Aspergillus niger*, citric acid accumulation, glycerol, isocitrate dehydrogenase

INTRODUCTION

Selected strains of the filamentous fungus *Aspergillus niger* are capable of accumulating large amounts of citric acid from glucose, when cultivated under nutrient conditions favouring a high glycolytic flux rate, which has been the basis for the industrial production of citric acid for nearly 50 years (Kubicek & Röhr, 1986; Röhr *et al.*, 1992). The biochemical basis of this efficient metabolite overflow has been a matter of investigation for many years. Several authors have interpreted the accumulation of citric acid as being due to an inhibition of the enzymes of its further metabolism, e.g. aconitase and isocitrate dehydrogenase (for review see Kubicek & Röhr, 1986), because the conversion of glucose to citric acid approaches almost quantitative yields (up to 90%). While the possibility of such an inhibition occurring *in vivo* has been clearly disproved for aconitase (Kubicek & Röhr, 1985), an inhibition of one of the enzymes catalysing isocitrate oxidation deserves re-appraisal.

Legisa and coworkers have published several papers in which they propose a major role for intracellular glycerol in the onset of citric acid accumulation by inhibiting the mitochondrial isoenzyme of the NADP-specific isocitrate dehydrogenase (Legisa & Matthey, 1986; Legisa & Kidric, 1989; Legisa *et al.*, 1992; Legisa & Gradisnik-Grapuln, 1995). They speculate that such an inhibition of isocitrate oxidation – because the NADP-linked enzyme accounts for more than 80% of the rate of mitochondrial citrate oxidation (Matthey, 1977; Jaklitsch *et al.*, 1991) – causes (because of the aconitase equilibrium) accumulation of citrate, which also inhibits the NADP-specific isocitrate dehydrogenase (Matthey, 1977; Meixner-Monori *et al.*, 1986), and therefore enhances citrate accumulation. The evidence for this model is essentially indirect, however, and its validity *in vivo* has never been tested critically.

In our laboratory, we routinely make use of 0.25 M glycerol in buffers to stabilize labile enzymes during their purification, and did so also during our work on the NADP-specific isocitrate dehydrogenase (Meixner-Monori *et al.*, 1986). During this work, we did not observe any effect of glycerol on the activity of the purified enzyme, which contrasts with the report of a *K_i* of

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8 mM of the enzyme for glycerol (Legisa & Matthey, 1986). This finding prompted us to re-assess the validity of the glycerol theory of citric acid accumulation.

In the present paper, we show that a rise in intracellular glycerol has no effect on the catabolism of citrate in *A. niger*, either by whole mycelia or by isolated mitochondria. Furthermore we show that the inhibition of NADP-specific isocitrate dehydrogenase is obviously an artefact, since it is observed only with crude enzyme preparations. We therefore conclude that glycerol is not an initiator of citric acid accumulation.

METHODS

Materials. For citric acid production we used commercially available crystalline sucrose (food grade), which was purified by ion-exchange chromatography as described previously (Kubicek & Röhr, 1977). All other chemicals used were standard analytical grade and provided by Merck.

Organism and culture conditions. The strain used in these experiments was *Aspergillus niger* ATCC 11414, a strain producing high yields of citric acid (Shu & Johnson, 1948). In selected experiments, *A. niger* strains B-60 (IMI 309 921) and N-400 (CBS 120.49) were also used. Methods for cultivation under non-citric-acid-producing conditions, and the maintenance and preparation of conidia for inoculation, have been described previously (Ma *et al.*, 1981). To produce citric acid, the strain was grown in the medium of Shu and Johnson as described previously (Kubicek & Röhr, 1977).

Enzyme assays. NADP-specific isocitrate dehydrogenase was assayed as described by Meixner-Monori *et al.* (1986). Marker enzymes for subcellular fractions were assayed as described by Lendenfeld *et al.* (1993). Succinate dehydrogenase (EC 1.3.99.1) was assayed by the spectrophotometric method described by Veeger *et al.* (1969). Enzyme activities are given as units ml⁻¹. One unit is defined as the amount of enzyme catalysing the formation of 1 µmol product min⁻¹ at a linear rate under the conditions given. Specific activities are given as U (mg protein)⁻¹. Protein concentrations were determined by the dye-binding procedure (Bradford, 1976).

Purification of NADP-specific isocitrate dehydrogenase. The enzyme was purified from mycelia grown for 36 h on 1% (w/v) glucose as carbon source as described previously (Meixner-Monori *et al.*, 1986).

Uptake of citric acid and citric acid oxidation by mycelia. To study the oxidation of [1,5-¹⁴C]citric acid by mycelia of *A. niger*, they were pregrown on 1% (w/v) glucose for 28 h (at which time they were approximately in the middle of their growth phase). The mycelium from one flask was harvested on a Buchner funnel (G1 porosity) without suction, and washed three times with a total of 200 ml of fresh nutrient medium. It was then suspended in fresh nutrient medium to give a mycelial suspension of approximately 2 g dry wt l⁻¹, and divided into two parts. One part was mixed with an equal volume of fresh nutrient medium, whereas the other part was mixed with an equal volume of fresh nutrient medium which also contained 1 M glycerol. Portions (10 ml) of both suspensions were distributed into several 100 ml Erlenmeyer flasks and pulsed with 0.37 MBq [1,5-¹⁴C]citric acid (4.1 GBq mmol⁻¹; Amersham). Each Erlenmeyer flask was sealed with a rubber stopper and connected, via a Teflon tube and a glass pipe (5 mm diam.), to a sealed 50 ml washing flask containing 5 ml 1 M Na₂CO₃. ¹⁴CO₂ trapped in the Na₂CO₃ solution was determined for a

period of 4 h by pipetting 0.5 ml samples into 10 ml LSC-cocktail (Pharmacia-LKB) and measuring their radioactivity by liquid scintillation counting. The radioactivity was related to the amount of mycelial biomass, which was determined from separate samples of the original mycelial suspension.

In order to measure the uptake of [1,5-¹⁴C]citrate, mycelial suspensions were prepared and 10 ml portions were distributed into 100 ml Erlenmeyer flasks as described above. After pulsing with citrate, 0.5 ml samples of the broth were withdrawn and mixed with 1 ml ice-cold 1 M sodium citrate (pH 5.0). The mycelia were then harvested by filtration through GF/C glass-fibre filters (Whatman) and the radioactivity was measured in a liquid scintillation system.

Cell fractionation. Damp mycelium (5 g) was washed with tap water, blotted dry between filter paper, suspended in 35 ml 10 mM Tris/HCl buffer, pH 7.5 (containing 2 mM EDTA, 50 mM dithiothreitol, 2 mM PMSF, 3 mM MgCl₂, and 1 M sorbitol), and mixed with 30 g glass beads (0.45–0.50 mm diam.; Braun) in a 75 ml beaker. The beaker was then placed into a Bead-Beater homogenizer, cooled by ice, and homogenized by eight 30-s treatments, interrupted by 3 min cooling-periods. The homogenate was withdrawn, the sedimented glass beads removed, and the cellular debris and nuclei centrifuged at 1000 g (4 °C, 10 min). Portions (8 ml) of the supernatant were then placed on 32 ml of a 0.33–2 M sucrose gradient and centrifuged at 80000 g for 2 h at 4 °C in a swing-out rotor. Fractions of 2 ml were then isolated from the top and assayed for protein, glycerol, and activity of marker enzymes (glucose-6-phosphate dehydrogenase, EC 1.1.1.49; α-mannosidase, EC 3.2.1.24; acid phosphatase, EC 3.1.3.2; citrate synthase, EC 4.1.3.7; succinate dehydrogenase). Fractions with high citrate synthase and succinate dehydrogenase activities only were again pelleted by centrifugation (see above), suspended in extraction buffer, and used as the source of mitochondria for further experiments.

Uptake of citric acid and citric acid oxidation by mitochondria. Portions (1 ml) of mitochondria, suspended in isotonic buffer (10 mM Tris/HCl buffer, pH 7.5; containing 2 mM EDTA, 50 mM dithiothreitol, 3 mM MgCl₂, and 1 M sorbitol), were pipetted into 5 ml vials (3 × 1.3 cm diam.), each of which was connected via a 1 mm Teflon tube to a second vial containing 0.5 ml 1 M Na₂CO₃, into which the tube tip was immersed. The mitochondrial suspensions were gently agitated by a 1 cm stirring bar. After pulsing with [1,5-¹⁴C]citric acid, 50 µl samples from the 1 M Na₂CO₃ solution were withdrawn for a period of up to 6 h, added to 4 ml LSC-cocktail (Pharmacia-LKB) and the radioactivity measured. Radioactivity released was related to the amount of mitochondrial protein, which was assayed by mixing equal volumes of the mitochondrial suspension and 1 M NaOH, vortexing for 1 min, removing insoluble contents by centrifugation (Eppendorf centrifuge, 10 min), and assaying the protein concentration of the supernatant by the method of Bradford (1976).

Oxidation of citrate by mitochondria. This was done as described by Watson & Smith (1967), using a Clark electrode (Oxygen Analyzer, Yellow Springs).

Analysis of biomass and citric acid formation. This was done as described by Röhr *et al.* (1981). Citric acid and glycerol in the culture fluid were determined by HPLC, using an Aminex HPX-87N column (300 × 7.8 mm; Biorad) equipped with a Carbo C precolumn, using water (HPLC-grade, Merck) at 65 °C as the eluent. During cell fractionation experiments, glycerol was determined by enzymic analysis (Eggstein & Kuhlmann, 1974) using a commercial test kit (Boehringer-Mannheim).

RESULTS

Manipulation of intracellular glycerol in *Aspergillus niger* by addition of extracellular glycerol

Our rationale for investigating the *in vivo* validity of the 'glycerol hypothesis' was to modulate the mitochondrial glycerol pool in *A. niger* and to study the resulting effect on citrate oxidation. As a prerequisite for this approach, appropriate conditions for modulation had to be established first. To this end, we added various concentrations of extracellular glycerol to young mycelia and studied the resulting total intracellular glycerol concentration during further incubation. Table 1 shows that the incubation of mycelia in 0.5 M glycerol for 4 h resulted in a rise of the total intracellular glycerol concentration up to 0.94 M, which compares well with glycerol concentrations observed during citric acid accumulation (Legisa & Matthey, 1986). In order to assess whether this increased glycerol pool also occurs in the mitochondria, mycelia from incubations in 0.5 M glycerol were subjected to subcellular fractionation, and the glycerol concentration of individual fractions was analysed (Fig. 1c). Most of the glycerol was found in a very light fraction, which also contained glucose-6-phosphate dehydrogenase activity, and which was therefore tentatively identified as the cytoplasmic fraction. However, two other fractions, one enriched with hydrolytic enzyme activities and the other with citrate synthase and succinate dehydrogenase, also contained detectable glycerol. When the total amounts of glycerol found in individual fractions were combined and compared, the cytoplasm was shown to contain 76 %, and the mitochondrial fraction 17 % of total cellular glycerol, respectively. Control mycelia (those which had not been incubated in glycerol), although containing less glycerol, contained an even higher proportion of total glycerol in the cytoplasmic fraction (84 %), whereas the proportion in the two other fractions was low. The specific glycerol content of the combined putative mitochondrial peak fractions (fractions 19–24) was 3.7 and 0.64 $\mu\text{mol (mg protein)}^{-1}$ for glycerol-loaded and non-loaded mycelia, respectively. Assuming an intracellular volume of the mitochondria of 0.8–1.8 $\mu\text{l (mg protein)}^{-1}$ (Sere, 1980), the glycerol concentrations would be between 2 and 4 M

Fig. 1. Subcellular fractionation of *A. niger*. Fractions (2 ml) were collected from a sucrose gradient, starting at the top of the tube, and were assayed for (a) glucose-6-phosphate dehydrogenase (●), citrate synthase (○), succinate dehydrogenase (■); (b) α -mannosidase (▲), acid phosphatase (□); (c) glycerol: (Δ), mycelia incubated in 0.5 M glycerol; (◆) mycelia without preincubation in glycerol. Enzyme activities are given in arbitrary units in order to fit them into the same graph. Values are from a single experiment, but consistent data were obtained in two other experiments.

Table 1. Effect of addition of extracellular glycerol on the intracellular glycerol pool in *A. niger*

Added glycerol (M)	Intracellular glycerol (M)*	
	2 h incubation	4 h incubation
0	0.32 ± 0.05	0.30 ± 0.05
0.1	0.36 ± 0.05	0.40 ± 0.07
0.2	0.42 ± 0.08	0.54 ± 0.08
0.3	0.47 ± 0.08	0.61 ± 0.10
0.4	0.48 ± 0.08	0.83 ± 0.12
0.5	0.54 ± 0.56	0.94 ± 0.16

* Mean intracellular concentration in whole cells ($\pm$ SD, $n = 3$), using a value for intracellular volume of 2.4 ml (g dry weight)⁻¹.

for glycerol-loaded cells and between 0.3 and 0.8 M for control cells. This indicates that mycelia not pre-loaded with glycerol already contain a mitochondrial glycerol concentration which is in excess of the K_i reported for inhibition of mitochondrial NADP-specific isocitrate dehydrogenase. Both concentrations should be sufficient to inhibit mitochondrial citrate oxidation.

Oxidation and accumulation of citrate by glycerol-loaded mycelia of *A. niger*

Having established that the addition of 0.5 M glycerol dramatically enhances the mitochondrial pool of glycerol in *A. niger*, we then examined its effect on the oxidation of citrate *in vivo*. For this purpose, we incubated the mycelia

Fig. 2. Release of $^{14}\text{CO}_2$ from $[1,5\text{-}^{14}\text{C}]$ citrate by mycelia (a) and mitochondria (b) of *A. niger*, preincubated (○) or not preincubated (●) in glycerol. Values are from a single experiment, but consistent data were obtained in five other experiments. The calculated rates varied by less than 10%.

in 0.5 M glycerol, and then added $[1,5\text{-}^{14}\text{C}]$ citrate and analysed the release of $^{14}\text{CO}_2$. Because of the labelling at the C-atoms 1 and 5, release of $^{14}\text{CO}_2$ can only result from mitochondrial oxidation. Preliminary experiments showed that more than 97% of the exogenously added labelled citrate had been taken up within 90 min of incubation. After a lag-time of about 45 min the mycelia started to release $^{14}\text{CO}_2$ at a linear rate. No differences could be detected in the rates of $^{14}\text{CO}_2$ release between mycelia preincubated or not preincubated with glycerol, suggesting that the catabolism of citrate was not affected (Fig. 2a). Also, the total intracellular citrate concentration did not significantly change during this experiment and remained at 1.3 ± 0.4 mM.

Metabolism and accumulation of citrate by glycerol-loaded mitochondria of *A. niger*

In order to support the results obtained with whole, intact mycelia, similar experiments were carried out with isolated mitochondria. For this purpose, we isolated the mitochondria from mycelia preincubated in 0.5 M glycerol and also from control mycelia. Although our procedure did not result in the preparation of coupled mitochondria, they took up citrate and oxidized it, and were thus considered suitable for this study. Fig. 2(b) shows that both types of mitochondria release $^{14}\text{CO}_2$ at comparable rates, and are thus not affected in their citrate oxidizing capacity by the glycerol content. Similar findings were obtained when the respiratory activity of the mitochondria with citrate and succinate was compared

Table 2. Effect of glycerol addition on the activity of NADP-specific isocitrate dehydrogenase from *A. niger*

One unit (U) of enzyme activity is the amount of enzyme catalysing the formation of $1 \mu\text{mol product min}^{-1}$. Activities were assayed in triplicate and the values are given as means $\pm$ SD.

Glycerol concn (mM)	Enzyme activity (U ml^{-1})		
	Cell-free extracts	Purified enzyme	Mixture of both*
0	1.24 ± 0.13	0.44 ± 0.07	0.82 ± 0.10
5.9	1.24 ± 0.15	0.48 ± 0.10	0.80 ± 0.13
11.9	1.12 ± 0.13	0.44 ± 0.08	0.75 ± 0.11
17.8	1.03 ± 0.10	0.42 ± 0.06	0.72 ± 0.09
33.4	0.87 ± 0.08	0.44 ± 0.06	0.57 ± 0.06
59.0	0.78 ± 0.08	0.46 ± 0.07	0.51 ± 0.05

* Cell free extract (0.8 U) and the purified enzyme 0.8 U were mixed in a total volume of 3.0 ml.

$[7.8$ and $7.6 \mu\text{mol oxygen consumed min}^{-1} (\text{mg protein})^{-1}$ by mitochondria from mycelia preincubated without and with 0.5 M glycerol, respectively], and this finding was not influenced by substituting 1 M glycerol for 1 M sorbitol in the buffer used for the incubation of mitochondria (data not shown). The latter was done to ensure that glycerol did not leak out of the mitochondria during incubation. From these data we conclude that glycerol has no effect on the mitochondrial oxidation of citrate in *A. niger*.

Effect of glycerol on NADP-specific isocitrate dehydrogenase from *A. niger*

In view of our findings that glycerol does not affect the mitochondrial oxidation of citrate, we wanted to find out whether the mitochondrial NADP-specific isocitrate dehydrogenase of *A. niger* is affected by glycerol. To this end we purified the enzyme to physical homogeneity and investigated its activity in the presence of various concentrations of glycerol (Table 2). With an actual Mg-isocitrate concentration of 0.1 mM, which is about the K_m of the enzyme, no inhibition could be observed even at concentrations of 59 mM, which represent a 7.5-fold excess over the K_i values reported by Legisa & Matthey (1986), and should therefore have resulted in an inhibition of at least 85%. In order to rule out that this effect was dependent on the strain used, we also purified and investigated the enzyme from the other *A. niger* strains, B-60 (high-citric acid producer; Röhr *et al.*, 1979) and N-400 (a moderate citric acid producer; unpublished data). Essentially similar results were obtained with both these strains.

Interestingly, glycerol appeared to inhibit the activity of NADP-specific isocitrate dehydrogenase in cell-free extracts (Table 2). Since the mitochondrial and the cytoplasmic isoenzymes of *A. niger* are identical (Meixner-

Monori *et al.*, 1986; Jaklitsch *et al.*, 1991), we suspected that this may be due to an artefact. To test this, we added the purified mitochondrial NADP-specific isocitrate dehydrogenase to cell-free extracts, and examined the inhibition of the resulting total activity by glycerol (Table 2). We found that the activity of the purified enzyme was also inhibited by glycerol when mixed with the crude cell-free extract. Controls, using cell-free extracts that had been heated for 10 min in a boiling water bath did not show this effect, whereas the effect was maintained during dialysis. We therefore conclude that this inhibition was caused by a protein present in the cell-free extracts, which either inhibited or inactivated NADP-specific isocitrate dehydrogenase activity.

DISCUSSION

The data presented in this paper show that a significant rise in the intracellular pool of glycerol has no effect on the oxidation of [1,5-¹⁴C]citrate within the citric acid cycle, hence disproving earlier theories (Legisa & Matthey, 1986; Legisa *et al.*, 1992) that glycerol may inhibit the mitochondrial NADP-specific isocitrate dehydrogenase. It should be noted that because of the labelling of citrate in positions 1 and 5, our approach quantified the CO₂ released during the 2-oxoglutarate dehydrogenase reaction (1 mol CO₂ per cycle) and not release by isocitrate dehydrogenation itself. This seems to be legitimate, however, since citrate has to pass the isocitrate dehydrogenation step for this purpose and, since the rates observed were similar, isocitrate dehydrogenation is either not limiting the flux through the cycle or, if limiting, is displaying the same activity irrespective of the presence of glycerol. Both possibilities strongly argue against an inhibition of citrate oxidation by glycerol. It should also be noted that other metabolic reactions are unlikely to affect the method used here: in theory, [1,5-¹⁴C]citrate may be cleaved by ATP-citrate lyase and used for lipid biosynthesis. Such a process would conserve the radioactive label in fatty acids, and subsequently release CO₂. We consider it unlikely that such a pathway would have interfered with our approach, since the rates of ¹⁴CO₂ evolution during the 6 h of the experiment were, after an initial lag, essentially linear, and less than 10% of the level of radioactivity measured in CO₂ was found in a chloroform/methanol extract of *A. niger* mycelium harvested at the end of the experiment (data not shown).

Consistent with the lack of effect on citrate oxidation *in vivo*, glycerol had also, in contrast to previous reports (Legisa & Matthey, 1986), no effect on the purified enzyme. Legisa *et al.* (1992) interpreted the inhibitory effect of glycerol in terms of structural similarities between citrate (which is a competitive inhibitor of the NADP-specific isocitrate dehydrogenase; Matthey, 1977; Meixner-Monori *et al.*, 1986) and glycerol (Legisa *et al.*, 1992). However, the inhibition by citrate is related to its ability to compete for the divalent metal ion required to chelate isocitrate (Meixner-Monori *et al.*, 1986), an effect which cannot be achieved by glycerol due to the absence of carboxylic groups in the glycerol molecule. Moreover, binding of the substrate to the active centre of the NADP-specific

isocitrate dehydrogenase requires both a chelated carboxylic group as well as a hydroxylic group (Ehrlich & Colman, 1987). All these data are consistent with our findings that glycerol does not inhibit this enzyme.

While the present data disprove the theory that glycerol inhibits mitochondrial citrate oxidation, they do not offer any other explanation for the triggering of citric acid accumulation. However, it should be pointed out here that, based on available data, there is no need to postulate an inhibition of citrate breakdown at either aconitase or one of the two isocitrate dehydrogenases. This is clearly illustrated by the following calculation. During the early phase of citric acid accumulation (20–45 h of fermentation), the biomass concentration of *A. niger* rises from 0.2 to 1.4 g l⁻¹ (Röhr & Kubicek, 1981). During the latter half of this period, the total citric acid concentration (i.e. intra- plus extracellular) rises from 0.08 to 1.8 mM (Kubicek & Röhr, 1981). Using published data for the protein and amino-acid composition of *A. niger* biomass during this phase (Kubicek *et al.*, 1979), the minimal metabolic flux from glucose to 2-oxoglutarate can be calculated as 1.4 µmol 2-oxoglutarate formed min⁻¹ (g biomass dry wt)⁻¹ (Zehentgruber, 1980; cited in Röhr & Kubicek, 1981). This value accounts for 73% of the total activities of both the NADP- and NAD-specific isocitrate dehydrogenases [1.9 U (g biomass dry wt)⁻¹] measured *in vitro*. Comparable flux rates are also obtained for fermentations with strains producing low levels of citric acid or with high-yielding strains under conditions of impaired citrate accumulation (i.e. in the presence of manganese ions). Given the fact that the *in vivo* concentration of the Mg-isocitrate complex is between 0.12 and 0.22 mM during this period (Kubicek & Röhr, 1985), and considering that the NAD-specific isocitrate dehydrogenase (which accounts for only about 15% of total isocitrate dehydrogenase activity *in vitro*; Jaklitsch *et al.*, 1991) has a substrate affinity similar to that of the NADP-specific enzyme (*K_m* 0.08 mM; Meixner-Monori *et al.*, 1986), the observed *in vivo* flux matches perfectly that which can be predicted from the *in vitro* data. All this is consistent with our hypothesis (cf. Röhr *et al.*, 1992; Kubicek *et al.*, 1993) that citric acid accumulation is initiated because of substrate overflow and not because of a limitation of its catabolism.

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