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# Regulation of Citric Acid Production by Oxygen: Effect of Dissolved Oxygen Tension on Adenylate Levels and Respiration in *Aspergillus niger**

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**Summary.** The mechanism of the control of citric acid accumulation by oxygen was investigated by means of pilot plant fermentation using *Aspergillus niger*. The critical dissolved oxygen tension (DOT) for oxygen uptake of this fungus was about 18–21 and 23–26 mbar for trophophase and idiophase, respectively. Minimal DOT for citric acid production was about 25 mbar. Citric acid production increased steadily between 40–150 mbar. Short time changes in the DOT produced immediate, irreversible changes in the rate of product formation. Adenine nucleotides paralleled growth but showed no evidence for control function in the oxygen effect on citric acid fermentation. A branched respiratory system was identified by experiments using specific inhibitors (antimycin, cyanide, azide, rotenone, amytal and salicylhydroxamic acid). Growth was sensitive towards inhibitors of the standard respiratory chain, but only slightly sensitive towards salicylhydroxamic acid (SHAM). Citric acid synthesis was highly sensitive towards SHAM during trophophase, but sensitive towards antimycine during idiophase. Interruptions in aeration cause an impairment of the SHAM sensitive oxidase during trophophase, and of the antimycin sensitive oxidase during idiophase.

## Introduction

Industrial production of various metabolites by filamentous fungi is susceptible to regulation by the dissolved oxygen tension (DOT) of the medium (Bull and Bushell 1975). As these products in most cases are produced by differentiated cells, it is evident that the critical DOT for growth and the critical DOT for product formation are distinct parameters and in general the latter is significantly higher. This is a common feature of fungal metabolism and points to a general role of oxygen in metabolic regulation.

*Dedicated to emeritus Professor Dr. Richard Brunner on the occasion of his 80th birthday

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In the case of citric acid production by *Aspergillus niger*, the influence of oxygen has been well established (Shu 1953; Clark and Lentz 1961; Khan and Ghose 1973), but the metabolic basis of this phenomenon has never been investigated.

A limited number of studies have dealt with the influence of the DOT on metabolism of *Aspergillus nidulans* (Carter and Bull 1969, 1971; Rowley and Pirt 1972), *Mucor geneviensis* (Rogers et al. 1974) and *Cokeromyces poitrasii* (Rogers and Gleason 1974). However all these studies were made by means of continuous culture under otherwise optimal nutrient conditions and thus bear little relevance to industrial metabolite productions, which are favoured by certain growth limiting conditions.

The present paper describes work with pilot plant citric acid production by *Aspergillus niger*. The aim of this study was to examine the metabolic pathways which are influenced by the DOT. Special interest was focussed on whether respiration or the adenine nucleotide system play any role in oxygen regulation of this fermentation.

## Materials and Methods

### *General*

A detailed description of citric acid production on pilot plant scale has been given previously in this journal (Kubicek and Röhr 1978). Unless otherwise stated, the same strain *(Aspergillus niger B 60)*, inoculum preparation, and analysis of the fermentation were used.

### *Measurement of DOT, Oxygen Uptake, and Carbon Dioxide Evolution*

Oxygen and carbon dioxide in the exit air were determined continuously as described previously (Kubicek et al. 1979), using the calculation method of Fiechter and Von Meyenburg (1969). DOT was monitored by means of a sterilizable polarographic type of electrode (Ingold).

### *Shake Flask Experiments*

When a spectrum of inhibitors was investigated for their possible influence on citric acid accumulation, the following procedure was used. Wide-mouthed Erlenmeyer flasks (100 ml) were inoculated with 20 ml of fermentor broth at a given stage of the pilot plant fermentation. After incubating the flasks on an orbital shaker at 250 rev/min the respective drugs were added by injection. The flasks were harvested after 5, 10, 15, and 20 h of incubation and analyzed for biomass concentration and citric acid. A series of flasks lacking the inhibitor was always included to check the validity of this procedure.

### *Manometric Techniques*

Respiratory measurements were made by means of an automatic Warburg apparatus (Longator) equipped with an external recorder, according to standard techniques (Umbreit et al. 1964).

### *Extraction of Adenylates*

Samples of 10–15 ml of fermentor broth were directly collected in a glass homogenizer tube containing 1 ml of 70% (w/v) perchloric acid. Care was taken that the samples did not touch the glass walls of the tube. Immediately after this the samples were

homogenized with several strokes of a teflon homogenizer at 4°C. The homogenates were then neutralized in the cold as described previously (Kubicek and Röhr 1978). Storage of the extracts at -20°C was possible, but repeated freezing and thawing was detrimental, resulting in lower levels of each nucleotide tested (ATP, ADP, AMP). Extracellular nucleotides were not determined. Preliminary experiments showed that negligible amounts of adenine nucleotides were present in the culture fluid.

*Assay of Adenine Nucleotides*

Adenine nucleotides were assayed by a modified procedure of the original fluorometric method of Lundin and Thorne (1974). Vials of commercially available luciferine-luciferase extract were reconstituted with 10 ml of 10 mM $MgSO_4 . 7H_2O$ und 1 mM EDTA in 1% (w/v) bovine serum albumin. Storage at -20°C was possible but resulted in partial inactivation of the enzyme. During analysis the enzyme solution was kept in an ice bath and direct light was avoided.

The assay was carried out using an Aminco fluorospectrophotometer with an external recorder. For each assay 0.3 ml luciferine-luciferase extract and 1.6 ml of 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM EDTA, were pipetted into a cuvette, which was then placed in a thermostabilized cuvette chamber (27°C). Twenty–100 µl of extract was injected rapidly to produce a flashpeak proportional to the amount of ATP present. An appropriate amount of standard was always injected into the same cuvette to compensate for the presence of certain substances from the fermenter broth, which partially inactivated the enzyme.

ATP was assayed directly. ADP and AMP were assayed after being converted to ATP using pyruvate kinase and myokinase (Williamson and Corckey 1973).

## Results

*Influence of DOT on the Kinetics of Citric Acid Fermentation*

A series of citric acid fermentations were run at a constant rate of aeration (0.5 vvm) at three different impeller speeds (1650, 600, and 450 rev/min, respectively). As shown in Fig. 1, this produced sufficiently different levels of DOT during the production phase of the fermentation.

The time courses of biomass formation, product accumulation, oxygen uptake rate (volumetric rate, $dO_2/dt$) and carbon dioxide evolution rate (volumetric rate, $dCO_2/dt$) are given in Fig. 2. It is shown that growth, acid production and oxygen uptake are favoured by increasing DOT in the medium. The effect on growth is in accordance with Zetelaki (1970) and Kristiansen and Sinclair (1979) but in contrast to Khan and Ghose (1973).

Considering the rates of product formation and gaseous metabolism per unit of biomass (Fig. 3) it is obvious that citric acid synthesis is affected specifically by changes in the DOT. No pronounced differences could be found with the specific rates of oxygen uptake or carbon dioxide evolution.

*Determination of the Critical DOT for Respiration and Citric Acid Production*

In an attempt to distinguish clearly between the influence of the DOT on oxygen uptake and on acid formation, we tried to determine the critical DOTs for both. As these

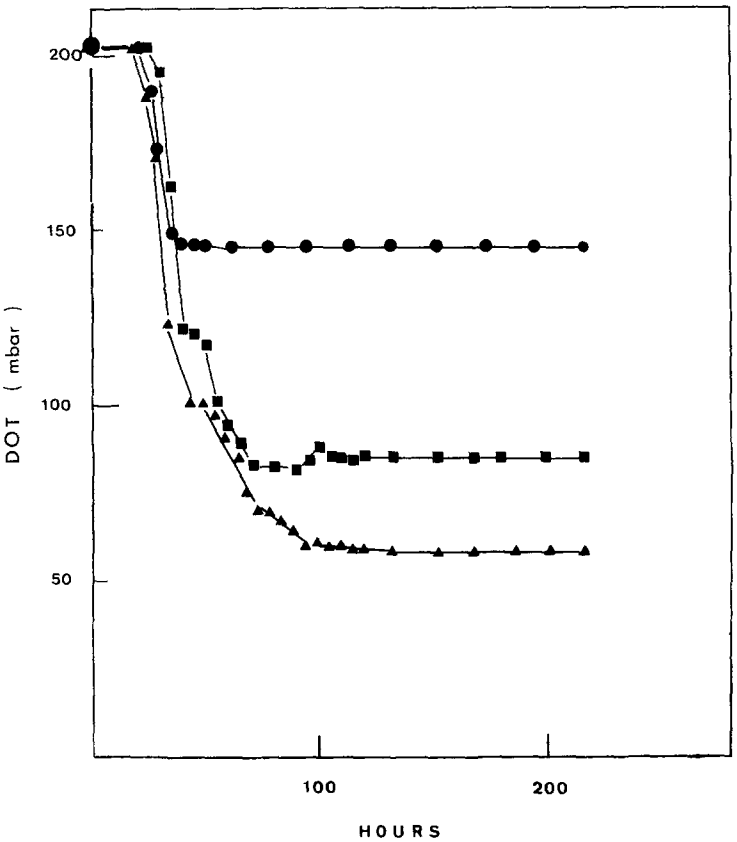


**Fig. 1.** Changes in dissolved oxygen tension (DOT) during citric acid fermentation under varying degrees of aeration: ( ● ) 1650 rev/min impeller speed, ( ■ ) 600 rev/min; ( ▲ ) 450 rev/min

values may change throughout the course of a batch fermentation, determinations were made during trophophase (at 60 h) as well as during idiophase (at 150 h). As shown in Fig. 4, values of 18–21 mbar (trophophase) and 23–26 mbar (idiophase) were obtained by calculating the critical DOT from an exhaustion curve according to Carter and Bull (1971). They are somewhat higher than those reported by Phillips and Johnson (1961), who reported a critical DOT of about 15 mbar, but this may be related to differences in the medium or the strain used.

The effect of lowering the DOT in a stepwise manner on oxygen uptake and citric acid production is shown in Table 1 for an idiophase culture. Citric acid production was found to be proportional to the DOT of the medium. Lowering the DOT below about 25 mbar resulted in complete loss of productivity.

*Effect of DOT Shifting*

As has been reported previously (Kubicek et al. 1979), changes in citric acid production rate could be produced within a few minutes by altering aeration, i.e., the DOT.

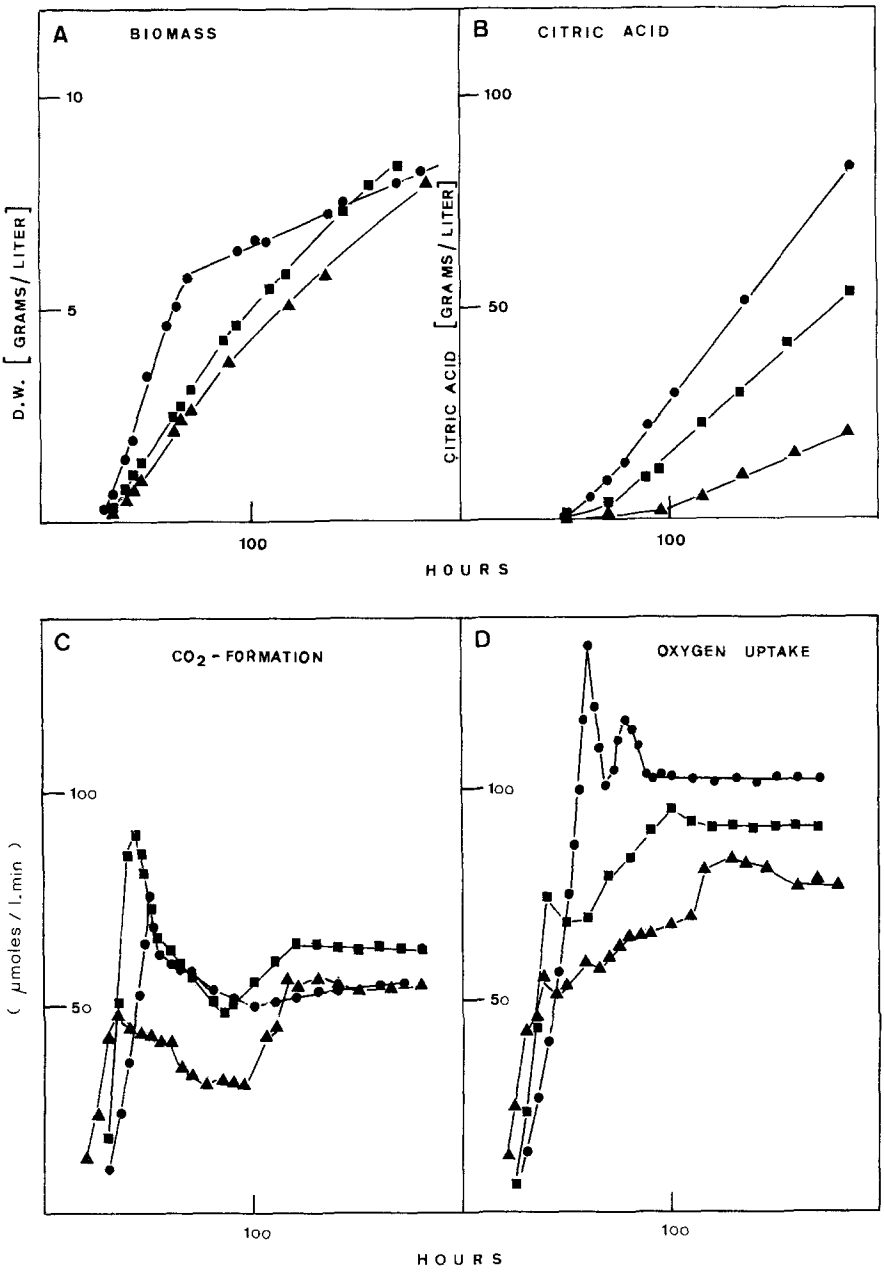


Fig. 2 A-D. Changes in several external parameters during citric acid fermentation under varying degrees of aeration. A biomass increase; B citric acid accumulation; C oxygen uptake rate; D carbon dioxide formation; symbols indicate conditions of aeration as in Fig. 1

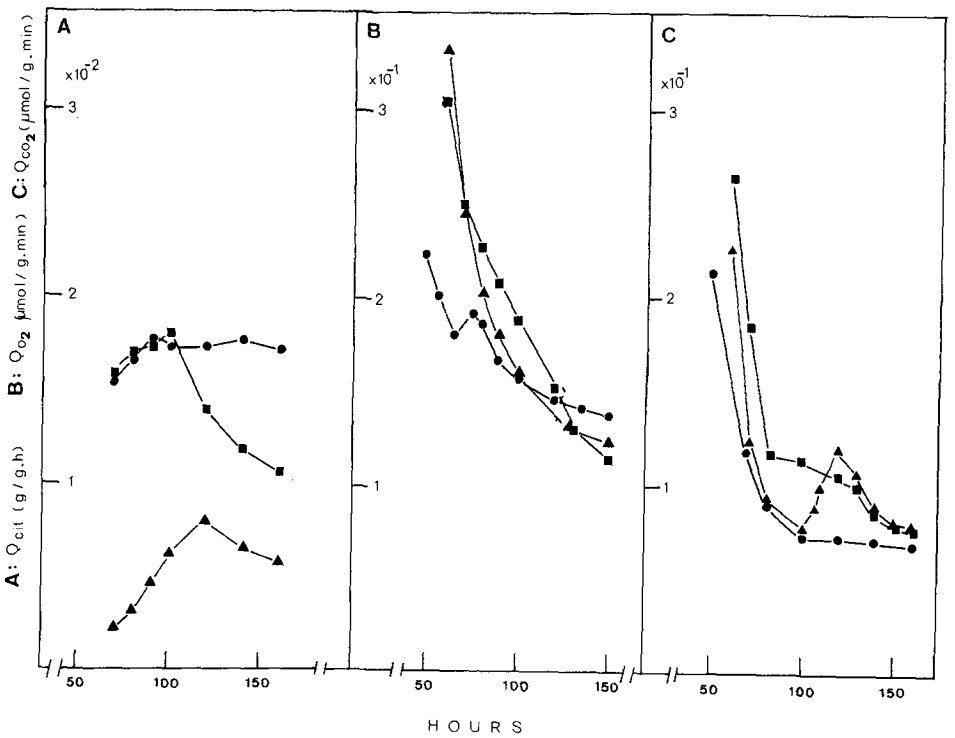


**Fig. 3 A-C.** Specific rates of citric acid accumulation (A), oxygen uptake (B) and carbon dioxide evolution (C) during citric acid fermentation under varying degrees of aeration. Symbols are as in Fig. 1.

During these few minutes overshoot/undershoot responses in the uptake of oxygen and the output of carbon dioxide could be demonstrated (Fig. 5).

When a culture, which was maintained at a lower DOT for 10–30 min, was again increased to its original DOT level, this regulatory switch resulted in favoured growth and lower oxygen uptake and citrate production, but had no significant effect on carbon dioxide output. Moreover, it was in part irreversible as during trophophase the original production of citric acid was restored after 4–6 h, whereas during idiophase the culture remained at a lower production rate.

The regulatory effect of changes in the DOT was dependent on several parameters such as the age of culture, the period of remaining at the lower DOT, the DOT values before and after the shift and nutrient parameters. The effect was more pronounced with older cultures and by increasing the time at the lower DOT.

*Effect of Interruption of Aeration*

An extreme case of suboptimal aeration in citric acid accumulation is a temporary interruption of aeration. Interruptions up to 20 min did not reduce the viability of the fungus, but resulted in a complete loss of ability to produce citric acid. When the fermentation was continued the growth rate increased. Similar properties were observed

**Table 1.** Effect of dissolved oxygen tension (DOT) on the volumetric rate of oxygen consumption ($dO_2/dt$) and citric acid production (dCIT/dt) of *Aspergillus niger*

| DOT (mbar) | $dO_2/dt$ | dCIT/dt |
|---|---|---|
| 159 | 106 | 32 |
| 132 | 102 | 28 |
| 93.5 | 90 | 23 |
| 80 | – | 21 |
| 50 | 85 | 14 |
| 39 | – | 9 |
| 32 | 74 | 3 |
| 18 | 63 | 0 |
| 8 | 57 | 0 |
| 5 | 43 | 0 |

Volumetric rates are given in μmol/l/min. The age of culture was 150 h. Respiration was measured from the exhaust gas of the fermentor. DOT levels were varied by stepwise decreases of the impeller speed. The reverse experiment (starting with a culture of low DOT) was not possible because of the irreversibility of the oxygen effect. However it could be shown that the size of the steps did not affect the results. The values given are means of two separate experiments

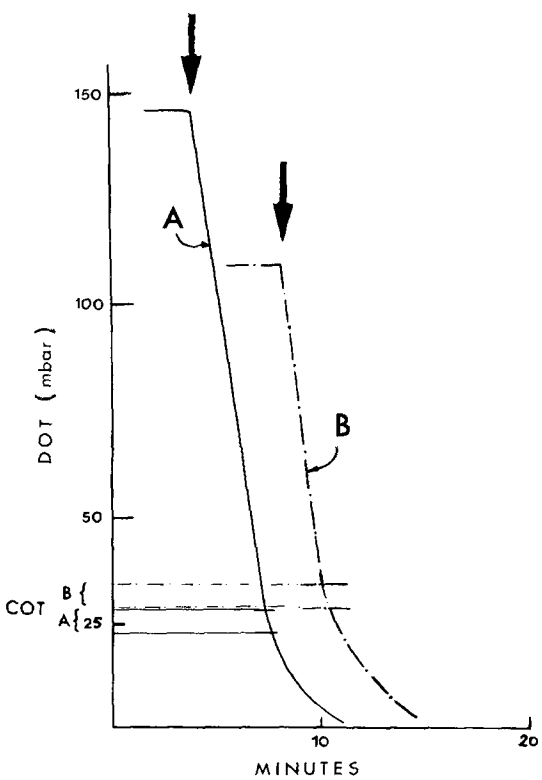


Fig. 4. Exhaustion curves. At the time marked by *arrow*, oxygen supply was cut off and oxygen uptake rate was calculated from the disappearance of oxygen in the fermentor. *(A)* trophophase culture (65 h), *(B)* idiophase culture (150 h); at oxygen tensions below the critical oxygen tension (COT) oxygen uptake was no longer independent of the oxygen tensions in the medium

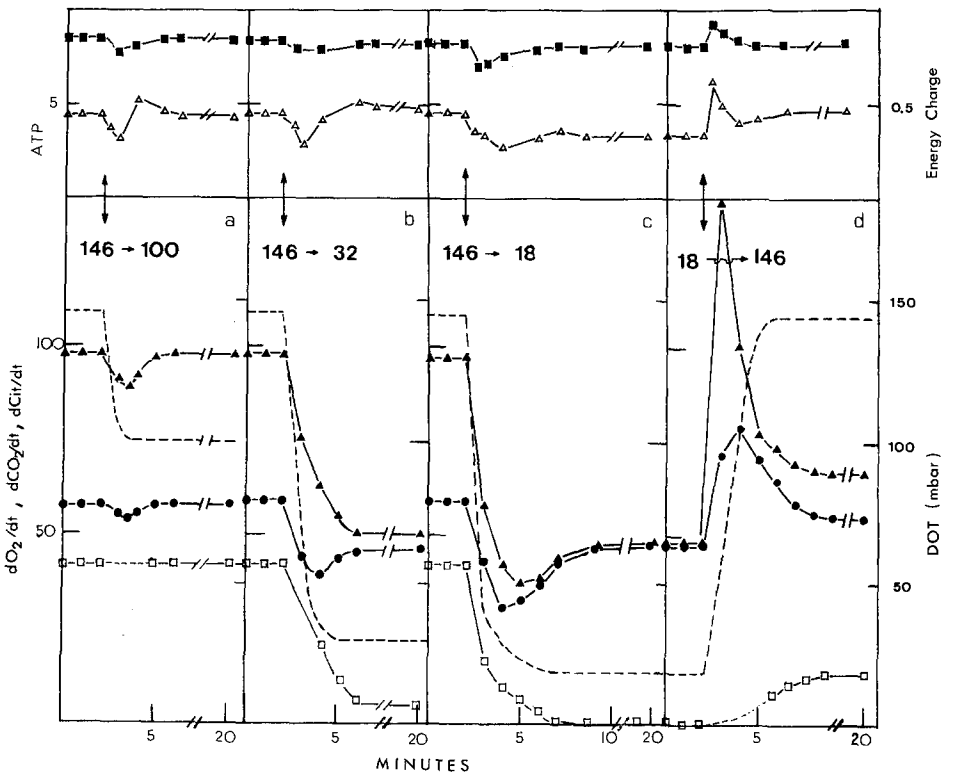


**Fig. 5 a-d.** Short time changes in DOT. Four examples are given to illustrate the effect of changes in DOT on citric acid accumulation (□), oxygen uptake (▲), carbon dioxide formation (●), ATP levels (△) and the energy charge function [ ■, E.C. = 0.5 ( 2 ATP + ADP / ATP + ADP + AMP )]. DOT was monitored continuously and is shown by the *dotted line*. The experiments have not been repeated, but are selected from a number of approximately 30 similar experiments, which all showed the same trends. Citric acid synthesis is given as the amount of $CO_2$ fixation, calculated as dCIT/dt = ( $dO_2/dt - dCO_2/dt$) x 2/3 (Kubicek et al. 1979). During transient changes in respiration no calculations were made because it is not known whether the equation, which forms the basis of this calculation, is applicable during this short period.

Gaseous metabolism rates and production rates are given as micromoles per liter and minute, DOT is given as mbar, ATP as micromoles per gram mycelial dry weight. *Arrows* indicate the time when DOT was changed (i.e., impeller speed was reduced). Final DOT levels (in mbar) are inserted into the figures. All experiments given were made with a 120 h old fermentation under standard optimal conditions. Each figure (**a** – **d**) refers to a separate experiment, except for **d**, which was a continuation of experiment **c**.

as in the case of DOT shifting: trophophase cultures recovered with regards to acidogenesis whereas with idiophase cultures this could never be observed.

### *Effect of DOT on Adenosine Phosphate Levels and Energy Charge*

In an attempt to find the intracellular regulator responsible for this control of citric acid synthesis by DOT, adenine nucleotides were studied. ATP or the ratio of the individual adenine nucleotides have dominant control functions in metabolism, and one

of the most important functions of oxygen in metabolism is as a substrate for the generation of energy, i.e., ATP.

The time course of ATP, ADP, and AMP levels throughout the fermentation has already been reported by Habison et al. (1979). In general, the levels were high during trophophase, and they decreased during idiophase. Energy charge was about 0.85 during trophophase, and decreased slightly during idiophase to about 0.80. No evidence was obtained for characteristic changes of adenosine phosphates at the onset of citrate production (Fig. 6).

The behaviour of ATP and the energy charge function during DOT shifting experiments is included in Fig. 5. ATP closely resembled oxygen uptake as some evidence for overshoots and undershoots was obtained. However, lowering the DOT levels suboptimal for citrate accumulation but well above the critical DOT for growth did not reduce either the energy charge or the ATP levels. Only lowering the DOT below the critical level for growth produced alterations in ATP and energy charge. This points to rather similar critical DOT values for growth and cellular ATP concentration.

The energy charge function was always almost constant during these experiments and sufficiently high to suggest that the adenine nucleotides exert a tight control of *Aspergillus niger* metabolic activity. On the other hand these investigations gave little evidence for the control of citric acid synthesis by oxygen via the adenine nucleotide system.

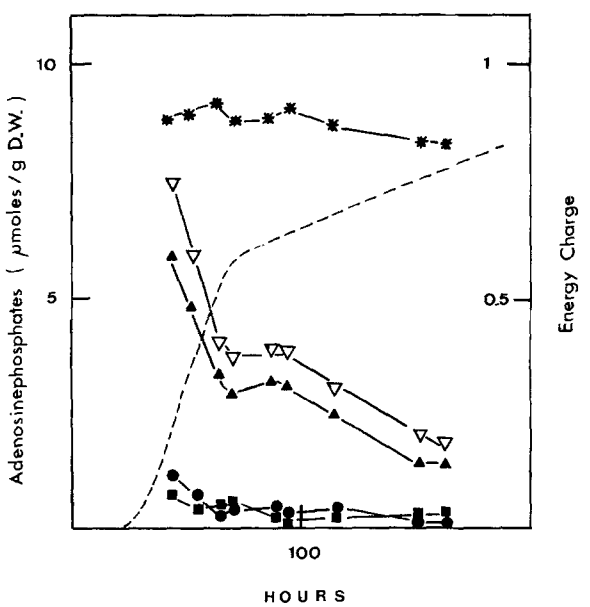


Fig. 6. Changes in adenylate levels and energy charge during citric acid fermentation. Concentrations of adenylates are given as micromoles per gram of mycelial dry weight, calculated from the volume of fermentor broth taken for extraction of the nucleotides. Each point is a mean value of two separate assays each of two separated extracted samples. The growth curve is shown by the dotted line. Data points of the growth curve are available from Fig. 2. ▼, total adenylate concentration; ▲, ATP; ●, ADP; ■, AMP; ★, energy charge calculated as in the legend to Fig. 5

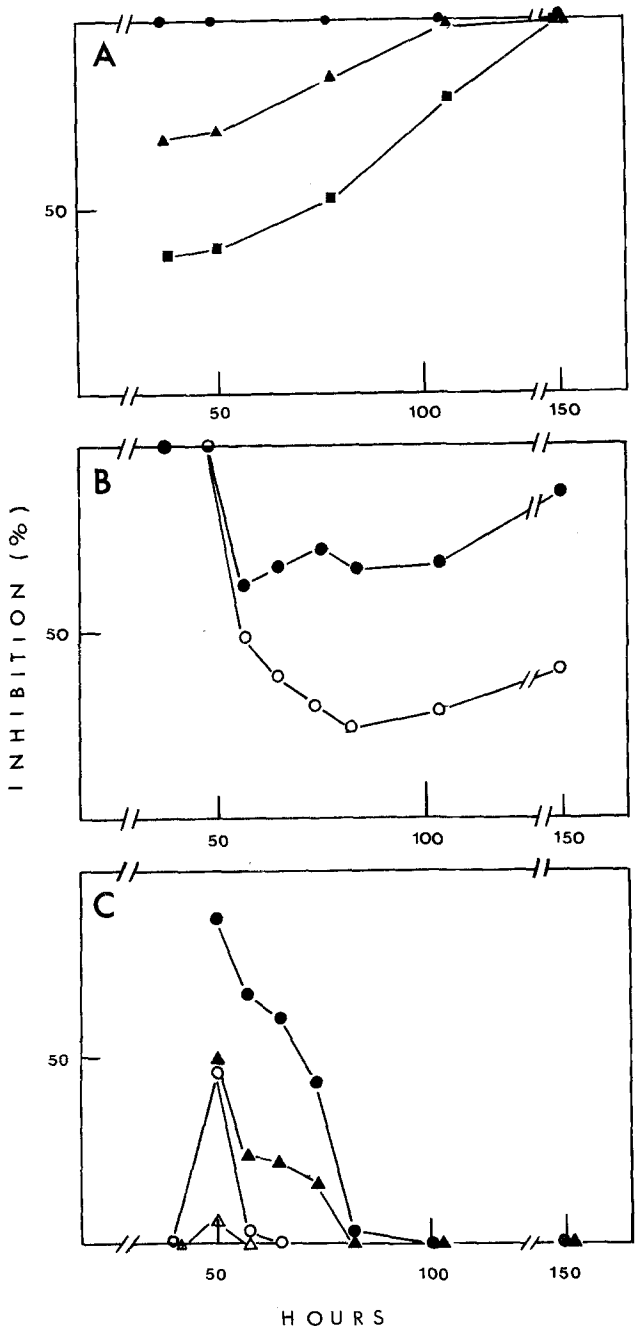


**Fig. 7 A-C.** Dynamics of cellular respiration as indicated by its sensitivity towards $NaN_3$ (A), antimycin (B) and SHAM (C). At appropriate times during fermentation, the culture was quickly transferred to a series of Erlenmeyer flasks with a total volume of 100 ml in portions of 20 ml. Details are as in the Materials and Methods. Because of the slow rate of growth during idiophase, the sensitivity of respiration was taken as a measurable value of growth. It has been shown during idiophase that growth and oxygen uptake behaved in a similar way. A. ■, $2.5 \times 10^{-5}$ M; ▲, $2.5 \times 10^{-4}$ M; ▶

*Respiratory Activities During Citric Acid Fermentation*

It has been demonstrated in the previous paragraphs of this paper that reducing the DOT resulted in partial loss of oxygen consumption by the fungus (cf. Fig. 5d). In order to investigate the activity of the respiratory chain 'in vivo' during citric acid fermentation, the influence of various inhibitors was tested at various stages of the fermentation with regards to growth and acidogenesis.

The results obtained are given in Fig. 7. Qualitative and quantitative changes in sensitivity were observed. During the first 50 h of fermentation only growth could be investigated, since no citric acid was present in the medium. During this phase, growth was highly sensitive towards azide and antimycin, but not towards rotenone or amytal (not shown) and salicylhydroxamate (SHAM). The effect of each drug on respiration was checked by manometric measurements.

With the onset of citric acid production, sensitivity of growth towards these inhibitors was increased. Furthermore sensitivity towards SHAM was observable, which was most pronounced with citric acid production and less with growth. Citric acid synthesis was also sensitive towards antimycin and azide.

With the beginning of idiophase (i.e., between 70–85 h) SHAM sensitivity disappeared, but sensitivity towards antimycin and azide increased. It should be noted that it was impossible to inactivate growth completely with antimycin, indicating an additional bypass of the respiratory chain at this stage. Azide, however, inhibited growth completely. In both cases citric acid synthesis proved more sensitive than growth.

*Influence of Inhibitors of Respiration on Cellular Adenosine Phosphate Pool*

These experiments (Fig. 8) were made in order to obtain some information about the contributions of the steps of the respiratory chain to energy metabolism in *Aspergillus niger*. The response of cellular adenine nucleotides and energy charge was investigated following the addition of azide, antimycin and SHAM. During trophophase, it was possible to inactivate the culture completely by addition of the drugs, which was reflected by a decrease in ATP and the energy charge, and rises in ADP and AMP levels. SHAM had only little effect on adenosine phosphates and the energy charge.

During idiophase, the lack of complete sensitivity towards antimycin was reflected in the maintenance of a constant, low level of ATP and energy charge after addition of the inhibitor. Azide however gave results comparable to trophophase.

*Inactivation of Respiratory Activities by Interruption of Aeration*

Two experiments were made where the influence of these inhibitors was tested before and after interruption of aeration, both for cultures during trophophase (at 60 h) and during idiophase (at 150 h).

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●, $5 \times 10^{-4}$ M $NaN_3$. No difference was observable between growth and citrate production. **B.** 2.5 $\times 10^{-6}$ antimycin; open symbols indicate growth, closed symbols citrate production. **C.** ●, 2.5 mM; ▲, 1.25 mM; symbols otherwise as indicated in **B.** 100% means that addition of inhibitor reduces growth or citrate production respectively to 0% within the following 10 hours compared with the control lacking the inhibitor

The results are given in Table 2. During trophophase, this treatment resulted in irreversible loss of the SHAM sensitive respiratory pathway. During idiophase, however, interruptions in aeration partly inactivated both antimycin and azide sensitive respiration.

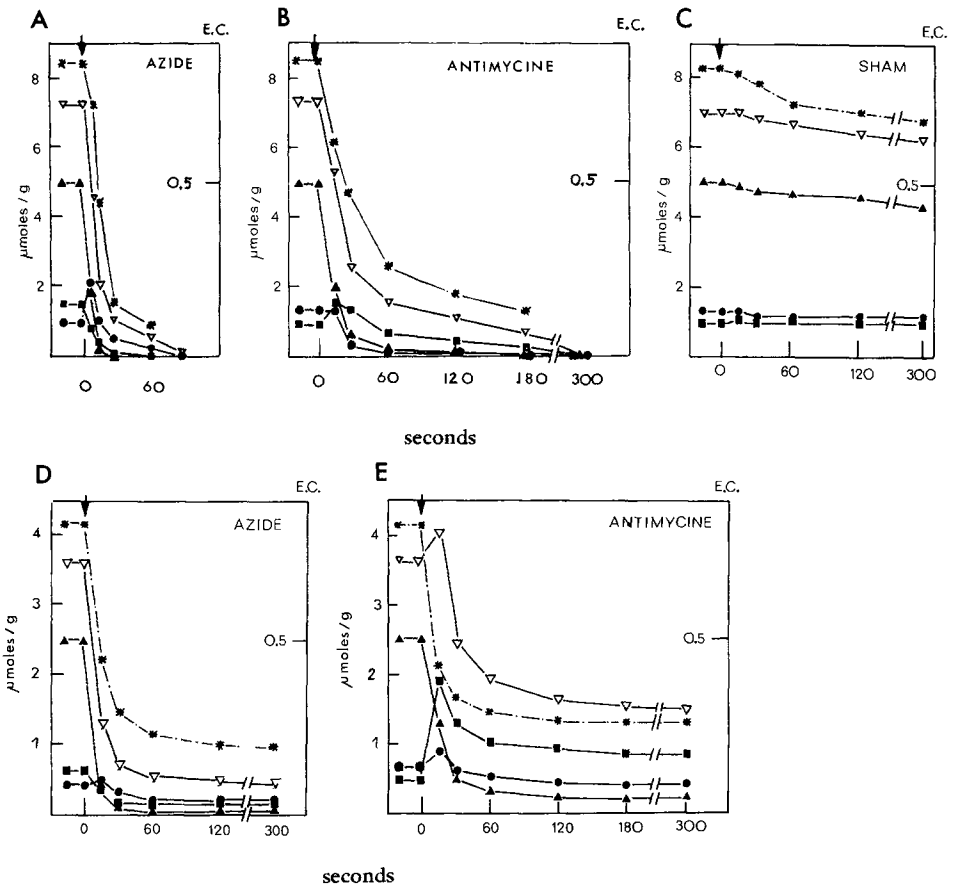


**Fig. 8 A-E.** Effect of addition of inhibitors of respiration on adenylate levels and energy charge. Experiment **A** - **C** was done during trophophase (60 h) and **D** - **E** during idiophase (150 h). The arrows on top of the figure indicate the time of addition of the inhibitor. The concentrations applied were $NaN_3$, $10^{-5}$ M; antimycin, $10^{-5}$ M; SHAM, $10^{-2}$ M. M refers to liters of fermentation broth. Addition of the drugs was performed by injecting an appropriate solution in either ethanol (antimycin), acetone (SHAM) or distilled water (azide). The addition of these solvents alone produced deviations in the pool levels of less than ± 5%. Symbols are as in Fig. 6

## Discussion

This work clearly shows that citric acid accumulation is favoured by increasing the DOT of the fermentation medium. Furthermore it demonstrates that oxygen acts as a direct regulator of citric acid accumulation, since a response was obtained within only few

minutes after altering the oxygen supply. These changes in citric acid production were accompanied by changes in the oxygen uptake rate, whereas carbon dioxide formation remained relatively unaffected and this suggests that there is a regulatory linkage between respiration and citric acid accumulation. The lack of coordination in the behaviour of the adenine nucleotides and citric acid synthesis, however, makes it probable that the oxygen effect is not exerted via the adenine nucleotides.

**Table 2.** Data refers to an experiment carried out with citric acid producing fermentations at trophophase (60 h) and idiophase (150 h) under otherwise optimal conditions as given in 'Materials and Methods'. Aeration was interrupted for 20 min by disconnecting the impeller, which resulted in a decay in the DOT below 5 mbar. 'Before Interruption' refers to data obtained immediately before interrupting aeration. 'After Interruption' refers to the steady state in gaseous metabolism, which was reached approximately 10–15 min after reinitation of aeration. Steady state was considered to be reached when the rates of oxygen uptake did not differ within three consecutive minutes.

Inhibitors were added by injecting the appropriate concentration of the drug to give the concentration per liter as indicated. The concentrations (M) were $NaN_3$ $10^{-5}$, Antimycin $10^{-5}$ and SHAM, $10^{-2}$. Solvents were used as described in Fig. 8.

Energy charge was calculated from the adenine nucleotide determinations, and is given as the means of five separate determinations.

Citric acid production was measured by the indirect method published recently (Kubicek et al. 1979) and checked by enzymatic analysis

| Trophophase (μmol/l/min) | Before Interruption | | After Interruption | |
|---|---|---|---|---|
| dCIT/dt | 25.4 | (100%) | 4 | (15.7%) |
| $dO_2/dt$ | 87.0 | (100%) | 72 | (82.7%) |
| $dO_2/dt$ + Antimycine | 24.0 | ( 27.6%) | 7.2 | ( 8.2%) |
| $dO_2/dt$ + Antimycin + SHAM | 8.7 | ( 10.0%) | 7.2 | ( 8.2%) |
| $dO_2/dt$ + $NaN_3$ | 0 | | 0 | |
| Energy charge | 0.905 | | 0.906 | |
| Idiophase (μmol/l/min) | | | | |
| dCIT/dt | 20.5 | (100%) | 3 | (14.4%) |
| $dO_2/dt$ | 98 | (100%) | 78 | (79% ) |
| $dO_2/dt$ + Antimycin | 78.4 | ( 80%) | 39.5 | (39.7%) |
| $dO_2/dt$ + Antimycin + SHAM | 78.4 | ( 80%) | 39.5 | (39.7%) |
| $dO_2/dt$ + $NaN_3$ | 0 | | 0 | |
| Energy charge | 0.857 | | 0.813 | |

A very interesting aspect of this work is the finding that a short interruption in aeration or a lowering of the DOT causes an irreversible alteration in both citric acid synthesis and respiration of *Aspergillus niger*. Some insights into the mechanism of this effect are provided by the studies using inhibitors of respiration. Evidence was obtained that *Aspergillus niger* has a branched respiratory chain. The presence of a standard respiratory chain was confirmed by the sensitivity towards antimycin, cyanide and azide. The absence of sensitivity towards site I inhibitors (rotenone, amytal) is puzzling, since the presence of site I respiration in *Aspergillus niger* has been demonstrated (Watson and

Smith 1967a). It may be possible that the inhibitors did not penetrate the cell. However absence of site I phosphorylation has been reported for some fungi (cf. Watson 1975). It should also be noted that oxidation of extramitochondrial NADH in fungi does not involve site I phosphorylation (Lambowitz et al. 1972; Kawakita 1970; Watson and Smith 1967b).

At least two types of alternative respiratory mechanisms were detected during this study. One is the recently characterized SHAM-sensitive bypass (Lambowitz and Slayman 1971; Edwards and Kwiecinsky 1973) which only appeared during late trophophase. This time citric acid accumulation was most sensitive towards inhibition by SHAM, whereas growth was only slightly affected. The role of this oxidase is still under discussion, but it is commonly accepted that it functions as a mechanism for non-phosphorylating reoxidation of glycolytic NADH under conditions of impaired energy metabolism thus permitting glycolytic ATP formation to proceed. During our studies this oxidase was very sensitive towards interruption of aeration.

The fact that only azide produced complete inhibition of respiration during idiophase could indicate a second bypass of the standard chain. This type of oxidase has recently been found in mutants of *Neurospora crassa* (Edwards and Unger 1978) and yeast like forms of *Histoplasma capsulatum* (Maresca et al. 1979); the lack of knowledge of a specific inhibitor of this pathway however makes it difficult to speculate about its nature.

In the past decade evidence has accumulated that microorganisms can use several bypasses of the electron transport chain to avoid wasteful overproduction of energy under conditions of impaired anabolism, i.e., restriction of growth (cf. Tempest and Nejssel 1979). Citric acid accumulation may be considered a good example for this situation, since during the course of this fermentation, growth becomes severely restricted due to trace metal deficiency, low pH and phosphate exhaustion. Since the process of citrate formation depends on active glycolysis, ATP formation mainly occurs at the substrate phosphorylation level. However, this produces NADH, which must be oxidized for glycolysis to continue.

We therefore conclude that the excess of oxygen during citric acid production is needed for maintenance of these 'uncoupled' turnover mechanisms. The observation that ATP is not involved in the oxygen effect on citrate synthesis would support this view.

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