Effect of Blue Light on Metabolism in *Penicillium isariiforme*

By W. D. J. GRAAFMANS*

Department of Plant Physiology, University of Amsterdam, The Netherlands

(Received 19 February 1977)

In blue light *Penicillium isariiforme* assimilates less CO₂ and excretes less citric acid into the medium than when grown in continuous darkness. This is due to a decreased supply of pyruvate which is carboxylated to form citric acid via oxaloacetate. Pyruvate carboxylase rather than phosphoenolpyruvate carboxylase or phosphoenolpyruvate carboxykinase is mainly responsible for CO₂ assimilation in this fungus. Of the glycolytic intermediates assayed, steady-state concentrations of pyruvate were low and concentrations of glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate were high in mycelium grown in the light compared with those in mycelium grown in the dark. Results of experiments with specifically-labelled glucose suggest that light stimulates the pentose phosphate pathway and inhibits the supply of pyruvate for citric acid synthesis via the Embden-Meyerhof-Parnas pathway.

INTRODUCTION

Morphogenesis in *Penicillium isariiforme* is governed to a large extent by the atmospheric concentrations of CO₂ and by light (Graafmans, 1973*a, b*, 1974). Previously I reported that cultures grown in the dark produced coremia when exposed to dim blue light and that this morphological change was correlated with a decrease in citric acid accumulation in the medium and a rise in the production of other low molecular weight intracellular intermediates, polysaccharides, lipids, nucleic acids and protein (Graafmans, 1974). This report presents a more detailed description of the effect of light on carbohydrate metabolism and its significance in relation to citric acid production.

METHODS

Unless mentioned otherwise, procedures were as described previously (Graafmans, 1973*a*, 1974). *Penicillium isariiforme* ATCC28274 was used.

Extraction and determination of glycolytic intermediates and adenine nucleotides. The mycelium was harvested by filtration, quickly rinsed in ice-cold water and frozen in liquid nitrogen before storage at -30 °C. Frozen tissue was ground to a powder in a mortar and samples were taken for the determination of moisture content by lyophilization. Metabolic intermediates were extracted by grinding with glass powder in the presence of 5% (w/v) HClO₄ followed by repeated freezing and thawing. The homogenate was centrifuged at 20000 *g* for 10 min, and the supernatant fluid was decanted and neutralized with KOH and Tris/HCl in the cold. Precipitated KClO₄ was removed by centrifuging. Adenine nucleotides and glycolytic intermediates were assayed by following the oxidation or reduction of NAD or NADP at 340 nm by means of direct or coupled enzymic reactions (Bergmeyer, 1963; Estabrook & Maitra, 1962; Maitra & Estabrook, 1964).

Carboxylating activity in mycelial extracts. Fresh mycelia were ground at 3 °C in a mortar with glass powder in 350 mM-Tris/HCl, pH 7.85, containing 18 mM-cysteine.HCl and 9 mM-diethyldithiocarbamate. The homogenate was centrifuged at 20000 *g* for 10 min and the supernatant fluid was passed through a Sephadex G-25 column to remove phenolic compounds and other low molecular weight substances. Carboxylating activity was based on the incorporation of NaH¹⁴CO₃ into malate. NADH and malate dehydrogenase were used to convert oxaloacetate to malate. The assay mixture contained (total volume 1.0 ml):

* Present address: Jacob Grootstraat 10, Middenbeemster, The Netherlands.

Fig. 1. Incorporation of CO₂ into cultures grown in the light (○) or in the dark (●). All cultures were grown in the dark for 5 days and then half of them were placed in the light. CO₂ incorporation was calculated from the radioactivity measured in the mycelium and the specific activity of the ¹⁴CO₂ administered in the incubation period of 60 s. Each result is the average of determinations for two separate cultures, pulsed and harvested at the same time. Specific activity ¹⁴CO₂, 63×10^4 d.p.m. (nmol CO₂)⁻¹; concentration 1 %.

100 mM-Tris/HCl, pH 8.4; 6 μ Ci NaH¹⁴CO₃; 20 mM-KHCO₃; 5 mM-MgCl₂; 20 mM-KCl; 1 mM-EDTA; 0.1 mM-NADH; 0.5 mg acetyl-CoA; 0.7 mg biotin; 55 units malic dehydrogenase; and up to 80 μ g extract protein. The concentrations of the various substrates were: 10 mM-pyruvate, 10 mM-phosphoenolpyruvate (PEP), 5 mM-ATP and 5 mM-ADP. Avidin was added at 0.33 mg ml⁻¹. Assays were carried out for 5 min at 25 °C in stoppered test tubes and were terminated by adding 0.5 ml 2 M-HCl. Residual ¹⁴CO₂ was removed by thoroughly shaking the mixture on a Vortex mixer. ¹⁴C-Radioactivity was measured by liquid scintillation. Carboxylating activity was proportional to the concentration of extract protein and to the incubation time. Protein was determined according to Cleland & Slater (1953).

Radiorespirometric methods. To avoid disturbing the physiological state of the cultures, ¹⁴C-labelled-substrate was added to mycelia in the original growth medium. The difference in the sucrose contents of the media of the cultures to be compared was less than 1 % at the time of labelling. At intervals, air samples were taken from the incubation flasks, passed into Hyamine hydroxide, in which ¹⁴CO₂ was trapped, and the radioactivity was measured by liquid scintillation.

Chemicals. Enzymes were from Boehringer. NaH¹⁴CO₃ and specifically-labelled glucoses were from The Radiochemical Centre, Amersham, and from NEN Chemicals, W. Germany. Avidin was from Sigma and D-biotin from Merck.

RESULTS

CO₂ fixation, carboxylating activity and the intracellular concentrations of adenine nucleotides and glycolytic intermediates

The synthesis of citric acid via anaplerotic mechanisms may be assayed conveniently by ¹⁴CO₂ fixation (Graafmans, 1973a). The lower ¹⁴CO₂ fixation rate in cultures grown in the light compared with those grown in the dark (Fig. 1) might be due to a diminished supply of substrate (pyruvate or PEP) and/or diminished synthesis or activity of the carboxylation enzymes. Cultures exposed to the light had slightly higher intracellular concentrations of PEP and lower concentrations of pyruvate than those grown in the dark (Fig. 2). Since the highest carboxylating activity of mycelial extracts was found in the presence of pyruvate and ATP (Table 1), pyruvate rather than PEP was probably the main substrate for carboxylation, via pyruvate carboxylase (EC 6.4.1.1), in whole mycelium. This enzyme has a requirement for biotin not normally shown by the alternative enzymes PEP carboxylase and PEP carboxykinase (Cannata & Stoppani, 1963). When avidin was added to carboxylation assays lacking biotin the activity was lowered, and was partially restored by adding biotin, thus suggesting that pyruvate carboxylase was indeed functioning.

Table 1. *Carboxylating activity of mycelial extracts*

Mycelium was grown for 5 days in the dark followed by 2 days in the light before extracts were prepared. $\text{NaH}^{14}\text{CO}_3$ was added to each assay mixture at 12.8×10^6 d.p.m. Carboxylating activity is expressed as d.p.m. per assay.

	Pyruvate carboxylase	PEP carboxylase	PEP carboxykinase
Alterations to standard assay mixture	Pyruvate + ATP	PEP	PEP + ADP
– Biotin	17870	15	164
+ Biotin	17878	46	181
– Biotin } + Avidin }	24	5	8
+ Biotin } + Avidin }	646	15	0
+ Biotin } – Acetyl-CoA }	14945	—	—

Fig. 2. Concentrations of adenine nucleotides and glycolytic intermediates in cultures grown in the light (○) or in the dark (●). All cultures were grown in the dark for 5 days and then half of them were placed in the light. G-6-P, Glucose 6-phosphate; G-1-P, glucose 1-phosphate; Fru-6-P, fructose 6-phosphate; Fru-1,6-diP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate.

Acetyl-CoA is an activator of pyruvate carboxylase from different sources (Utter & Keech, 1960; Ruiz-Amil *et al.*, 1965; Budd, 1971) and, likewise, if acetyl-CoA was omitted from the standard assay mixture, the measured activity was lower (Table 1). Moreover, this confirms that $^{14}\text{CO}_2$ was incorporated in malate and not in malonyl-CoA.

Because of a possible limiting supply of pyruvate, the kinetics of carboxylation with respect to pyruvate concentration were studied. Pyruvate carboxylase from both light-exposed and dark-grown mycelium had an apparent K_m value of 0.18 mM for pyruvate. As 0.18 μmol pyruvate (ml cell water) $^{-1}$ is equivalent to 1.1 μmol pyruvate (g dry wt mycelium) $^{-1}$ and as the steady-state concentrations of pyruvate in light-exposed mycelium are well below this value (Fig. 2), the amount of pyruvate might well be a limiting factor for citric acid synthesis. The theoretical amount of CO_2 fixation can be calculated on the basis of this K_m value and the concentration of pyruvate in the mycelium. For light-exposed and dark-grown mycelium on day 7, the theoretical values [2.11 and 6.27 nmol CO_2 min $^{-1}$ (mg dry wt) $^{-1}$ respectively] are well above the experimental values [0.38 and 1.10 nmol CO_2 min $^{-1}$ (mg dry wt) $^{-1}$] although the ratio of theoretical to experimental values for light-exposed and dark-grown mycelium is similar.

To determine the total pyruvate carboxylase content of light-exposed and dark-grown mycelium, CO_2 fixation was studied using mycelial extracts from cultures harvested at different times. A minimum value of about 60 nmol CO_2 min $^{-1}$ (mg soluble protein) $^{-1}$ was found and, as the protein extracted was 21 % of the dry matter, this corresponded to 12.8 nmol CO_2 min $^{-1}$ (mg dry wt) $^{-1}$. CO_2 fixation in whole mycelium was between 0.30 and 1.25 nmol CO_2 min $^{-1}$ (mg dry wt) $^{-1}$ (Fig. 1). Although the true values for CO_2 fixation *in vivo* are probably above these figures, because of dilution of the added $^{14}\text{CO}_2$, it seems likely that the full potential of the enzyme is not being expressed in whole mycelium.

Experiments with specifically-labelled glucoses

To test if there was a diminished supply of pyruvate via the Embden–Meyerhof–Parnas (EMP) pathway, radiorespirometric experiments were performed with specifically-labelled glucoses to compare carbohydrate metabolism in light-exposed and dark-grown cultures (Wang, 1972). If glucose is metabolized by way of the EMP pathway, $^{14}\text{CO}_2$ evolution from [1- ^{14}C]glucose and [6- ^{14}C]glucose will be equivalent. If the pentose phosphate pathway is also operative, more $^{14}\text{CO}_2$ will be lost from [1- ^{14}C]glucose than from [6- ^{14}C]glucose.

There was a very low yield of $^{14}\text{CO}_2$ from [6- ^{14}C]glucose compared with that from [1- ^{14}C]glucose under both conditions (cf. Graafmans, 1976). The evolution of $^{14}\text{CO}_2$ from [1- ^{14}C]glucose was always much higher in light-exposed cultures. Thus the pentose phosphate pathway is operative and is stimulated by light.

Separate experiments with [3,4- ^{14}C]glucose verified this conclusion. The extent of $^{14}\text{CO}_2$ evolution from [3,4- ^{14}C]glucose was indicative of carboxylation of pyruvate decarboxylase: after exposure to light, less $^{14}\text{CO}_2$ was evolved from [3,4- ^{14}C]glucose than in the dark.

DISCUSSION

Pyruvate was identified as the substrate for carboxylation. The apparent K_m of 0.18 mM pyruvate for pyruvate carboxylase is somewhat lower than the values of 0.29 mM and 0.28 mM reported for *Neocosmospora vasinfecta* (Budd, 1971) and *Aspergillus niger* (Feir & Suzuki, 1969) respectively. The steady state concentrations of pyruvate in light-exposed mycelium were well below the K_m for pyruvate. Therefore, unless compartmentation is a major factor, pyruvate may be rate-limiting for citric acid synthesis. The significant drop in pyruvate concentration in dark-grown cultures suggests pyruvate may be limiting in these cultures as well. In keeping with these conclusions, assimilation of CO_2 declines in dark-grown cultures (Fig. 1) and after 7 days growth in the dark the accumulation of citric acid also decreases (Graafmans, 1974). A limitation of enzyme activity by substrate availability

would not be unusual in view of the situation encountered in other systems (cf. Wright, 1966).

The experiments with specifically-labelled glucoses support the hypothesis that the flow of carbon via the EMP pathway is inhibited in the light. Other experiments have shown that polysaccharide synthesis in *P. isariiforme* is stimulated in the light (Graafmans, 1976), which was to be expected in view of the increase in dry weight under these conditions (Graafmans, 1974). This, combined with a stimulation of the pentose phosphate pathway, implies an increasing need for glucose 6-phosphate. As sucrose uptake is the same in both the dark and the light (Graafmans, 1974), less endogenous substrate will be channelled via the glycolytic EMP pathway in the light.

A stimulation of the pentose phosphate pathway under differentiating conditions has also been reported for several other fungi. Turian & Bianchi (1972), Ng, Smith & Anderson (1972) and Wong & Willets (1974) suggested that the pentose phosphate pathway may be important in the conidiation of *Neurospora crassa*, the conidiophore differentiation of *A. niger* and the morphogenesis of sclerotia in *Sclerotinia sclerotiorum*, respectively.

Metabolism through the pentose phosphate pathway would provide both pentose sugars for nucleic acid synthesis and NADPH for the biosynthetic reactions associated with differentiation.

REFERENCES

- BERGMEYER, H. U. (1963). *Methods of Enzymatic Analysis*. New York and London: Academic Press.
- BUDD, K. (1971). Bicarbonate fixation by cell-free extracts and by mycelium of *Neocosmospora vasinfecta*. *Journal of General Microbiology* **67**, 99–106.
- CANNATA, J. J. B. & STOPPANI, A. O. M. (1963). Phosphopyruvate carboxylase from bakers' yeast. *Journal of Biological Chemistry* **238**, 1196–1207.
- CLELAND, K. W. & SLATER, E. C. (1953). Respiratory granules of heart muscle. *Biochemical Journal* **53**, 547–556.
- ESTABROOK, R. W. & MAITRA, P. K. (1962). A fluorimetric method for the quantitative microanalysis of adenine and pyridine nucleotides. *Analytical Biochemistry* **3**, 369–382.
- FEIR, H. A. & SUZUKI, I. (1969). Pyruvate carboxylase of *Aspergillus niger*: kinetic study of a biotin-containing carboxylase. *Canadian Journal of Biochemistry* **47**, 697–710.
- GRAAFMANS, W. D. J. (1973*a*). The influence of carbon dioxide on morphogenesis in *Penicillium isariiforme*. *Archiv für Mikrobiologie* **91**, 67–71.
- GRAAFMANS, W. D. J. (1973*b*). The influence of carbon dioxide on sporulation and citric acid synthesis in dark-grown *Penicillium isariiforme*. *Acta botanica neerlandica* **22**, 543–545.
- GRAAFMANS, W. D. J. (1974). Metabolism in *Penicillium isariiforme* on exposure to light, with special reference to citric acid synthesis. *Journal of General Microbiology* **82**, 247–252.
- GRAAFMANS, W. D. J. (1976). *Enige biochemische aspecten van de morfogenese bij Penicillium isariiforme*. Ph.D. thesis, University of Amsterdam.
- MAITRA, P. K. & ESTABROOK, R. W. (1964). A fluorimetric method for the enzymatic determination of glycolytic intermediates. *Analytical Biochemistry* **7**, 472–484.
- NG, W. S., SMITH, J. E. & ANDERSON, J. G. (1972). Changes in carbon catabolic pathways during synchronous development of conidiophores of *Aspergillus niger*. *Journal of General Microbiology* **71**, 495–504.
- RUIZ-AMIL, M., DE TORRONTGUI, G., PALACIÁN, E., CATALINA, L. & LOSADA, M. (1965). Properties and function of yeast pyruvate carboxylase. *Journal of Biological Chemistry*, **240**, 3485–3492.
- TURIAN, G. & BIANCHI, D. E. (1972). Conidiation in *Neurospora*. *Botanical Review* **38**, 119–154.
- UTTER, M. F. & KEECH, D. B. (1960). Formation of oxaloacetate from pyruvate and CO₂. *Journal of Biological Chemistry* **235**, PC 17.
- WANG, C. H. (1972). Radiorespirometric methods. In *Methods in Microbiology*, pp. 185–230. Edited by J. R. Norris and D. W. Ribbons. New York and London: Academic Press.
- WONG, A. L. & WILLETS, H. J. (1974). Polyacrylamide-gel electrophoresis of enzymes during morphogenesis of sclerotia of *Sclerotinia sclerotiorum*. *Journal of General Microbiology* **81**, 101–109.
- WRIGHT, B. E. (1966). Multiple causes and controls in differentiation. *Science* **153**, 830–837.