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Biochemical investigation of the slow-growing non-perithecial (*sgp*) mutants of *Aspergillus nidulans*

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SUMMARY

Using polarography the uptake of oxygen by intact and homogenized mycelium of a wild-type strain and strains of slow-growing non-perithecial (*sgp*) mutants was compared. It was found that whilst the oxygen uptake of intact, wild-type mycelium increased on the addition of glucose or succinate as substrate, uptake of oxygen by mutant mycelium increased only when glucose was used as substrate and was unaffected by succinate. When, however, homogenates of mutant mycelium were used oxidation of the succinate occurred. It was concluded that the inability of the mutants to utilize succinate was due to their impaired ability to take up the compound across the hyphal wall and this was confirmed using radioactively labelled substrates. It is tentatively suggested that the abnormal growth of the *sgp* mutants on glucose medium and their impaired permeability to tricarboxylic acid cycle intermediates may be due to reduced availability of high energy compounds caused by a lesion in their oxidative phosphorylation system.

1. INTRODUCTION

The isolation of five groups of *sgp* mutants of *Aspergillus nidulans* which grow slowly on glucose as sole carbon source and which fail to grow on media containing intermediates of the tricarboxylic acid (TCA) cycle has already been described (Houghton, 1970). The present paper describes investigations into the underlying causes of the characteristic behaviour of the mutants.

The mutants were originally isolated by their inability to grow on non-fermentable carbon sources (Nagai, Yanagishima & Nagai, 1961) and so to establish whether lesions were present in the respiratory pathway, their ability to utilize atmospheric oxygen was investigated. Radioactive substrates were used to determine whether the mutants were blocked in any of the reactions of the TCA cycle and whether they were able to take up TCA cycle intermediates across the hyphal wall.

2. MATERIALS AND METHODS

(i) *Strains.* The strains used were the wild-type 13 and the *sgp* mutants as previously described (Houghton, 1970).

(ii) *Media.* The liquid medium was a slightly modified form of the minimal

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medium (MM) of Pontecorvo *et al.* (1953). Homogeneous mycelial suspensions were produced by a method similar to that described by Roberts (1963). The liquid medium was inoculated with conidial suspension and the flasks were incubated on a gyratory shaker (New Brunswick Scientific Co., Brunswick, N.J.) at about 200 rotations/min. Cultures treated in this way produced a suspension of small mycelial pellets which could easily be pipetted and harvested on a sterile Buchner funnel covered by filter paper. The pellets could then be washed with sterile distilled water and suspended in 0·04 M potassium phosphate buffer, pH 6·5 (Roberts, 1963). Mycelial homogenates and mitochondrial preparations were prepared by a similar technique to that described by Watson & Smith (1967).

(iii) *Polarography.* A Y.S.I. 53 Biological Oxygen Monitor was used for the polarographic measurement of response in oxygen uptake by intact and homogenized mycelium to the addition of glucose and succinate. Suspensions of intact mycelial pellets in phosphate buffer were prepared and their dry weights estimated. Homogenates were also prepared and their protein contents estimated by the biuret reaction after solubilization with sodium deoxycholate (Jacobs *et al.* 1956). Aliquots (3 ml) of these suspensions were used in the reaction vessel, substrate solutions (0·042 μmoles glucose, sodium succinate or 20 μl distilled water) were added by way of the access slot.

(iv) *Utilization of radioactive substrates.* (*a*) *Mycelial homogenates.* Mycelial homogenates were prepared and their protein contents estimated and adjusted using 0·5 M mannitol–4 mM EDTA buffer (pH 7·0) to give final protein contents of 4·0 mg./ml. Samples (0·15 ml) of each homogenate were added to centrifuge tubes containing 0·49 ml. incubation mixture. This consisted of potassium phosphate buffer (pH 7·4) + additives (0·37 ml); distilled water (0·02 ml); radioactive substrate (10 μC in 1 ml). The phosphate buffer was prepared by the method of Gomari (1955) and contained $MgSO_4.7H_2O$ (2mM); KCl (10 mM); cytochrome *c* (0·03 mM); ATP (1 mM). 0·1 ml samples were removed from the tube immediately on addition of the homogenate and at regular intervals thereafter and added to 0·4 ml. absolute ethanol and quickly boiled to terminate metabolic reactions. The labelled compounds present in the incubation mixture samples were separated by two-dimensional paper chromatography and located and identified by autoradiography (Moses, 1960). The amount of radioactivity in each spot on the chromatograms was measured with a Geiger Muller Tube (supplied by 20th Century Electronics Ltd.) connected to an I.D.L. Scaler which gave direct readings in counts per minutes.

(*b*) *Intact mycelium.* Suspensions of small mycelial pellets in phosphate buffer were prepared and their weights determined. Aliquots (0·15 ml) were added to centrifuge tubes containing incubation mixture. At the end of the incubation period the tubes were centrifuged at 800 *g* for 10 min. The supernatant was discarded and the pellets washed by resuspension and centrifugation in two changes of distilled water. The pellets were finally resuspended in 0·5 ml absolute ethanol and quickly boiled. The suspensions were then homogenized using a Soniprobe (Dawe Instruments Ltd., London). Samples of the sonicated suspension were

chromatographed and the labelled compounds located and their radioactivity measured as previously described.

The radioactive substrates used in this study were supplied by the Radiochemical Centre, Amersham, Bucks. These were: (U-$^{14}C_6$) D-glucose; (1,4-$^{14}C_2$) succinic acid; (1,4-$^{14}C_2$) fumaric acid.

All incubations were carried out at 37 °C.

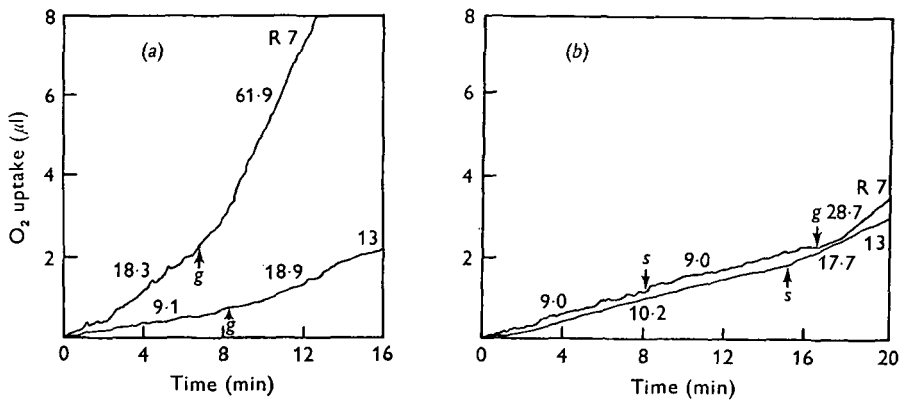

Fig. 1. Polarographic traces obtained using intact mycelium of strains R 7 and 13. (*a*) Oxygen uptake with time. Glucose (*g*) added at times indicated. (*b*) Oxygen uptake with time. Succinate (*s*) and glucose (*g*) added at times indicated. The values marked on the traces are rates of oxygen uptake in μl/h/mg dry wt. mycelium.

3. RESULTS

(i) *Polarography of intact mycelium.* The addition of glucose to the reaction vessel caused a marked increase in the oxygen uptake of both wild-type and mutant mycelium. The polarography tracings of strains 13 and R 7, a mutant of group *sgp-3*, are given in Fig. 1*a*. Other mutant strains gave qualitatively the same results, although the quantitative measurements of uptake often varied, even the same strain was found to consume different volumes of oxygen in different experiments. Nevertheless, although the figures may not be taken as representing absolute values of oxygen uptake, they serve to illustrate the magnitude of the change in the rate of uptake following substrate addition. When water was used instead of substrate no increase in oxygen uptake occurred.

When succinate was used as substrate only the wild-type strain showed an increase in oxygen uptake, no effect on the oxygen uptake of mutant mycelium was observed. Subsequent addition of glucose to the mutants produced an increase in uptake, indicating that the absence of response was due to the use of succinate as substrate rather than to deficiency in respiratory activity (Fig. 1*b*).

(ii) *Polarography of mycelial homogenates.* The addition of succinate caused an increase in oxygen uptake by both wild-type and mutant strains (Fig. 2). Similarly, the addition of pyruvate and fumarate caused an increase, although this was less marked.

(iii) *Polarography of mitochondrial suspensions.* With suspensions of mitochondria from wild-type and mutant strains the addition of succinate was followed by a sharp increase in oxygen uptake (Fig. 3).

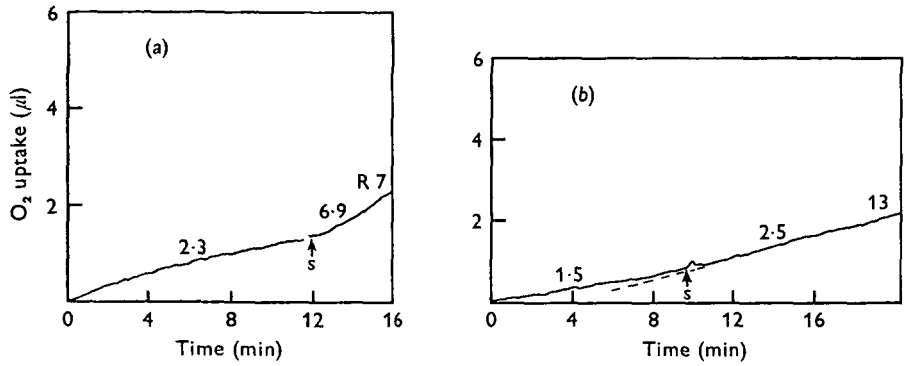

Fig. 2. Polarographic traces obtained using mycelial homogenates of (*a*) strain R 7, (*b*) strain 13. Succinate (*s*) added at the times indicated. The values marked on the traces are rates of oxygen uptake in μl/h/mg dry wt. mycelium.

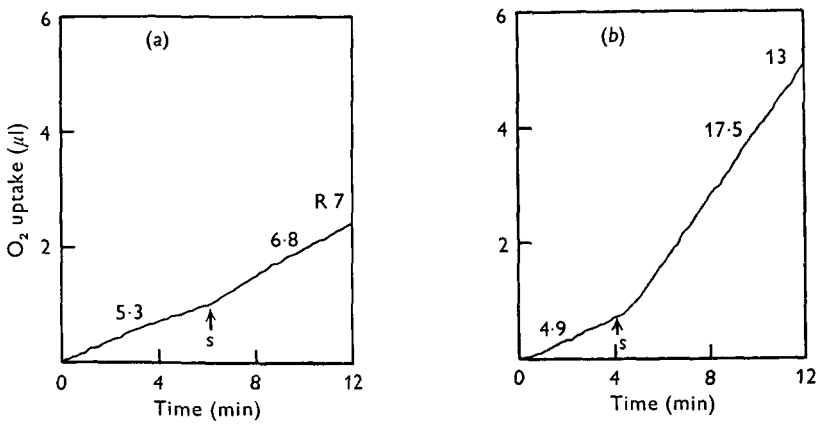

Fig. 3. Polarographic traces obtained using mitochondrial suspensions of (*a*) strain R 7, (*b*) strain 13. Succinate (*s*) added at the times indicated. The values marked on the traces are rates of oxygen uptake in μl/h/mg dry wt. mycelium.

(iv) *Utilization of radioactive substrates.* (*a*) *Mycelial homogenates.* Mycelial homogenates of wild-type and mutant strains were incubated in the presence of glucose. The measurements of radioactivity distributed into soluble intermediates after 1 h for wild-type strain 13 and *sgp-4* mutant strain R 10 are given in Table 1, other mutant strains gave the same results. The distribution was found not to alter significantly in subsequent samples. The incorporation of radioactivity into intermediates such as oligosaccharides and sugar phosphates indicated that wild-type and mutant strains were capable of metabolizing glucose by either the glycolytic, pentose phosphate or similar pathway. The presence of labelled lactate implied the

formation of pyruvate which was converted to lactate by a dehydrogenase. The presence of succinate in the R10 sample indicated that the enzymic reactions necessary for the conversion of pyruvate to TCA cycle intermediates were operative.

Table 1. *Distribution of radioactivity from* (U-$^{14}C_6$) *glucose in soluble intermediates of mycelial homogenates of wild-type and mutant strains after* 60 *min incubation*

(Results are expressed as counts/min. The radioactivity of the initial glucose was determined from a sample removed prior to incubation.)

Radioactive intermediate	13	R10	Radioactive intermediate	13	R10
Initial glucose	51594	59040	Sugar phosphates	6768	2406
Residual glucose	43062	55218	Lactate	168	42
Fructose	210	132	Succinate	0	150
Oligosaccharides	168	0			

Table 2. *Distribution of radioactivity from* (1,4-$^{14}C_2$) *succinate in soluble intermediates of mycelial homogenates of wild-type and mutant strains after* 60 *min incubation*

(Results expressed in counts/min.)

Radioactive intermediate	13	R10	Radioactive intermediate	13	R10
Initial succinate	60918	61956	Aspartate	138	54
Residual succinate	36894	36504	Asparagine	366	72
Fumarate	2490	1920	Glutamate	504	18
Malate	9468	6270	Lactate	2262	7758
Citrate	180	156			

The distribution of radioactivity from labelled succinate amongst the soluble intermediates of mycelial homogenates of strains 13 and R10 after 1 h's incubation is given in Table 2. Radiocarbon was incorporated into intermediates associated with the TCA cycle and into amino acids in both strains. This experiment clearly demonstrated that the *sgp* mutants had the enzymic activity necessary for the metabolism of TCA cycle intermediates. The inability of the mutants to grow on TCA cycle acids was not the result of a blocked cycle. This was confirmed using mitochondrial preparations, radiocarbon was incorporated into the same range of compounds as when homogenates were used.

(*b*) *Intact mycelium.* Mycelial pellets of wild-type and mutant strains were incubated in the presence of glucose. It was found that because of the small quantities of labelled substrate available and the low permeability of the hyphal walls, an incubation of 24 h was necessary for measurable amounts of substrate to be taken up and metabolized (Table 3). There was no marked difference in the ability of wild-type and mutant strains to take up and metabolize glucose. The radiocarbon was incorporated into intermediates of the TCA cycle and into amino acids.

The results given in Table 4 show that there is a marked difference in the ability of intact mycelium of wild-type and mutant strains to take up succinate.

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The wild-type mycelium was found to contain about ten times as much radiocarbon per mg mycelium as the mutants. The small amount of labelled succinate taken up by the mutants, however, was metabolized. Even when the differences in growth rates of the strains are taken into account, the difference in succinate uptake is still significant.

When the uptake of fumarate was compared, the wild-type was found to take up a much greater amount than the mutants.

Table 3. *Distribution of radioactivity from* (U-$^{14}C_6$) *glucose in soluble intermediates of intact mycelial pellets of wild-type and mutant strains after* 24 *h incubation*

(Results expressed in counts/min/mg mycelium. Initial glucose = 361774.)

Radioactive intermediate	13	R10	Radioactive intermediate	13	R10
Glucose	1425	1620	Aspartate	11	11
Fructose	776	840	Asparagine	17	5
Sugar phosphate	96	125	Glutamate	169	259
Succinate	136	260	Glutamine	10	35
Fumarate	11	15	Alanine	31	49
Malate	24	38			
Citrate	3	18	Total	2709	3275

Table 4. *Distribution of radioactivity from* (1,4-$^{14}C_2$) *succinate in soluble intermediates of intact mycelial pellets of wild-type and mutant strains after* 24 *h incubation*

(Results expressed in counts/min/mg mycelium. Initial succinate = 235902.)

Radioactive intermediate	13	R10	Radioactive intermediate	13	R10
Succinate	879	72	Asparagine	21	0
Fumarate	41	0	Glutamate	34	12
Malate	75	16	Glutamine	21	0
Citrate	6	4	Alanine	15	0
Aspartate	0	5	Total	1092	109

4. DISCUSSION

The results indicate that the *sgp* mutants, although having the enzymic capacity to oxidize TCA cycle acids, are severely impaired in their ability to take them up from growth media. Their growth rate and morphology on glucose is abnormal (Houghton, 1970), although the ability to take up glucose and oxidize it by way of the TCA cycle does not appear to be affected. The mutants also differ from wild-type in that they do not produce perithecia, their conidial heads are sparser, smaller and paler and the conidia show reduced viability and are unusually sensitive to the lethal effects of ultraviolet irradiation (Houghton, 1970). Although the impaired uptake of TCA cycle acids would suggest a permeability defect was responsible for the abnormal behaviour of the mutants, this would provide no explanation for the slow and abnormal growth on glucose which suggests a lesion in some central area of metabolism.

A possible lesion that might explain both the permeability defect and the slow growth on glucose is that the energy supply of the mutants is, in some way, defective. In the absence of a utilizable energy supply the active uptake of TCA cycle acids would cease and the mutants would be unable to grow on these compounds as carbon sources. The small amounts of labelled succinate taken up by the mutants could be explained by simple diffusion or by very slow active transport. Since the uptake of sugars such as glucose is thought to be by passive facilitated diffusion (Cirillo, 1961), the *sgp* mutants would be able to take up normal amounts of glucose. The aerobic metabolism of the glucose could then proceed by way of the TCA cycle. However, if the energy produced by these reactions was not efficiently conserved in the formation of ATP, or if the mutants were unable to store or utilize ATP, the growth of the mutants would be slow. Most biosynthetic reactions require an energy source and the reduced amounts of melanin-type and conidial head pigment produced by the mutants could be explained by the reduced availability of utilizable energy. This could also explain the absence of perithecial production since this is an energy-consuming process, and might also be responsible for the reduced number and lower viability of mutant conidia. The reduced shoulders observed in the ultraviolet survival curves of the *sgp* mutants (Houghton, 1970) may be due to the lower activity of repair mechanisms (Lennox & Tuveson, 1967) caused by reduced energy availability.

In the absence of direct experimental evidence it is difficult to postulate whether it could be ATP production, storage or utilization that could be defective. However, the endogenous rate of oxygen uptake by intact and homogenized mutant mycelium and the exogenous rate of intact mycelium in the presence of glucose was generally higher than that of wild-type. This might suggest that it is ATP synthesis, as a result of oxidative phosphorylation that is defective. A lesion in oxidative phosphorylation would effectively uncouple ATP synthesis from electron transport and, in the absence of rate limitation by ADP or inorganic phosphate availability, lead to accelerate oxygen uptake.

Mutants have been isolated from *Saccharomyces cerevisiae* which are thought to be oxidative phosphorylation-deficient (Kovac, Lachowicz & Slonimski, 1967; Kovac & Hrusovska, 1968) and these appear to show some characteristics in common with the *sgp* mutants. It is obvious, however, that some research is necessary before the exact nature of the *sgp* lesion is fully understood.

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