Oxidative Phosphorylation and Respiratory Control in Mitochondria from *Aspergillus niger*

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1. Tightly coupled mitochondria were isolated from *Aspergillus niger* by using an all-glass homogenizer followed by differential centrifugation. 2. The mitochondria oxidized the common intermediates of the tricarboxylic acid cycle, NADH₂ and the ascorbate-tetramethyl-*p*-phenylenediamine system. 3. High P/O ratios and control of respiration by ADP were obtained with all substrates tested. The average P/O ratios observed were: 1.5–1.8 with succinate as substrate [respiratory control ratio (RC) 2–4]; 0.8–1.0 with ascorbate-tetramethyl-*p*-phenylenediamine (RC 1.2–1.5); 1.4–1.8 with NADH₂ (RC 2–3); 2.4–2.8 with α -oxoglutarate (RC 3–5). 4. Bovine serum albumin (0.05–0.2%) was essential for tightly coupled respiration to be observed. 5. Coupled oxidation of exogenous NADH₂ was relatively insensitive to rotenone and Amytal. 6. The mitochondria responded to specific inhibitors and uncoupling agents in a manner similar to that of mammalian mitochondria. 7. It was concluded that the isolated mitochondria from *A. niger* show respiratory properties similar to those reported for intact yeast and mammalian mitochondria.

Oxidative phosphorylation by isolated mitochondria from fungi has not been extensively studied, and information about the respiratory activities of fungal mitochondria has almost exclusively been obtained from the study of particles from yeasts.

Utter, Keech & Nossal (1958) reported the preparation of yeast mitochondria that had low P/O values with succinate as substrate. The extraction techniques adopted by Vittols & Linnane (1961), Gregollin & Scaella (1964) and Zvyagilskaya & Kotelnikova (1964) resulted in mitochondrial preparations that were capable of phosphorylation coupled to oxidation of substrates other than succinate. All these methods employed a mechanical procedure for the disruption of the yeast cells, and control of respiration by ADP, where reported, was poor. The technique of digestion of the fungal cell wall with snail (*Helix pomatia*) digestive juice (Eddy & Williamson, 1957; Heyman-Blanchet, Zajdela & Chaix, 1959) was applied by Ohnishi & Hagihara (1964) and by Duell, Inoue & Utter (1964) to the isolation of intact yeast mitochondria. The latter workers, from a comparison of the morphological and respiratory properties of the mitochondria, concluded that the enzymic procedure was superior to the mechanical one for the preparation of intact mitochondria.

The report by Ohnishi, Kawaguchi & Hagihara (1966a) on the preparation and properties of mitochondria from the yeast *Saccharomyces carlsberg-*

ensis indicated a close similarity between the yeast and mammalian electron-transport systems. Further studies on yeast mitochondria, and especially the concept of site I phosphorylation, have been developed by Ohnishi, Sottocasa & Ernster (1966b). The latter authors considered site I phosphorylation to be absent from *Saccharomyces* yeasts, but Stekhoven (1966) has presented evidence for the existence of at least three phosphorylation sites in the respiratory chain of mitochondrial preparations from *S. carlsbergensis*.

Apart from the yeasts, the mitochondrial fraction isolated from *Aspergillus oryzae* by Iwasa (1960) required the addition of a nucleotide fraction for maximum phosphorylation efficiency (P/O 1.6) with succinate. Other fungi from which phosphorylating mitochondria have been isolated include *Allomyces macrogynus* (Bonner & Machlis, 1957) and *Neurospora crassa* (Hall & Greenwalt, 1964; Weiss, 1965).

The object of the present investigation was the preparation of tightly coupled and hence relatively intact mitochondria from the filamentous fungus *Aspergillus niger*, and a comparison of the electron-transport chain with that of yeast and mammalian mitochondria.

MATERIALS AND METHODS

ADP (sodium salt), oligomycin and antimycin A were obtained from Sigma Chemical Co. (London) Ltd. (London,

S.W. 6). Stock solutions of oligomycin and antimycin A were prepared in ethanol. Rotenone was a gift from W. J. Craven and Co. Ltd. (Evesham, Worcs.). Analyses of the two samples of rotenone used in the experiments were given as 96.3% and 93.3%. The samples were recrystallized from ethanol. For mitochondrial respiratory studies, additions of oligomycin, antimycin A and rotenone were made in small amounts (5–20 μ l.) of ethanolic solution: these amounts of ethanol alone did not appreciably affect respiration. Sodium Amytal was a gift from Eli Lilly and Co. Ltd. (Basingstoke, Hants.). Hexokinase (grade 2) was obtained from Seravac Laboratories Ltd. (Maidenhead, Berks.). All other reagents were purchased from British Drug Houses Ltd. (Poole, Dorset).

Growth of organism. *Aspergillus niger* Van Tieghem, strain I.M.I. 59374, was obtained from the Commonwealth Mycological Institute, Kew, Surrey. Stock cultures of this fungus were maintained on potato-glucose agar slopes in screw-cap bottles at 27°. Subcultures were made periodically by transferring large numbers of conidia to agar slopes. Liquid cultures were inoculated with 2% of their volume of a heavy conidial suspension in sterile water (about 10⁶ conidia/ml.), obtained from 5–7-day cultures.

For experimental work, the culture medium used was: glucose, 25g.; NH_4NO_3 , 3g.; KH_2PO_4 , 2g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 12mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 16mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2mg.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5mg.; $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$, 20mg.; distilled water to 1l. The pH of the medium was 4.5. The bottles were plugged with non-absorbent cotton wool and sterilized with steam (15 min. at 15 lb./in.²). Mycelium for mitochondrial studies were grown in static liquid culture (650 ml. of culture medium in 2l. Roux bottles) for 48 hr. at 27°. The yield of mycelium was usually 15–25 g./bottle. Young spores produced a good yield of mycelium within 48 hr., but with older spore inoculations 76 hr. was required for comparable growth. No marked difference in mitochondrial activity was noted for 48 hr. or 72 hr. mycelium.

Isolation of mitochondria. This was carried out at 0–4°. An all-glass homogenizer was used, the pestle shaft of which was connected by rubber tubing to the drive motor of a Waring Blender.

The mycelium was washed with cold tap water and then distilled water. The mycelium was then homogenized by using an extraction medium of 0.5 M-mannitol–4 mM-EDTA, pH 7.0, and a ratio of 1–1.5 g. of mycelium to 10 ml. of extraction medium. The homogenate was filtered through a double layer of moist muslin. The pH of the filtrate was usually 6.5–6.8 and was not further adjusted. The filtrate was centrifuged at 800g for 5 min. The residue was resuspended in extraction medium and recentrifuged at 800g for 5 min. The supernatants from the first and second centrifugations were combined and centrifuged at 12000g for 10 min. The mitochondrial pellet was carefully resuspended in 0.5 M-mannitol–0.5 mM-EDTA, pH 6.5, the suspension centrifuged at 800g for 5 min. and the resulting supernatant centrifuged at 12000g for 8 min. The final mitochondrial suspension was in 0.5 M-mannitol.

Usually 7–8 g. of mycelium was used for each experiment; this yielded sufficient mitochondria for five to seven determinations with the oxygen electrode.

Measurement of oxygen uptake. A Gilson Oxygraph (model KM; Gilson Medical Electronics, Middleton, Wis., U.S.A.) with a rapidly oscillating platinum electrode and a

polarizing voltage of 0.65 v was used for the polarographic measurement of O_2 uptake. The capacity of the incubation vessel was 2.2 ml. An electrode treated with collodion solution (ether-methanol-collodion, 4:0.2:1, by vol.) was used to avoid daily cleaning difficulties and underestimation of O_2 uptake (Hagihara, 1961). After reagents were added by micro-pipettes via a side arm, the reaction medium was rapidly stirred with a glass rod. The servo recorder pen was left on throughout the experiment except during the addition of the mitochondrial suspension.

The standard reaction medium in a final volume of 1.9 ml. contained: mannitol (0.5 M), EDTA (0.5 mM), KCl (5.26 mM), potassium phosphate (5.26 mM) and bovine serum albumin (4 mg.); the final pH was 6.5. Experiments were carried out at 25°. Substrates were added as given in the Results section. Phosphorylation was measured by the method of Chance & Williams (1955). Solutions of ADP were standardized assuming a molar extinction coefficient of 15400 at 259 m μ (pH 7) (control assays of Sigma Chemical Co. Ltd.).

Manometric assay of oxidative phosphorylation. The O_2 uptake was determined by standard Warburg techniques and phosphorylation measured by the disappearance of inorganic phosphate from the reaction medium. Sucrose was used in the suspension and reaction media, as mannitol interfered with phosphate determination by the method of Lowry & Lopez (1946).

The main compartment of each Warburg flask contained (final concentrations): sucrose (166 mM), KCl (4.2 mM), potassium phosphate (8.3 mM), EDTA (0.4 mM), bovine serum albumin (5 mg.) and mitochondrial suspension (0.5 ml.; 4–7 mg. of protein). The side arm held ADP (2.1 mM), glucose (20.8 mM), hexokinase (2.5 mg.) and substrate (8.3 mM-succinate, 8.3 mM- α -oxoglutarate, 2.1 mM-NADH₂ or 8.3 mM-ascorbate plus 0.08 mM-tetramethyl-p-phenylenediamine); 0.1 ml. of 40% (w/v) KOH was placed in the centre well. The final volume was 2.4 ml.; the pH was 6.5 and the temperature 30°. The flasks were equilibrated for 8 min., the contents of the side arm were tipped and O_2 uptake was determined for 20–25 min. The reaction was terminated by pipetting 1 ml. of the reaction mixture into 1 ml. of ice-cold 10% (w/v) trichloroacetic acid and adjusting the volume to 5 ml. with 0.1 M-sodium acetate. The precipitated protein was removed by centrifuging and the inorganic phosphate determined as above.

Mitochondrial protein was estimated by the biuret reaction after solubilization with sodium deoxycholate (Jacobs, Jacob, Sanadi & Bradley, 1956). Crystalline bovine serum was used as a standard.

RESULTS

The control of respiration by ADP is a very sensitive criterion of the metabolic state of extracted mitochondria. The respiratory control ratio, as defined by Chance & Williams (1955), is the ratio of respiration rate with ADP (state 3) to that after all the added ADP has been phosphorylated (state 4).

Mitochondria extracted from *A. niger* exhibited high respiratory control ratios. Typical polarographic traces are shown in Figs. 1 and 2 with succinate, NADH₂ and α -oxoglutarate as substrates. In Fig. 1, endogenous respiration (state 1)

Fig. 1. Respiratory control with succinate. The standard reaction medium described in the Materials and Methods section was used. The following additions were made: A, mitochondrial suspension (2.3 mg. of protein); B, succinate (5.26 mM); C, ADP (0.134 mM); C', ADP (0.134 mM). In this and subsequent Figures the values marked on the traces are rates of O_2 uptake in $m\mu g.$ atoms of O/min., and RC represents the respiratory control ratio.

Fig. 2. Respiratory control with α -oxoglutarate and $NADH_2$. The standard reaction medium described in the Materials and Methods section was used. The following additions were made: (a) mitochondrial suspension (approx. 1.8 mg. of protein); A, α -oxoglutarate (5.26 mM); B, ADP (0.139 mM); B', ADP (0.139 mM); (b) mitochondrial suspension (approx. 1.5 mg. of protein); A, $NADH_2$ (2.63 mM); B, ADP (0.087 mM); B', ADP (0.087 mM).

Table 1. Oxidative and phosphorylative capacities of *A. niger* mitochondria with different substrates

Final concentrations of substrates were 5.26 mM, except for $NADH_2$ (2.63 mM) and malate (1.05 mM); tetramethyl-*p*-phenylenediamine was added to a final concentration of 25 μM ; the standard reaction medium described in the Materials and Methods section was used. Respiratory control and ADP/O ratios were derived by the method of Chance & Williams (1955).

Substrate	Respiratory rate (state 3) O/min./mg. of protein)	Respiratory control ratio	ADP/O
Succinate	0.09–0.12	2–4	1.5–1.8
$NADH_2$	0.07–0.10	2–3	1.4–1.8
α -Oxoglutarate	0.06–0.10	3–5	2.4–2.8
Ascorbate–tetramethyl- <i>p</i> -phenylenediamine	0.07–0.10	1.2–1.5	0.8–1.0
Citrate	0.05–0.08	1.5–2.0	1.7–2.3
Glutamate–fumarate	0.05–0.08	2–3	2.4–2.7
Glutamate	0.04–0.06	2–3	2.2–2.6
Pyruvate–malate	0.04–0.06	2–2.5	2.0–2.4

method of Chance & Williams (1955). Similar results were obtained when α -oxoglutarate and $NADH_2$ were substrates (Figs. 2a and 2b).

In several experiments, the respiratory control ratio was 5–6 with α -oxoglutarate as substrate, but with most preparations the ratio was 3–4. Succinate and $NADH_2$ oxidations had respiratory control ratios 2–4. Owing to the low ratio of the ascorbate–tetramethyl-*p*-phenylenediamine system (1.2–1.5) it was sometimes difficult to observe clearly a second state 4–state 3–state 4 cycle, but the first cycle was always obtained. The ratios were similar to those reported by Ohnishi *et al.* (1966a) for yeast mitochondria, but with NAD-linked substrates the ADP/O ratios were slightly higher (Table 1). The results were an indication that site I phosphorylation was present in the isolated mitochondria from *A. niger*.

Oxidative phosphorylation was also measured by the Warburg technique. The P/O ratios agreed fairly well with those determined by the polarographic method (Table 2). The use of sucrose in the suspension and reaction medium did not have a marked effect on respiratory control or ADP/O ratios as measured by the polarograph.

Effect of bovine serum albumin. Bovine serum albumin was required for tight coupling of phosphorylation to be observed in mitochondria from *A. niger*. Mitochondria could be prepared without albumin provided that it was incorporated in the reaction medium, and the presence of albumin (0.05–0.2%) throughout the preparation procedure did not improve the respiratory control ratio. In

was increased about threefold on the addition of succinate (state 4). An immediate acceleration in respiration was observed when ADP was added (state 3) and this high rate continued until all the ADP was depleted (state 4). This state 4–state 3–state 4 cycle, characteristic of tightly coupled mitochondrial systems, was repeated on a further addition of ADP. Respiratory control and ADP/O (P/O) ratios were calculated graphically by the

Table 2. *Manometric assay of oxidative phosphorylation*

Final concentrations of substrates were 8.3 mM, except for malate and NADH₂ (2.1 mM). Tetramethyl-*p*-phenylenediamine was added to a final concentration of 80 μ M; the reaction time was 25 min. and the temperature was 30°; 4–7 mg. of mitochondrial protein was used for the different experiments. P/O ratios were corrected for endogenous oxygen and phosphate uptakes. Other experimental conditions were as described in the Materials and Methods section.

Substrate	P _i uptake (μ moles/ 25 min.)	O ₂ uptake (μ g.atoms of O/25 min.)	P/O ratio
α -Oxoglutarate	10.56	4.06	2.59
Pyruvate-malate	6.00	2.45	2.44
Citrate	5.52	2.45	2.25
Succinate	9.12	5.61	1.62
NADH ₂	6.74	4.69	1.44
Ascorbate-tetramethyl- <i>p</i> -phenylenediamine	6.34	6.46	0.98
Ascorbate-tetramethyl- <i>p</i> -phenylenediamine + antimycin A (0.2 μ M)	6.60	6.82	0.97

Table 3. *Stability of mitochondria from A. niger*

Mitochondria were suspended in 0.5 M-mannitol or in 0.5 M-mannitol containing bovine serum albumin (0.2%) at 0° and samples were assayed polarographically at the times indicated. The standard reaction medium described in the Materials and Methods section was used; the substrate was succinate (5.26 mM) and ADP was added to a final concentration of 0.1 mM; 1.5 mg. of mitochondrial protein was used in each assay.

		Respiratory rate		Respira- tory control ratio	ADP/O ratio	
		Incuba- tion time (hr.)	(μg.atoms of O/min.)			
			State 4			State 3
No serum albumin	0	54	134	2.5	1.6	
	1	65	152	2.3	1.6	
	3	70	157	2.2	1.6	
	4	70	134	1.9	1.2	
	24	58	58	1.0	—	
With serum albumin	0	50	128	2.6	1.7	
	4	45	121	2.7	1.7	
	24	49	45	1.0	—	

Fig. 3. Effect of the addition of bovine serum albumin to the reaction medium. (a) No albumin in reaction medium; (b) 0.05% bovine serum albumin in reaction medium. The following additions were made to both (a) and (b): mitochondrial suspension (1.9 mg. of protein); A, succinate (5.26 mM); B, ADP (0.108 mM); B', ADP (0.054 mM).

some experiments 0.05% albumin in the reaction medium was sufficient to obtain good respiratory control, whereas in other preparations the amount had to be increased to 0.2%. This effect may be attributed to the presence of an increased amount of uncoupling compounds in these preparations. To avoid such difficulties 0.2% albumin was added to all reaction media. In Fig. 3, acceleration of res-

piration by ADP was not followed by a clear state 4–state 3–state 4 cycle. Under identical experimental conditions but with albumin added to the reaction medium, coupled phosphorylation and respiratory control were clearly obtained (Fig. 3b).

Stability. Mitochondrial suspensions were kept at 0°, and the respiratory properties were little changed after 3 hr. (Table 3). Stability could be maintained for a longer period (at least 4 hr.) if albumin was added to the suspension medium (Table 3). However, storage at 0° for 24 hr., even with albumin, resulted in loss of phosphorylation ability.

Effect of pH and Mg²⁺ ions. In the pH range 6–8, oxidation of succinate increased with increasing pH. In one series of experiments, the state 4 respiration at pH 8 was almost three times that at pH 6.0 (Table 4). This rapid state 4 respiration resulted in low respiratory control and ADP/O ratios at the higher pH. The optimum pH of 6.5 for maximum respiratory control ratios is lower than that normally employed for mammalian mitochondrial studies.

Mg²⁺ was omitted from the reaction medium as it was found to greatly stimulate state 4 respiration of succinate and produced low respiratory control and ADP/O ratios (Mg²⁺ concentration, 5 mM). In contrast, α -oxoglutarate oxidation was not stimulated by Mg²⁺ and in some experiments there was a distinct improvement in respiratory control. These effects of Mg²⁺ may be due to stimulation of adenosine-triphosphatase activity, but this has not been investigated.

Effect of uncoupling agents and inhibitors. Potassium cyanide (0.4 mM) and antimycin A (0.2 μ M)

produced almost 100% inhibition of respiration with succinate, α -oxoglutarate and NADH_2 as substrates. Antimycin A ($0.2\mu\text{M}$) had little effect on the ascorbate-tetramethyl-*p*-phenylenediamine system when measured polarographically or manometrically (Table 2).

The effects of Amytal and rotenone on the respiration of mitochondria from *A. niger* have been reported (Watson & Smith, 1967). Oxidation and phosphorylation with NADH_2 as substrate was relatively insensitive to concentrations of Amytal and rotenone that were sufficient to strongly inhibit the coupled oxidation of α -oxoglutarate (Table 5). Succinate oxidation was virtually unaffected by Amytal (2mM) and rotenone ($30\text{m}\mu\text{moles/mg. of protein}$). A higher concentration of rotenone was required to inhibit state 3 respiration with mitochondria from *A. niger* than with mammalian mitochondria. These results may be compared with those reported by Ohnishi *et al.* (1966*b*) for yeast mitochondria.

2,4-Dinitrophenol (0.13mM) abolished respiratory control with succinate (Fig. 4) and NADH_2 ,

but not with α -oxoglutarate (Fig. 5). Stimulation of state 4 respiration by dinitrophenol was about 40% with α -oxoglutarate as substrate and coupled oxidation was still evident, as shown on the addition of ADP. The ADP/O ratio was about 1 in this cycle, suggesting an efficient substrate-level phosphorylation not uncoupled by dinitrophenol (Hunter, 1951; Judah, 1951; Azzone & Ernster, 1961). The effect of oligomycin is shown in Fig. 6 with succinate and NADH_2 and in Fig. 7 with α -oxoglutarate. With all three substrates oligomycin abolished phosphorylation, and the further addition of dinitrophenol produced complete uncoupling in that oxygen uptake was accelerated to the state 3 respiration rate. Ohnishi *et al.* (1966*a*) reported that

Fig. 4. Effect of 2,4-dinitrophenol on respiration with succinate as substrate. The standard reaction medium described in the Materials and Methods section was used. The following additions were made: mitochondrial suspension (1.5mg. of protein); A, succinate (5.26mM); B, 2,4-dinitrophenol (0.13mM); C, ADP (0.131mM).

Table 4. Effect of pH on succinate oxidation

The final concentration of succinate was 5.26mM , and that of ADP was 0.1mM ; approx. $2.5\text{mg. of mitochondrial protein}$ was used; the standard reaction medium described in the Materials and Methods section was used, except that the concentration of bovine serum albumin was 0.05% .

pH	Respiratory rate ($\mu\text{g. atoms of O/min.}$)		Respiratory control ratio	ADP/O ratio
	State 3	State 4		
6.0	133	72	1.9	1.8
6.5	216	107	2.0	1.8
7.0	208	120	1.7	1.7
7.5	253	144	1.8	1.3
8.0	262	192	1.4	1.2

Table 5. Effect of Amytal and rotenone on α -oxoglutarate and NADH_2 oxidation

Respiration was measured polarographically. The standard reaction medium described in the Materials and Methods section was used, with α -oxoglutarate (5.26mM) or NADH_2 (2.63mM), plus ADP (0.126mM). ADP/O and respiratory control ratios were measured after the addition of inhibitor. With α -oxoglutarate as substrate, the further addition of ADP was inhibitory, but with NADH_2 as substrate respiration and phosphorylation were still clearly evident.

Inhibitor	Substrate	Respiratory rate (state 3) ($\text{m}\mu\text{g. atoms of O/min./mg. of protein}$)		Inhibition (%)	ADP/O ratio	Respiratory control ratio
		Before addition of inhibitor	After addition of inhibitor			
Amytal (3mM)	α -Oxoglutarate	72	16	78	—	—
	NADH_2	73	62	15	1.6	2.0
Rotenone ($30\text{m}\mu\text{moles/}$ mg. of protein)	α -Oxoglutarate	78	31	60	—	—
	NADH_2	81	65	20	1.7	2.6

Fig. 5. Effect of 2,4-dinitrophenol on respiration with α -oxoglutarate as substrate. The standard reaction medium described in the Materials and Methods section was used. The following additions were made: mitochondrial suspension (approx. 2mg. of protein); A, α -oxoglutarate (5.26mM); B, 2,4-dinitrophenol (0.13mM); C, ADP (0.087mM); C', ADP (0.087mM).

Fig. 7. Release of oligomycin inhibition by 2,4-dinitrophenol with α -oxoglutarate as substrate. The standard reaction medium described in the Materials and Methods section was used. The following additions were made: mitochondrial suspension (1.5mg. of protein); A, α -oxoglutarate (5.26mM); B, ADP (0.131mM); C, oligomycin (2.5 μ g. in 5 μ l. of ethanol); B', ADP (0.087mM); D, 2,4-dinitrophenol (0.13mM).

Fig. 6. Release of oligomycin inhibition by 2,4-dinitrophenol with succinate and NADH_2 as substrates. The standard reaction medium described in the Materials and Methods section was used. The following additions were made: (a) mitochondrial suspension (1.8mg. of protein); A, succinate (5.26mM); B, ADP (0.131mM); C, oligomycin (2.5 μ g. in 5 μ l. of ethanol); B', ADP (0.131mM); D, 2,4-dinitrophenol (0.13mM); (b) mitochondrial suspension (1.8mg. of protein); A, NADH_2 (2.63mM); other additions as in (a).

for mitochondria from the yeast *S. carlsbergensis* a much higher concentration of oligomycin (20 μ g./mg. of protein) was required to change state 3 to state 4 respiration, and this inhibition could be overcome by dinitrophenol. Such effects were not observed when α -oxoglutarate was the substrate. It therefore appears that α -oxoglutarate oxidation differs in detail in mitochondria from *S. carlsbergensis* and from *A. niger*.

DISCUSSION

Electron microscopy has revealed the presence of abundant mitochondria in many species of fungi (Hawker, 1965). However, with the exception of the yeasts, there has been little progress in the knowledge of oxidative phosphorylation in fungal mitochondria. A major difficulty has been the development of a procedure for disruption of the fungal cell wall that does not cause functional damage, such as loss of phosphorylating ability, to the mitochondria. Digestion of the cell wall by snail-gut juice was used by Duell *et al.* (1964) and Ohnishi *et al.* (1966a) for the preparation of intact yeast mitochondria. The present paper describes a mechanical method for breakage of the cell wall, by the use of which mitochondria have been isolated from *A. niger* that appeared to be reasonably intact as demonstrated by good control of respiration by ADP and high P/O ratios.

Observation of tightly coupled respiration required the addition of bovine serum albumin to the reaction medium. It has been established that bovine serum albumin restores the phosphorylating capacity of aged or damaged mammalian mitochondria. The mechanism of the action of bovine serum albumin in restoring oxidative phosphorylation is not fully understood (Pullman & Racker, 1956; Polis & Shmukler, 1957; Helinski & Cooper,

1960). Insect mitochondria also require bovine serum albumin for maximum phosphorylation (Sacktor, 1954; Lewis & Slater, 1954; Sacktor, O'Neill & Cochran, 1958), apparently in order to bind long-chain fatty acids released during the homogenization procedure (Lewis & Fowler, 1960; Wojtezak & Wojtezak, 1960). More recently, Weinbach & Garbus (1966a,b) have shown that the uncoupling effect of substituted phenols can be completely overcome by serum albumin.

Among the main metabolites of the fungi are phenolic compounds (Birkenshaw, 1965). Release of these compounds during the extraction could result, in the absence of serum albumin, in the uncoupling of mitochondria from *A. niger*, and the ability of serum albumin to restore phosphorylation and respiratory control may be due to binding of such substances.

The isolated mitochondria showed maximal phosphorylation and respiratory control at pH 6.5. The presence of Mg^{2+} in the reaction medium greatly stimulated respiration with succinate, but not with α -oxoglutarate. Similar effects of Mg^{2+} have been reported for brain mitochondria (Voss, Campello & Bacilla, 1961; Bacilla, Campello, Vianna & Voss, 1964). Ozawa, Seta & Handa (1966) concluded that NAD-linked substrates had maximum phosphorylation rates in the presence of high Mg^{2+} concentrations but that Mg^{2+} lowered the respiratory control and ADP/O ratios with all substrates tested.

The oxidation of $NADH_2$ by *A. niger* mitochondria showed good respiratory control, and was sensitive to antimycin A, oligomycin and dinitrophenol. Under normal conditions of assay, exogenous $NADH_2$ is not oxidized by intact liver mitochondria (Lehninger, 1951). However, mitochondria isolated from various sources, e.g. yeasts (Duell *et al.* 1964; Ohnishi *et al.* 1966a,b), higher plants (Wiskich & Bonner, 1963), algae (Lloyd, 1965) and pigeon heart (Blanchaer, Lundquist & Griffith, 1966), oxidize $NADH_2$, despite appearing intact as judged by electron microscopy, respiratory control and phosphorylation efficiency. The outer membrane of plant mitochondria differs in appearance from that of liver mitochondria (Parsons, Bonner & Verboon, 1965) and the particles may well have different permeability properties.

The sensitivity of the respiratory chain of *A. niger* to potassium cyanide and antimycin A and the P/O ratios 1.7 for succinate and 0.9 for ascorbate-tetramethyl-*p*-phenylenediamine show a close relationship to the results with mammalian and yeast mitochondria.

Coupled oxidation was abolished by Amytal and rotenone with NAD-linked substrates, but these inhibitors had little effect on exogenous $NADH_2$ oxidation (Watson & Smith, 1967). This phenomenon has also been observed for mitochondria

from *Torulopsis utilis* and *Endomyces magnusii* (Ohnishi *et al.* 1966b). At this stage it can only be suggested that coupled oxidation of exogenous $NADH_2$ proceeds via a non-phosphorylating pathway between $NADH_2$ and cytochrome *b*. Mitochondria from *Saccharomyces* yeasts are insensitive to rotenone both with exogenous $NADH_2$ and with NAD-linked substrates (Ohnishi *et al.* 1966b; Stekhoven, 1966). Ohnishi *et al.* (1966a) reported that *S. carlsbergensis* mitochondria were unaffected by 3mM-Amytal. However, with a higher concentration of Amytal (5mM), Stekhoven (1966) found that citrate oxidation was inhibited by 75% whereas exogenous $NADH_2$ oxidation was not inhibited.

Substrate-level phosphorylation was presumably present in mitochondrial preparations from *A. niger* as α -oxoglutarate oxidation was not completely uncoupled by dinitrophenol (Fig. 5), in contrast with $NADH_2$ and succinate oxidations. However, dinitrophenol completely relieved the inhibition of respiration by oligomycin with all three substrates. The concentration of oligomycin required for inhibition was similar to that for animal mitochondria, but was very much lower than that required for mitochondria from *S. carlsbergensis* (Ohnishi *et al.* 1966a). Even in the much-studied mammalian system there is still doubt whether substrate-level phosphorylation can be uncoupled by dinitrophenol (Charles, Tager & Slater, 1963; Scholte & Tager, 1965). Using a preparation of heart sarcosomes, Davis (1965) has shown that oligomycin can inhibit α -oxoglutarate oxidation and that, in the presence of 1mM- Mg^{2+} , dinitrophenol relieves this inhibition.

As a result of this work we conclude that there is a close similarity between *A. niger* mitochondria and those of yeasts and animals.

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