The Effect of Metabolic Inhibitors on the Development of Respiration in Anaerobically Grown Yeast

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(Received 7 October 1965)

1. Iodoacetate and fluoride did not prevent the development of respiration in aerobically grown yeast. 2. The effect of dinitrophenol suggested that phosphorylation developed simultaneously with respiration in anaerobically grown yeast, but the effect of oligomycin suggested that the phosphorylation and oxidation were not tightly coupled. 3. Inhibitors of electron transport showed that both the respiratory peak and the subsequent respiration were cyanide-sensitive, but the peak respiration was insensitive to antimycin. 4. Of the inhibitors of protein or RNA synthesis tested, only *p*-fluorophenylalanine inhibited the development of respiration. The results are not consistent with a new synthesis of mitochondria. 5. 2-Phenylethanol inhibited the development of respiration in anaerobically grown yeast and also yeast growth. Other inhibitors of DNA synthesis had no effect on the development of respiration. 6. The relevance of the results to mitochondrial morphogenesis is discussed.

Tustanoff & Bartley (1964*a*) described the time-course of the development of respiration and cytochrome oxidase in yeast grown anaerobically with glucose as substrate when the harvested cells were exposed to oxygen. The time-course under the conditions of these authors was influenced by: (a) the presence and concentration of a utilizable carbon source; (b) the availability of amino acids; (c) the physiological age of the culture; (d) the concentration of the glucose during the growth of the yeast. The need for an amino acid source had already been described by Slonimski (1953). All the available evidence suggests that the yeast grown anaerobically on glucose synthesizes new enzymes and respiratory pigments when exposed to aerobic conditions. Inhibitors that affect the utilization of the carbon source for the production of energy, or interfere with protein synthesis, might be expected to alter the time-course of development of respiration if added in the adaptation medium.

This paper described the effects of the following classes of inhibitor on the adaptation process: (1) inhibitors of glycolysis; (2) inhibitors of oxidation and electron transport; (3) inhibitors of protein synthesis; (4) inhibitors of RNA synthesis; (5) inhibitors of DNA synthesis and cell division.

METHODS

Maintenance and growth of yeast. Saccharomyces cerevisiae strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey)

was used in the present work. The organism was maintained aerobically on agar slopes containing inorganic salts, 2.25% (w/v) of Difco malt extract, 0.05% of Difco yeast extract and 0.5% of sucrose. The yeast was grown for 48 hr. at 30° and subcultured monthly.

Growth of anaerobic cells. The medium for the anaerobic bulk growth of the yeast contained (per l.): glucose, 20 g.; Difco yeast extract, 10 g.; Oxoid casein hydrolysate (acid), 5 g.; KH_2PO_4 , 9 g.; CaCl_2 , 0.3 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $(\text{NH}_4)_2\text{SO}_4$, 6 g.; 70–72% (w/w) sodium lactate, 5 ml.; wheat-germ oil, 0.15 ml.; Tween 80, 5 ml.; ergosterol dissolved in ethanol, 20 mg. in 5 ml. The inoculum of yeast was 0.93 mg. dry wt. of cells/g. of hexose in the medium. Growth was in a wide-based conical stoppered flask (Fernbach flask) with a gas inlet and outlet that was continuously flushed with a slow stream of O_2 -free N_2 and shaken at 30°. The harvested cells were washed three times with ice-cold water and finally suspended in water at a concentration of 100 mg. wet wt./ml.

Adaptation of cells to respiration. Usually this was done in Warburg vessels at 25°. The vessels contained KH_2PO_4 (66 mm) and usually glucose (33 mm). Casein hydrolysate, when present, was added at 20 mg./vessel. The aqueous suspension of yeast (0.2 ml.; 4 mg. dry wt.) was added to the side arm of the vessel. The final volume was 3.0 ml., and the centre well contained 0.2 ml. of 10*N*-NaOH and filter paper. The yeast was tipped from the side arm after 10 min. of equilibration at 25° and the subsequent gas changes were measured over several hours. Inhibitors were added to the main compartment of the Warburg vessel, which also contained substrate and other cofactors. Thus the inhibitor was present during the whole period of adaptation. The Q_{O_2} values given are calculated over a 15 min. period at the times indicated. Occasionally the yeast was aerated in bulk in a Fernbach flask and samples were removed periodically for

analysis. When CO_2 production was to be measured the vessels were gassed with N_2 and the centre well contained a stick of yellow phosphorus. Yeast growth was followed turbidimetrically.

Measurement of cytochrome c oxidase. The yeast was collected by centrifuging at 0° , washed with ice-cold water and then re-centrifuged. The washed pellet of yeast was passed through a chilled French pressure cell at a pressure of 23000 lb./in.². Cell walls and debris were removed by centrifuging at 0° for 5 min. at 1500g. The cloudy supernatant was used for the measurement of cytochrome c oxidase according to the method of Minnaert (1961).

Measurement of uptake and utilization of amino acids by yeast during adaptation. Cells were grown anaerobically on 5.4% (w/v) glucose for 12 hr. The harvested yeast cells were then adapted in the standard aerobic medium (Tustanoff & Bartley, 1964a,b) supplemented with acid-hydrolysed casein (7 mg./ml. of incubation medium) and $1.1 \mu\text{C}$ of ^{14}C -labelled algal-protein hydrolysate/ml. Alternate vessels also contained *p*-fluorophenylalanine (18 mM). The Warburg vessels were removed at 30 min. intervals and rapidly cooled

to 0° . The contents of the vessels were centrifuged at 0° and the clear supernatant was analysed for its radioactivity (diminution in radioactivity was taken as a measure of amino acid uptake by the yeast), and the KOH from the centre well was removed and the trapped CO_2 converted into BaCO_3 for measurement of its radioactivity (increase in radioactivity in the KOH was taken as a measure of amino acid oxidation by the yeast).

RESULTS

Effect of inhibitors of glycolysis on the respiratory adaptation and $Q_{\text{N}_2}^{\text{CO}_2}$ (glucose) of yeast cells. The yeast was grown anaerobically in glucose (1.34% or 5.4%, w/v) or in galactose (5.4%, w/v). The results of tests with iodoacetate, azide and fluoride on subsequent development of respiration and on the rate of fermentation after exposure to oxygen are given in Tables 1 and 2. There was no clear correlation between the effects of the inhibitors on the development of respiration and the effect on fermentation. This is illustrated, for example, by the effect of iodoacetate, which inhibited fermentation completely but still allowed the development of respiration, although the onset was delayed substantially. This may merely reflect the time taken to decrease the glucose concentration by pathways other than fermentation. Fluoride (1 mM) had little effect, but azide (1 mM) abolished both fermentation and the development of respiration. These experiments give no support to the idea that fermentation is necessary for the development of respiratory enzymes.

Effect of inhibitors of electron transport on the development of respiration during adaptation to aerobic conditions. As shown in Table 3, the respiration developed by yeast in adaptation to aerobic conditions is completely cyanide-sensitive. This was confirmed by adding cyanide at the peak of adaptive respiration; all respiration ceased. Both 4-hydroxy-2-nonylquinoline *N*-oxide and antimycin A had little or no effect on the development of the initial peak of respiration in cells grown on glucose or galactose, but subsequently antimycin

Table 1. *Effect of inhibitors of glycolysis on the development of respiration in anaerobically grown yeast*

The yeast was grown anaerobically for 12 hr. at 30° on the medium described in the Methods section with glucose (1.34%) as the carbon source. The harvested, washed yeast was incubated in Warburg vessels in the adaptation medium described in the Methods section with 20 mg. of hydrolysed casein in each vessel. The inhibitor, when added, was present in the main compartment. The yeast (0.2 ml., 4 mg. dry wt.) was tipped from the side arm after 10 min. equilibration at 25° and the subsequent gas changes were measured over several hours. The maximum Q_{O_2} for the control was 48 achieved after 2-18 hr.

Inhibitor	Maximum respiration achieved (% of control)	Time taken to develop max. respiration (% of time taken by control)
Iodoacetate (1 mM)	90	145
Sodium azide (1 mM)	4	—
Sodium fluoride (1 mM)	104	114

Table 2. *Effect of glycolytic inhibitors on anaerobic production of carbon dioxide from glucose*

The yeast was grown anaerobically for 12 hr. at 30° on 5.4% glucose or 21 hr. at 30° on 5.4% galactose. Cells were harvested and washed and added from the side arm into the adaptation medium, as described in Table 1, but the manometers were gassed with N_2 and the centre well contained a stick of yellow phosphorus.

Growth medium	Inhibitor	$Q_{\text{N}_2}^{\text{CO}_2}$ at 1 hr.	$Q_{\text{N}_2}^{\text{CO}_2}$ at 2 hr.
Glucose (5.4%)	None	380	380
Glucose (5.4%)	Sodium azide (0.7 mM)	0	0
Glucose (5.4%)	Sodium fluoride (5.0 mM)	340	220
Galactose (5.4%)	None	390	390
Galactose (5.4%)	Iodoacetate (1 mM)	0	0
Galactose (5.4%)	Sodium fluoride (1 mM)	320	320

Table 3. *Effect of inhibitors of respiration on the development of respiration in anaerobically grown yeast*

The yeast was grown on 1.34% glucose or 5.4% galactose as described in Tables 1 and 2. Adaptation was carried out as described in Table 1. The maximum Q_{O_2} for the control grown on 1.34% glucose was 48 after 2.18 hr. of adaptation; that for yeast grown on 5.4% galactose was 42 after 2.6 hr.

Growth medium	Inhibitor	Maximum respiration achieved (% of control)	Respiration 1 hr. after peak (% of control)	Time taken to develop max. respiration (% of time taken by control)
Glucose (1.34%)	Cyanide (1.0 mm)	0	0	—
Glucose (1.34%)	4-Hydroxy-2-nonylquinoline <i>N</i> -oxide (0.08 mm)	110	56	100
Galactose (5.4%)	4-Hydroxy-2-nonylquinoline <i>N</i> -oxide (0.08 mm)	100	100	100
Glucose (1.3%)	Antimycin A (0.11 μ g./ml.)	88	6	100
Galactose (5.4%)	Antimycin A (1.5 μ g./ml.)	93	6	100

Table 4. *Effect of inhibitors of protein and RNA synthesis on the development of respiration in anaerobically grown yeast*

Details of growth and adaptation were as given in Tables 1 and 2.

Growth medium	Inhibitor	Maximum respiration achieved (% of control)	Respiration 1 hr. after peak (% of control)	Time taken to develop max. respiration (% of time taken by control)
Glucose (1.34%)	Chloramphenicol (0.35 mm)	125	60	100
Galactose (5.4%)	Chloramphenicol (0.36 mm)	105	105	95
Glucose (2.7%)	<i>p</i> -Fluorophenylalanine (12 mm)	43	95	112
Glucose (2.7%)	<i>p</i> -Fluorophenylalanine (12 mm) added to yeast 30 min. before transfer to adaptation medium	28	75	120
Glucose (5.4%)	<i>p</i> -Fluorophenylalanine (18 mm)	33	75	120
Glucose (1.34%)	8-Azaguanine (4.4 mm)	115	100	115
Glucose (1.34%)	Bromodeoxyuridine (2.2 mm)	155	140	100

caused an almost complete loss of respiration. Presumably electrons from the substrate responsible for the respiratory peak are fed into the developing electron-transport chain at the level of cytochrome *c*, whereas subsequently direct reduction of cytochrome *c* becomes impossible and electrons enter before the point at which antimycin acts. The synthesis of cytochrome oxidase is inhibited by the presence of antimycin, being only 60% (*k*0.00071 compared with *k*0.00136) of that found in the uninhibited yeast at the time of the respiratory peak. This observation makes it unlikely that antimycin is unable to penetrate the yeast until after the respiratory peak and supports the idea that the pathway of electron transport at the time of the respiratory peak does not have to pass through an antimycin-inhibited step. Cells grown anaerobically on galactose retain respiratory activity and mitochondria; this respiration was completely abolished by the addition of antimycin, showing that anti-

mycin does penetrate into the cells before the development of the respiratory peak. The behaviour of cells grown on glucose in response to 4-hydroxy-2-nonylquinoline *N*-oxide was similar to the response to antimycin, but cells grown on galactose were unaffected by the inhibitor.

Effect of 2,4-dinitrophenol and oligomycin on the development of respiration in anaerobically grown yeast. Dinitrophenol lowered the respiratory peak by 40%, but subsequently the respiration was only 22% of the control. This suggests that the development of the electron-transport chain requires simultaneous development of oxidative phosphorylation. However, since oligomycin had no effect on the size of the respiratory peak, or on the subsequent respiration, phosphorylation cannot be tightly coupled to respiration.

Effect of inhibitors of protein and RNA synthesis on the development of respiration in anaerobically grown yeast. Table 4 shows the effect of several

Table 5. *Effect of inhibitors of DNA synthesis on the development of respiration in anaerobically grown yeast*

Details of growth and adaptation were as given in Tables 1 and 2.

Growth medium	Inhibitor	Maximum respiration achieved (% of control)	Respiration 1 hr. after peak (% of control)	Time taken to develop max. respiration (% of time taken by control)
Glucose (1.34%)	Colchicine (3.3 mM)	115	80	118
Glucose (2.7%)	Mitomycin C (400 µg./ml.)	108	108	100
Glucose (2.7%)	Mitomycin (400 µg./ml.) added to yeast 30 min. before adding other components of incubation medium	100	108	106
Glucose (2.7%)	2-Phenylethanol (2.5 mg./ml.)	11	23	—
Glucose (2.7%)	2-Phenylethanol (2.5 mg./ml.) added to yeast 30 min. before adding other components of incubation medium	11	23	—

inhibitors of protein synthesis on the development of respiration. Of these, only *p*-fluorophenylalanine had a marked effect and this was more pronounced with the 'respiratory peak' than with the subsequent respiration. The disappearance of radioactive amino acids added to the medium was linear during the incubation period but was 20% lower when the *p*-fluorophenylalanine was present. The production of radioactive carbon dioxide was also linear in both cases but was 37% lower when the inhibitor was present. Bromodeoxyuridine stimulated the respiration. Assuming that the inhibitors are effective at the concentrations used, the results give no support to a mechanism of development of respiration that depends on a major new synthesis of respiratory enzymes from amino acids. However, unpublished work of A. W. Linnane shows that much higher concentrations of chloramphenicol inhibit respiratory adaptation. The results are consistent, however, with a mechanism in which already elaborated precursors are spatially integrated, or in which prosthetic groups are added to apoenzymes. However, the process of developing respiration apparently requires some utilization of amino acids, since (a) amino acids stimulate the process, and (b) the inhibition of the development of respiration brought about by *p*-fluorophenylalanine is roughly paralleled by the inhibition of uptake and utilization of amino acids (see the Methods section).

Effect of inhibitors of DNA synthesis on the development of respiration in anaerobically grown yeast. Table 5 shows the effect of colchicine, mitomycin C and 2-phenylethanol on the development of respiration. Colchicine had no effect, nor did mitomycin C. Mitomycin C is known to inhibit DNA synthesis and this was tested by measuring the increase in DNA in the yeast under the adaptation conditions. Without mitomycin the DNA content of the medium increased by 75%. When the

Fig. 1. Effect of 2-phenylethanol on the growth of yeast. Yeast was grown aerobically on the synthetic medium described by Polakis & Bartley (1965). The glucose concentration initially was 2%. The change in E_{540} was used as an index of growth. $\square$, 0.5% of 2-phenylethanol added to the growth medium; Δ , 0.25% of 2-phenylethanol added to the growth medium; $\circ$, 0.125% of 2-phenylethanol added to the growth medium; $\blacktriangle$, growth without 2-phenylethanol.

yeast cells were preincubated with mitomycin C for 30 min. before being added to the adaptation medium, the synthesis of DNA was inhibited by 80% during the subsequent period of adaptation. When mitomycin C was added at the start of the incubation period, the DNA synthesis was inhibited by 50%. However, in the control and in both tests with mitomycin C, the formation of cytochrome oxidase was the same ($k0.0024$). Thus the development of respiration does not appear to be correlated with cell division. By contrast, 2-phenyl-

ethanol (2.5 mg./ml.) inhibited the development of respiration, but the mode of action of this substance is unknown. Although DNA synthesis is inhibited by 2-phenylethanol in Gram-negative bacteria (Berrah & Konetzka, 1962) there is no evidence that it has the same effect on yeast. However, as shown in Fig. 1, 2-phenylethanol is a potent inhibitor of yeast growth, acting presumably by inhibiting DNA synthesis.

DISCUSSION

Schatz (1965) has suggested that anaerobically grown yeast contains precursors of mitochondria that can be isolated as granules. These granules contain some dehydrogenases, but no cytochromes or cytochrome oxidase. Such findings are against the synthesis of mitochondria from low-molecular-weight compounds. The present findings support those of Schatz (1965), since substances that inhibit protein synthesis or RNA synthesis in other organisms did not abolish the development of respiration when anaerobic yeast was adapted to aerobic conditions. Similarly, the presence of inhibitors of glycolysis had little effect. The process of forming mitochondria is therefore one requiring little energy, since glycolysis is the sole route of energy production until respiration develops. Possibly the proteins of cytochrome oxidase and other respiratory enzymes exist in the form of apoenzymes, which require porphyrin, lipid or a metal to complete the mitochondrial structure. The participation of lipid has been suggested by Hebb, Montgomery & Slebodnik (1958), and Tustanoff & Bartley (1964a) suggested that repression by glucose was particularly effective for enzymes of a

lipoprotein nature. The repressive effect of glucose could be mediated through an enzyme responsible for the association of lipid with protein. None of the present observations are inconsistent with this idea. The inhibitory effect of 2-phenylethanol was surprising. The synthesis of cytochrome oxidase and the development of respiration does not require cell division. This has been demonstrated by Slonimski (1953); his finding is supported by the effects of mitomycin C described in the present paper, namely inhibition of DNA synthesis but not of the formation of cytochrome oxidase. Yeast mitochondria contain DNA as one of their components (Schatz, Haslbrunner & Tuppy, 1964), and the divergent results with mitomycin C and 2-phenylethanol may be explained if it is assumed that 2-phenylethanol inhibits the synthesis of both nuclear and mitochondrial DNA, whereas mitomycin C inhibits only the synthesis of nuclear DNA.

E.R.T. thanks the Life Insurance Medical Research Fund for the receipt of a Fellowship.

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