The Effect of Oxygen on the Growth and Metabolism of the Aerobic Thermophilic Actinomycete *Micromonospora vulgaris*

By D. M. WEBLEY

The Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen, Scotland

SUMMARY: In the presence of pure oxygen at one atmosphere pressure *Micromonospora vulgaris* is unable to form in liquid media a surface pellicle of growth-bearing aerial mycelium. The production of bottom growth is either unaffected or stimulated by oxygen. Vegetative mycelium which develops from spores in Warburg vessels has an oxygen uptake that is similar in the presence of air or oxygen. Aerial mycelium, harvested from growths in air, shows marked differences as regards oxygen uptake in the presence of air or oxygen; oxygen is inhibitory. Some evidence is presented to show that the effect of oxygen may be connected with the inactivation of essential —SH groups of thiol enzymes.

Full and detailed accounts of the isolation, cultivation, temperature/growth relationships and reproduction of the thermophilic actinomycete *Micromonospora vulgaris* were given by Erikson (1952, 1953*a, b*). She showed that this organism possesses the most complex reproductive pattern of any actinomycete yet studied, and considers that 'the device of producing a secondary aerial mycelium capable both of further growth and of producing spores, more than doubles the expected life of a mycelial organism at elevated temperatures'. Erikson & Webley (1953), in a study of the respiration of the different growth phases of this structurally complex organism, found that a high rate of oxygen uptake at 60° was associated with the presence of aerial mycelium harvested from 1- to 2-day cultures grown on liquid medium at 60°, while bottom growth harvested under similar conditions was inactive in this respect. It has now been possible to show that pure oxygen exerts an inhibitory effect on the production of aerial mycelium (in growth experiments) and metabolism of aerial mycelium (respiration experiments; Webley, 1953) of this aerobic organism. The production of vegetative growth is unaffected by these differences in the gas phase.

METHODS

Organism. The experiments presented here were performed with strain H of *Micromonospora vulgaris* (Erikson, 1953*a*).

Technique of growth. Material from 2-day-old growth on liquid CPS medium was used throughout (see Erikson & Webley, 1953).

Preparation of spore suspensions and aerial mycelium for the Warburg experiments. Spore suspensions were obtained by shaking the pellicle of growth finally obtained, after washing free of medium, in 5 ml. of M/75 phosphate buffer (pH 7.0) on a 'Microid' shaker for 5 min. This process resulted in the detachment of a high proportion of the spores which could be separated from

the aerial mycelium by filtration through sterile glass wool. The spores were washed in a centrifuge and finally suspended in 2–3 ml. buffer. The resulting suspension was uniform and could be pipetted into the Warburg vessels in a similar manner to bacterial suspensions. The material remaining on the glass wool, which consisted mainly of aerial mycelium, was used for experiments with aerial mycelium. In the latter case, instead of dry-weight determinations being carried out at the end of the experiment as in previous work (Erikson & Webley, 1953), it was found less tedious to use wet weight as a means of estimation of the amount of material in the Warburg vessels. For this purpose, the sterilized Warburg vessels were first dried and weighed. The aerial mycelium remaining on the glass wool, after the preparation of spore suspensions, was next transferred aseptically into the tared vessel (care being taken to avoid the inclusion of any visible pieces of glass wool). Finally, buffer, substrates, etc., were added and the vessels attached to the manometers. Full details of running the Warburg at 60° were given previously (Erikson & Webley, 1953). For the replacement of air by oxygen the technique described by Umbreit, Burris & Stauffer (1951) was used. The manometers were first taken out of the bath and the vessels allowed to cool to room temperature before evacuation.

For convenience in this paper the final values for oxygen uptake in Figs. 2–4 are expressed as μ l. oxygen uptake/100 mg. wet weight material and, in the case of Tables 1, 2 and 3 as μ l. oxygen uptake/100 mg. wet weight/30 min. interval (i.e. rate of oxygen uptake during each interval). In consequence of the inherent variability of an organism of such complexity of structure it was found that in spite of all precautions not all the vessels gave uniformity in the amounts of oxygen uptake under similar conditions. Various attempts were made to overcome these difficulties, including: (1) repetition of experiments; (2) tipping substances in from the side arm or adding gases only after it was seen that the rate of oxygen uptake in the experimental vessels was initially the same as the control; (3) proportional comparison of rate of oxygen uptake before and after tipping in substances or gassing of the material in the vessels.

These details were adhered to throughout the work reported here.

Growth experiments in various gas mixtures. For this purpose, either 250 ml. filter flasks or 20 ml. filter test-tubes were employed. In the case of the flasks, 20 ml. medium was usually used while 5 ml. was sufficient for the tubes. The flasks and tubes were sterilized with a cotton-wool plug in the side tubes and necks. They were then inoculated with one or two drops of spore suspension prepared from 2-day surface growth. The cotton-wool plugs were replaced by tight-fitting sterile rubber bungs, while over the side arms were placed short pieces of pressure tubing carrying screw clips (the cotton-wool plugs being left in the side arms). The various attachments for the tubes and flasks were sterilized separately. For filling with oxygen or oxygen + nitrogen mixtures the technique described by Umbreit *et al.* (1951) for Warburg manometers, was used. Each flask or tube was alternately evacuated and filled through the

side arms with the gas 3 times before the screw clip was finally closed and the flasks and tubes placed in the 60° incubator. The flasks were examined daily for surface and bottom growth.

RESULTS

Effect of pure oxygen at one atmosphere on the respiration of M. vulgaris

Vegetative mycelium developing from spores in Warburg vessels. Vegetative mycelium harvested from older cultures has previously been shown to have no measurable O₂ uptake, whereas young vegetative mycelium developing within the Warburg vessels from a suspension of developing spores is active in this respect (Erikson & Webley, 1953). Fig. 1 shows the early stages of development, as measured by O₂ uptake, of a spore suspension growing in the presence of air or oxygen. It will be seen that oxygen exerted an accelerating effect on the rate of oxygen uptake of the developing mycelium (see also growth experiments).

Table 1. *Effect of pure oxygen (1 atm. pressure) on the respiration of the aerial mycelium of M. vulgaris after mechanical removal of spores*

Surface growth remaining on glass wool after removal of spores in previous experiment. 50 mg. wet weight weighed into sterile Warburg vessel. Further additions 0.5 ml. m/75 phosphate (pH 7.0) + 2.0 ml. CPS medium. After 90 min. in air at 60°, air replaced by pure oxygen at 1 atm. pressure.

Time interval (min.)	μ l. O ₂ uptake/100 mg. wet weight mycelium during interval
	In air
0-30	52.2
30-60	142.2
60-90	272.6
90-120	Air replaced by oxygen
120-150	154.8
150-180	82.8
180-210	50.2
210-240	32.2

Aerial mycelium. When aerial mycelium remaining after the preparation of the spore suspension in the above experiment was tested in air and in oxygen, a marked inhibitory effect by the oxygen was observed (Table 1). That this inhibition was not due to exhaustion of substrate is seen from Fig. 2, where the oxygen uptake in air has been compared with that in oxygen. In this experiment, the rate of oxygen uptake (266 and 280 μ l. oxygen/100 mg. wet weight/hr.) in air before replacement of air by oxygen, shows only a difference of about 6% between the vessels as compared with the final difference after the replacement of air by oxygen (Fig. 2). The inhibition increases with time (Table 1 and Fig. 2) and is irreversible. Thus on transferring back to air after exposure to oxygen the oxygen uptake of the aerial mycelium does not rise again (Table 2).

Dickens (1946*a*) observed a similar effect of oxygen at high pressures on the metabolism of brain tissue; this effect was also irreversible. Dickens was, however, able to protect the tissue by the previous addition of certain divalent

metals. These metals, in order of efficiency, were $Mn > Co > Mg > Ca$. Table 8 gives the results of a similar experiment carried out with the aerial growth of *Micromonospora vulgaris*. There was little evidence of a protective action on the part of these divalent metals against oxygen inhibition in the present case.

To summarize from the above experiments, the following points appear to have been established: (1) Pure oxygen (1 atm.) did not inhibit the oxygen

Fig. 1

Fig. 2

Fig. 1. Effect of pure oxygen (1 atm. pressure) on the respiration of vegetative mycelium developing in Warburg vessels. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60°, washed once with *m*/75 phosphate buffer (pH 7.0). Shaken for 5 min. in buffer on Microid shaker subsequently filtered through sterile glass wool. Filtrate centrifuged and deposit of spores resuspended in 2 ml. *m*/75 phosphate buffer (pH 7.0); 0.5 ml. of this suspension placed in each vessel + 2 ml. CPS medium. Gas phase: air or oxygen, as shown.

Fig. 2. Effect of pure oxygen (1 atm. pressure) on the respiration of the aerial mycelium of *M. vulgaris*. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60° washed once with *m*/75 phosphate buffer (pH 7.0), shaken for 5 min. in buffer on Microid shaker subsequently filtered through sterile glass wool; 75 mg. (wet weight) of surface growth remaining on glass wool weighed into Warburg vessels. Further additions 0.5 ml.-*m*/15 phosphate buffer (pH 7.0) + 1.5 ml. CPS + distilled water to give total vol. 2.5 ml. Gas phase: air or oxygen, as shown.

uptake of vegetative mycelium of *Micromonospora vulgaris* developing from spores in Warburg vessels. On the contrary, oxygen exerted a stimulating

Table 2. *Effect of replacing pure oxygen (1 atm. pressure) by air on the respiration of the aerial mycelium of M. vulgaris*

M. vulgaris (strain H); grown in liquid CPS medium for 2 days at 60° washed once with M/75-phosphate buffer (pH 7.0). Shaken for 5 min. in buffer on Microid shaker, subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into sterile Warburg vessel. Further additions 0.5 ml. phosphate (pH 7.0) + 2.0 ml. CPS medium. After 150 min. in pure oxygen (1 atm. pressure) at 60°, oxygen replaced by air.

Time interval (min.)	μ l. O ₂ uptake/100 mg. wet weight mycelium during interval
	In oxygen
0-30	87.0
30-60	74.6
60-90	45.3
90-120	40.0
120-150	28.0
150-180	Oxygen replaced by air
180-210	21.0
210-240	21.0
240-270	16.0

Table 3. *Effect of the presence of certain divalent metals on the inhibition of the respiration of M. vulgaris by oxygen*

M. vulgaris (strain H); growth in liquid CPS medium for 2 days at 60° washed once with M/75-phosphate buffer (pH 7.0). Shaken for 5 min. in buffer on Microid shaker, subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into sterile Warburg vessels. Further additions 0.5 ml. M/15-phosphate buffer (pH 7.0) + 1.5 ml. CPS medium + 0.5 ml. 0.001 M-MnSO₄, or 0.001 M-CaCl₂ or, 0.001 M-Co acetate or distilled water. After 90 min. incubation in bath at 60° vessels taken off and air replaced by oxygen except in case of control.

Period of observation from start (min.)	Added solutions				
	Distilled water	Distilled water	0.0002 M		
			MnSO ₄	Co acetate	CaCl ₂
			O ₂ uptake by mycelium during corresponding time intervals μ l. O ₂ /100 mg. wet wt. mycelium		
			In air		
0-30	164.4	107.6	155.7	115.6	104.3
30-60	220.8	186.5	242.8	75.2	192.7
60-90	188.4	212.8	235.1	208.5	191.5
Vessels taken off bath; air replaced by oxygen except control					
	Air	O ₂	O ₂	O ₂	O ₂
180-210	97.2	45.9	67.2	66.7	66.8
210-240	102.0	22.4	30.4	30.7	32.9
240-270	—	—	—	—	—
270-300	124.8	22.3	31.7	27.2	20.4

effect on the rate of oxygen uptake. (2) Pure oxygen (1 atm.) had a marked inhibitory effect on respiration of aerial mycelium of *M. vulgaris*. Certain divalent metals did not protect the material against this effect of oxygen.

Growth of M. vulgaris in air and oxygen

Confirmation of the inhibitory action of pure oxygen upon aerial growth can be gained in a spectacular fashion, simply by growing the organism in air or in oxygen in the manner described above in Methods. In pure oxygen there is a very marked suppression of aerial mycelium while bottom growth is stimulated by the presence of oxygen. A gas mixture of 20% (v/v) O₂ + 80% (v/v) N₂, i.e. an artificial air without carbon dioxide, prepared by the methods of Umbreit *et al.* (1951) gave exactly the same type of growth as normal air; thus the effect of pure O₂ was not connected with the absence of CO₂.

Relationship of oxygen inhibition with inactivation of —SH enzymes

As a result of his work on the effect of high oxygen pressures on brain metabolism, Dickens (1946*b*) found that many of the oxygen-sensitive enzymes were those in which —SH groups were known to be present. With this in mind, the action of various —SH enzyme inhibitors was tested on the respiration of the aerial mycelium of *Micromonospora vulgaris*. From Fig. 3 it will be seen that 0.001M-sodium iodoacetate markedly inhibited the oxygen uptake of the aerial mycelium. On the other hand, 0.001M-sodium azide (an inhibitor of metal-containing enzymes) has no inhibitory effect on the respiration. Barron (1951) considered that *p*-chloromercuribenzoate is a much more specific inhibitor for —SH containing enzymes than iodoacetate. It is quite evident from Fig. 4 that *p*-chloromercuribenzoate at M/70,000 exerted a marked effect on the oxygen uptake of the aerial mycelium. Final proof of the presence of —SH containing enzymes is obtained if the inhibition by known —SH inhibitors is reversed by certain —SH containing compounds; of these, glutathione is the most effective (Barron, 1951). All attempts to annul the inhibitions produced by *p*-chloromercuribenzoate by means of glutathione were unsuccessful.

*Accumulation of keto bodies in the presence of pure oxygen
in respiration experiments*

Throughout the work with a pure oxygen gas-phase in Warburg experiments, it was consistently observed that keto bodies accumulated in the presence of oxygen. This was demonstrated by the use of the 2, 4-dinitrophenylhydrazine test described by Jowett & Quastel (1937) and the ammonia nitroprusside test described by James & Norval (1938). The latter test is claimed to be more specific, especially for pyruvic acid (James & James, 1940), than the 2, 4-dinitrophenylhydrazine reaction. Dickens (1946*a*) suggested that in the case of brain metabolism oxygen may poison the pyruvate oxidase system by attacking the essential —SH groups of the enzyme. In view of the accumulation of keto bodies (possibly pyruvic acid) during the respiration of the aerial mycelium of *Micromonospora vulgaris* in pure oxygen at 1 atm. pressure, it may well be that oxygen is here too causing a similar inactivation. It should also be mentioned that a similar accumulation of keto bodies was observed in the presence of the highly specific —SH inhibitor *p*-chloromercuribenzoate at M/70,000 and M/175,000 concentration.

Fig. 3

Fig. 4

Fig. 3. Effect of sodium azide and sodium iodoacetate on the respiration of the aerial mycelium of *M. vulgaris*. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60° washed once with M/75 phosphate buffer (pH 7.0). Shaken for 5 min. in buffer on Microid shaker, subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into Warburg vessels. Further additions 0.5 ml. phosphate buffer + 1.5 ml. CPS + 0.5 ml. distilled water or 0.5 ml. 0.005 M-sodium azide or 0.005 M-sodium iodoacetate in side arm; side arm contents tipped after 90 min. Gas phase: air.

Fig. 4. Effect of *p*-chloromercuribenzoate on the oxygen uptake of *M. vulgaris* (aerial mycelium) at 60°. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60° washed once with M/75 phosphate buffer (pH 7.0); shaken for 5 min. in buffer on Microid shaker and subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into Warburg flasks. Further additions 0.5 ml. phosphate buffer + 1.5 ml. CPS and various volumes of M/7000-*p*-chloromercuribenzoate in side arm to give final concentrations of M/70,000 or M/175,000. Distilled water added to give final volume of 2.5 ml.

DISCUSSION

Very little work seems to have been done on the production and metabolism of bottom and surface growths of aerobic micro-organisms in pure oxygen at one atmosphere pressure. Darby & Goddard (1950) found that in pure oxygen there was no difference in the rate of respiration of *Myrothecium verrucaria* in Warburg vessels as compared with that in air. As they grew their organism on a rotary shaker their material would have produced very little (if any) aerial mycelium. In the present work also, the growth and respiration of vegetative bottom growth was not adversely affected by the presence of pure oxygen (see Fig. 1; and growth experiments). On the other hand, the actual production of aerial mycelium was markedly suppressed by oxygen while the oxygen uptake of aerial mycelium was inhibited by the presence of pure oxygen at 1 atm. pressure. This gives rise to the question: does the physiology of the aerial mycelium vary from that of the vegetative growth? That the aerial growth of *Micromonospora vulgaris* is likely to be physiologically different from the vegetative growth is supported by the differences in morphology and the presence of lipid staining material in the former, which is absent from the latter (Erikson, 1952).

As regards the mechanism of inhibition, in many ways it parallels that described by Dickens (1946*a*) for the variations of brain tissue metabolism brought about by high oxygen pressures. Thus, inhibition increases with time and is irreversible. On the other hand, protection by divalent metals against inhibition as demonstrated by Dickens could not be shown to take place with *Micromonospora vulgaris*. It is possible, however, that the protective action of these metals might have been masked in the presence of the complex substrate mixture (CPS medium) which it was found necessary to use in Warburg experiments. The evidence obtained also suggests that the inhibition caused by pure oxygen may be connected with inactivation of thiol-containing enzymes. Thus, the oxygen uptake of the aerial mycelium is very sensitive to the highly specific —SH inhibitor *p*-chloromercuribenzoate and accumulation of keto bodies takes place in the presence of this inhibitor or of oxygen. Unfortunately, it has not been found possible to annul inhibition by *p*-chloromercuribenzoate by means of —SH compounds (Barron, 1951). Here again it is possible that the reaction of *p*-chloromercuribenzoate with —SH groups in enzymes functioning at 60° is somewhat different from its reaction with —SH enzymes functioning at more normal temperatures.

I am very grateful to Mrs D. Oxford for her advice and suggestions throughout this work and for supplying the material for the Warburg experiments. My thanks are due, also, to Miss Irene Taylor and Miss M. Michael for technical assistance, and to Dr G. K. Fraser for his interest throughout the work.

REFERENCES

- BARRON, E. S. G. (1951). Thiol groups of biological importance. *Advanc. Enzymol.* **11**, 201.
- DARBY, R. T. & GODDARD, D. R. (1950). Studies on the respiration of the mycelium of the fungus *Myrothecium verrucaria*. *Amer. J. Bot.* **37** (5), 379.
- DICKENS, F. (1946*a*). The toxic effect of oxygen on brain metabolism and on tissue enzymes. I. Brain metabolism. *Biochem. J.* **40**, 145.
- DICKENS, F. (1946*b*). The toxic effect of oxygen on brain metabolism and on tissue enzymes. II. Tissue enzymes. *Biochem. J.* **40**, 171.
- ERIKSON, D. (1952). Temperature/growth relationships of a thermophilic actinomycete, *Micromonospora vulgaris*. *J. gen. Microbiol.* **6**, 286.
- ERIKSON, D. (1953*a*). The reproductive pattern of *Micromonospora vulgaris*. *J. gen. Microbiol.* **8**, 449.
- ERIKSON, D. (1953*b*). Variation of mycelial pattern in sporogenous and asporogenous actinomycetes in Symposium Actinomycetales, p. 102. *VIIth Congr. int. Microbiol., Rome*. Oxford: Blackwell Scientific Publications Ltd.
- ERIKSON, D. & WEBLEY, D. M. (1953). The respiration of a thermophilic actinomycete *Micromonospora vulgaris*. *J. gen. Microbiol.* **8**, 455.
- JAMES, G. M. & JAMES, W. O. (1940). The formation of pyruvic acid in Barley respiration. *New Phytol.* **39**, 266.
- JAMES, W. O. & NORVAL, I. P. (1938). The respiratory decomposition of pyruvic acid by barley. *New Phytol.* **38**, 454.
- JOWETT, M. & QUASTEL, J. H. (1937). Effects of hydroxylmalonate on the metabolism of brain. *Biochem. J.* **31**, 276.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1951). *Manometric Methods and Tissue Metabolism*. Minneapolis: Burgess Publishing Co.
- WEBLEY, D. M. (1953). High temperature metabolism of a thermophilic actinomycete *Micromonospora vulgaris*. *Proc. VIIth Congr. int. Microbiol., Rome* (in the Press).

(Received 9 March 1954)