OXYGEN INHIBITION IN *AZOTOBACTER VINELANDII* PYRUVATE OXIDATION

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SUMMARY

The oxygen inhibition of respiration in *Azotobacter vinelandii* has been investigated, and shown to increase with time and to be reversible. The decrease in oxygen uptake with time has been found to correlate with accumulation of keto acids, identified by chromatography as pyruvic and α-ketoglutaric acids. Oxaloacetic acid has been found to accumulate in cultures oxidizing malate, but no relation between this and oxygen poisoning is indicated.

Other inhibitors producing similar effects to oxygen included methylene blue and arsenite. Neither –SH group stabilising reagents nor 2,3-dimercaptopropanol was able to reverse the inhibitory effects of oxygen.

Cell-free extracts were sensitive to oxygen when the substrate was pyruvate but not when succinate was used.

It is concluded that pyruvic oxidase is sensitive to oxygen, and that α-ketoglutaric oxidase is also implicated, although the mechanism of action is not yet clear.

The similarity of the inhibition under study to the oxygen-induced toxicity of X-radiation is indicated.

INTRODUCTION

The toxic action of molecular oxygen has been investigated in relation to several problems—the Pasteur effect, the toxicity to animals of high pressures of oxygen, and enzyme inhibition by oxygen. Much of the work on the first two aspects has, in fact, been done to indicate the particular enzymes sensitive to oxygen.

An oxygen inhibition of glycolytic activity associated with rat-brain mitochondria has been explained as a poisoning of glyceraldehyde-3-phosphate dehydrogenase[1]. In pea seed extracts, a similar effect has been noted[2] and ascribed to oxidation of essential sulphydryl groups in the enzyme protein. The effects on *A. vinelandii*, where the oxygen uptake itself is affected, appear to differ from these cases of inhibition of glycolysis. In yeast, for example, oxygen has little effect on the systems responsible for oxygen uptake[3], although the Pasteur effect is well-known.

In order to explain the toxic effects of oxygen at high pressure on animal metabolism, workers have examined the oxygen sensitivity of several enzymes. Among

Abbreviations: EDTA, ethylenediaminetetraacetic acid; BAL, 2,3-dimercaptopropan-1-ol; TPP, thiamin pyrophosphate.

those shown to be sensitive were: succinic dehydrogenase[4,5], xanthine oxidase[6,7] and *d*-amino acid oxidase[6]. In these instances, inhibition was demonstrated at very high pressures, from 4.4–7 atm, except for xanthine oxidase[7] where 1 atm was used. Oxidation of pyruvate and lactate were also sensitive to oxygen at high pressure[7]. Results of attempts to protect the systems appear contradictory; DICKENS[8] noted that magnesium, cobalt and manganese ions were protective to brain preparations, but that –SH group stabilizing compounds were not. On the other hand, he pointed out a general correlation between those enzymes sensitive to oxygen and those requiring –SH groups for activity.

In plant tissues, several steps in the tricarboxylic acid cycle have been implicated as sites of oxygen toxicity. In peas, exposure to 5 atm oxygen caused accumulation of "citrate" (citrate plus isocitrate) and a decrease in α-ketoglutarate, suggesting inhibition of citrate metabolism[9]. In potato tubers, BARKER AND MAPSON[10] observed very similar patterns, and concluded that citrate oxidation was the stage affected. In apples, complex changes were observed, but the pattern of accumulation of pyruvate and citrate with the loss of α-ketoglutarate and oxaloacetate was again obtained[11].

In microbial systems, oxygen has been shown to inhibit pyruvate oxidation by *Staphylococcus aureus*[12]. Oxygen uptake by the aerial mycelium of *Micromonospora vulgaris* is similarly affected, in this case with the accumulation of ketone materials[13]. The aerobic inhibition of anaerobic organisms is outside the scope of this paper.

In purified enzyme systems, oxygen has been shown to cause inactivation of cytochrome *c* reductase[14] and of yeast cytochrome b_2 (see ref. 15), in the latter case by oxidation of –SH groups with release of flavin. The iron-protoporphyrin chelating enzyme is also inactivated by oxygen[16].

In Azotobacter, the inhibition of oxygen uptake by oxygen pressures above about 0.2 atm has been noted by several workers[17–20] and an overall mechanism suggested[3]. This paper presents evidence to show that one site of inhibition is at the level of pyruvate oxidation and that other sites may exist.

METHODS

Bacteriological

A. vinelandii strain O (kindly provided by Prof. P. W. WILSON) was used as the test organism. It was preserved by vacuum drying, and cultures for broth inoculation carried on a medium (M6) containing: glucose, 10 g; $Na_2HPO_4 \cdot 10H_2O$, 0.3 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaSO_4 \cdot 2H_2O$, 0.05 g; NaCl, 0.05 g; Na_2MoO_4, 5 mg; EDTA iron complex, 15 mg; potato extract[21], 5 ml; agar, 20 g; deionized water to make 1 l; final pH 7.2. Cells were grown overnight in the M22 medium of PARKER AND SCUTT[20], lightly centrifuged (1000 × *g*, 5 min), adjusted to give desired optical density at 600 mμ (Unicam SP 600 spectrophotometer) and aerated for a further 2 h.

Where cells adapted to other substrates were required, the supernatant of the first centrifugation detailed above was centrifuged again (3500 × *g*, 5 min). The cells thus recovered were resuspended in medium containing the desired substrate at a known density and reaerated until adaptation was complete[22]. These media contained, per litre: K_2HPO_4, 0.436 g; KH_2PO_4, 0.341 g; $CaSO_4 \cdot 2H_2O$, 0.03 g; $MgSO_4 \cdot 7H_2O$,

0.2 g; NaCl, 0.2 g; Na_2MoO_4, 3 mg; EDTA iron complex, 15 mg; trace elements (Mn = 0.25 p.p.m., Cu = 0.05 p.p.m., Zn = 0.1 p.p.m., B = 0.25 p.p.m.); final pH 7.2. The various carbon sources were added at the following rates, per litre: sodium succinate, 23.7 g; malic acid 11.7 g; sodium pyruvate, 2.5 g; sucrose, 10 g; glycerol, 10.8 g. The medium was then adjusted to pH 7.2 and sterilised by filtration.

Under these conditions, cultures metabolizing sucrose reached Q_{O_2} (N) values of 20000 to 40000. Experiments where cultures failed to reach these values (nitrogen contents being estimated from optical density) were rejected.

Where larger quantities of cells were required, 20 l cultures were grown on M22 with added potato extract with forced aeration in a stainless steel batch culture apparatus maintained at 30°. Cells were recovered with a Sharples centrifuge, or with a Servall SS-1 modified for continuous flow operation, and stored at — 20°.

Analytical

Pyruvate was determined as the 2,4-dinitrophenyl hydrazone complex in alkali, by a method modified from JOWETT AND QUASTEL[23]. The reagents were those specified by these authors, but a 30 min interval was allowed after addition of alkali to complete precipitation of insoluble material, which was then centrifuged off before reading optical density at 580 mμ. This method, though less sensitive than those using lower wave-lengths, gave low blank values in aqueous solution and was satisfactory over the range 0–100 μg.

Oxaloacetate was determined as pyruvate after decarboxylation with aniline citrate, and inorganic phosphate by the method of TAUSSKY AND SHORR[24].

Oxygen uptake was determined manometrically at 30° by conventional Warburg techniques. pCO_2 was maintained constant in the flasks at 0.005 atm using the diethanolamine buffer system of PARDEE[25]. Uptakes were corrected, where necessary, for autoxidation of diethanolamine as noted by KREBS[26], although the values were negligible over a 3 h period.

Peroxide was estimated by oxidation of *o*-dianisidine catalysed by horseradish peroxidase, using the following reagents:

Dye solution: 0.8 ml 0.1 % *o*-dianisidine in ethanol is diluted to 100 ml with 0.01 *M* phosphate buffer, pH 6.0, containing 15 mg blood albumen per 100 ml. The protein was found necessary to protect the colloidal coloured product from precipitation.

Peroxidase: Stock solution contained 0.5 mg/ml, which was diluted to 25 μg/ml for use.

A 0.5 ml sample, containing up to 6 μg hydrogen peroxide, is mixed with 1.5 ml of dye solution, followed by the addition of 1 ml of peroxidase solution. After incubation at 35° for 30 min, the colour is read at 460 mμ. The calibration curve is linear up to 6 μg of peroxide per 0.5 ml sample and is reproducible from day to day.

Chromatography

Keto acids were converted to their 2,4-dinitrophenyl hydrazones and chromatographed as such. The extract was incubated 30 min at 30° with 0.5 % 2,4-dinitrophenylhydrazine in 2 *N* HCl, and overnight at 5°. The solvent used to remove the hydrazones from aqueous solution was either ethyl acetate or diethyl ether; solvent volumes followed those of TAUBER[27].

For the isolation of other non-keto tricarboxylic acid cycle acids, column chromatography on Amberlite IR-410 (formate cycle) was used. The organic acids were recovered by elution with 6 *M* formic acid and concentrated *in vacuo* at or below room temperature.

The keto acid hydrazones were chromatographed with the following solvent systems: (*i*) butanol – ethanol – water (70/10/20)[28]; (*ii*) isopropanol – water – ammonium hydroxide (sp. gr. 0.880) (200/20/10)[29]; (*iii*) 1 % sodium carbonate[30]; and (*iv*) 0.1 *M* glycine–NaOH buffer, pH 8.5, in the presence of a trace of phenol[31]. Solvents were all used for ascending chromatography, (*i*) and (*ii*) on Whatman No. 4 paper and (*iii*) and (*iv*) on Whatman No. 1. Spots were identified against markers of pure (vacuum distilled) pyruvic acid, α-ketoglutaric acid, oxaloacetic acid and glyoxylic acid dinitrophenylhydrazones, and with colour reaction with ethanolic NaOH.

Tricarboxylic acid cycle acids were chromatographed on Whatman No. 4 paper, by the ascending technique with butanol saturated with 2.3 *M* formic acid[32] and spots identified with iodate–iodide–starch[29]. Ammonium salts were chromatographed on Whatman No. 1 paper using ethanol–ammonia–water (160/10/30)[33] as solvent and Nessler's reagent as indicator.

Sugars were chromatographed on Whatman No. 4 paper using butanol–isopropanol–water (20/140/40) as ascending solvent[29] and silver nitrate–sodium hydroxide as detecting reagent.

Preparation of cell-free extracts

Cell-free extracts of *A. vinelandii* were prepared by alumina grinding as described by McILWAIN[34]. The ground cell material was suspended in 0.015 *M* phosphate buffer, pH 7.0, containing 0.01 *M* nicotinamide, at the rate corresponding to 1 g cells per 10 ml of buffer. The crude extracts were centrifuged for 10 min at 3500 × *g* at 5° to remove whole cells, larger cell fragments and the grinding alumina.

Reagents

Sodium pyruvate was prepared as described by ROBERTSON[35].

Thiamin pyrophosphate, reduced glutathione, α-lipoic acid and oxaloacetic acid were obtained from Nutritional Biochemicals Corporation and peroxidase from Worthington Biochemical Corporation.

Calf liver catalase was prepared according to BONNICHSEN[36].

The alumina used for preparation of cell-free extracts was Aloxite 600, washed once with 0.01 *M* EDTA, pH 8.0, and rinsed several times with deionised water before drying at 80°.

Distilled water used was deionised over Bio-Deminrolit resin (The Permutit Company).

Gas mixtures

The mixtures contained oxygen at partial pressures from 0.1 to 1.0 atm; the diluent gas to 1 atm was argon (99.8 % pure, British Oxygen Company). They were stored over concentrated sodium chloride solution and displaced into the flasks with it. The flasks were evacuated to 6 cm Hg pressure and refilled three times.

The same procedure was followed for larger incubations on the shaker.

RESULTS

Characteristics of the inhibition

Oxygen uptake by *A. vinelandii* reaches a clearly defined maximum at 0.2–0.3 atm oxygen, then declines thereafter up to 1.0 atm[17–20]. The general form of the curve is shown in Fig. 3. The inhibition at 1.0 atm oxygen increases with time (Fig. 1), with either sucrose or pyruvate as the substrate, uptake falling to some 50 % or less of the initial rate after 3 h. In most experiments, uptake usually fell almost to zero after 12 h exposure. In this respect, the results with *A. vinelandii* are essentially similar to those noted for *M. vulgaris*[13].

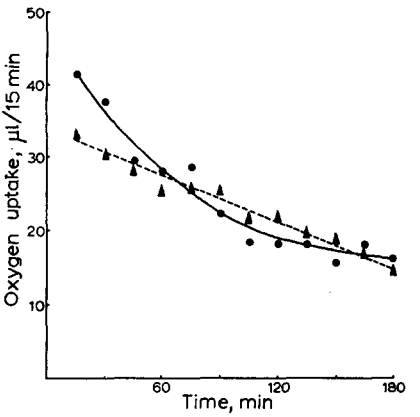

Fig. 1. Curves showing the decline in oxygen uptake rate in *A. vinelandii* cultures incubated in 1.0 atm O_2 for 3 h. Each flask contained: main compartment, 1.0 ml bacterial culture; centre well, 0.2 ml CO_2 buffer for 0.005 atm CO_2. ●—●, sucrose substrate (M22 medium of PARKER AND SCUTT[20]), culture density $E^{0.5\text{ cm}}_{600\text{ m}\mu} = 0.06$. ▲---▲, pyruvate substrate (as given in METHODS), culture density $E^{0.5\text{ cm}}_{600\text{ m}\mu} = 0.125$.

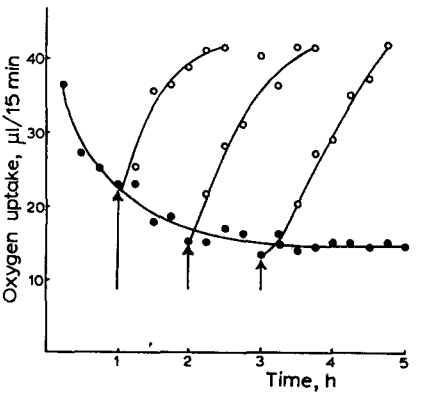

Fig. 2. Curves showing reversibility of oxygen inhibition of *A. vinelandii* cultures incubated in 1.0 atm O_2. Flask contents as for Fig. 1, with sucrose substrate. Culture density, $E^{0.5\text{ cm}}_{600\text{ m}\mu} = 0.06$. ●—●, 1.0 atm O_2 throughout; O—O, after replacement with 0.3 atm O_2 in argon at the points marked ↑.

The inhibition is quite reversible up to 3-h, replacement of the gas phase with one where pO_2 is 0.3 atm is followed by a return of oxygen uptake to that found intially in the control, usually within 45–60 min. This reversibility over short term exposures is shown in Fig. 2. The ease of reversibility is in contrast to that reported for *M. vulgaris*[13], but similar to that found for *S. aureus*[12]. Brain tissue, however, has been found to behave similarly to *M. vulgaris* in not responding to change in the gas phase after inhibition had set in[7,8].

Accumulation of compounds

Accumulation of unspecified ketone type materials has been reported in *M. vulgaris*[13] and increases in known ketoacids have been demonstrated in other tissues[9,10] after exposure to oxygen at atmospheric or higher pressures. Cultures of *Azotobacter* were incubated on sucrose as substrate for periods varying from 3–16 h under 1 atm oxygen and the culture supernatants examined for keto compounds. Positive results were obtained and the substance (as the 2,4-dinitrophenylhydrazone) shown to extract

from ethyl acetate into 10 % sodium carbonate, indicating a keto-acid. The hydrazone was isolated and identified chromatographically as that of pyruvic acid using four different solvent systems. The possibility that pyruvic acid was produced by spontaneous decarboxylation of oxaloacetic acid was examined, but no trace of oxaloacetic acid could be detected, despite immediate treatment of the supernatants. Pyruvic acid does not appear to accumulate as a result of a deficiency of oxaloacetic acid since the addition of this acid did not produce any stimulation of oxygen uptake in a culture where the inhibition was established.

In the cultures examined after 16 h incubation, α-ketoglutaric acid was also demonstrated chromatographically, the second pair of solvents listed in METHODS distinguishing this from oxaloacetic acid. α-Ketoglutaric acid was not detected before 6–8 h incubation under oxygen.

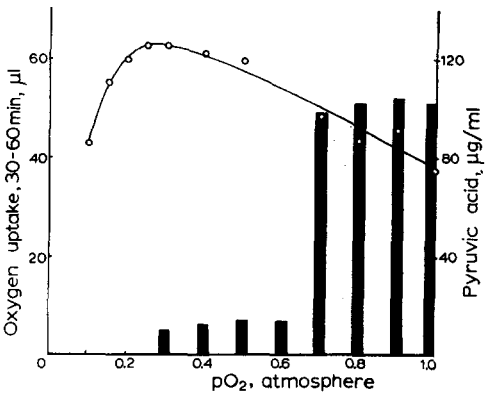

Fig. 3. Relationship between oxygen partial pressure and pyruvic acid accumulation in cultures of *A. vinelandii* exposed to 1.0 atm O_2 for 3 h. Flask contents as for Fig. 1, with sucrose substrate. Culture density, $E^{0.5\text{ cm}}_{600\text{ m}\mu} = 0.08$. Histograms represent the pyruvic acid found after 3 h; O—O, oxygen uptake between 30 and 60 min.

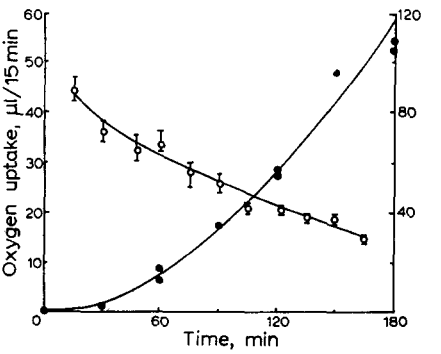

Fig. 4. The relationship between decline in oxygen uptake and the accumulation of pyruvic acid in cultures of *A. vinelandii* exposed to 1.0 atm O_2 for 3 h. Flask contents as for Fig. 1, with sucrose substrate. Data for oxygen uptake (O—O) is given as mean of remaining flasks, and pyruvate production as measured at 30 min intervals (●—●). Culture density, $E^{0.5\text{ cm}}_{600\text{ m}\mu} = 0.08$.

Significant amounts of phosphorylated compounds did not accumulate in the sucrose-incubated cultures under oxygen, since the inorganic phosphate of the medium could be recovered quantitatively without prior hydrolysis.

The relation between keto-acid accumulation and oxygen partial pressure is shown in Fig. 3. Pyruvic acid was absent from cultures incubated at partial pressures below 0.3 atm, where the oxygen uptake is still increasing. Significant amounts of pyruvic acid were not produced below a pO_2 of 0.6–0.7 atm, although the oxygen uptake was considerably reduced below this partial pressure.

The association of pyruvic acid accumulation with decline in oxygen uptake is shown in Fig. 4 over a period of 3 h. The obvious negative correlation between rate of oxygen uptake and pyruvic acid accumulation is strong evidence for the increasing inactivation of the system responsible for pyruvate oxidation. Over the 3 h experimental period, about 30 % of the sugar metabolized (computed from oxygen uptake) appeared as pyruvic acid.

The supernatants of cultures metabolizing sucrose under 1 atm oxygen for 3 h were then tested for accumulating sugars or tricarboxylic acid cycle intermediates. No such substances were detected, although the presence of pyruvic acid in the media was confirmed by its appearance on the organic acid chromatograms.

Cultures adapted to various substrates were examined for accumulation of compounds after incubation for 3 h under 1.0 atm oxygen. The test substrates were pyruvate, succinate and malate. Only in cultures oxidizing malate was an appreciable amount of accumulating keto-acid detected. Isolation of this compound showed it to be oxaloacetic acid, by comparison with authentic material on chromatograms and with the delicate chemical test of KALNITSKY AND TAPLEY[37]. The accumulation of oxaloacetic acid under different oxygen partial pressures was directly proportional to the oxygen uptake (Fig. 5). Were the accumulation of keto-acid caused by oxygen poisoning, increasing amounts would be expected at the higher partial pressures of oxygen where the oxygen uptakes are reduced. Since keto-acid accumulation and oxygen uptake were linearly related at all pressures, it seems unlikely that oxaloacetic acid accumulates owing to oxygen poisoning.

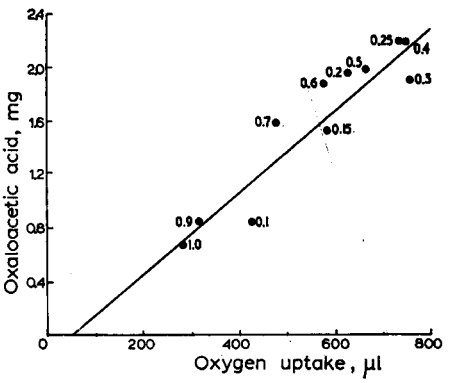

Fig. 5. Correlation between oxygen uptake and oxaloacetate production in cultures of *A. vinelandii* oxidizing malate under different oxygen partial pressures. Flask contents as for Fig. 1, with malate substrate (medium composition as in METHODS). Numbers adjacent to points indicate the oxygen partial pressure. Culture density, $E^{0.5\text{ cm}}_{600\text{ m}\mu} = 0.11$. Correlation co-efficient (r) = 0.94^{***} (10 d.f.).

This production of oxaloacetic acid by cultures of *A. vinelandii* has not, to the author's knowledge, been previously reported.

With succinate or pyruvate as the substrate, no keto-acid accumulation was found; no other acids accumulated when cultures metabolized the substrates under oxygen, though the oxidation of these three compounds is strongly inhibited by oxygen[3]. Citrate was not used as a substrate, since the organism was completely impermeable to it and resisted all attempts to produce an adapted strain.

WOLIN *et al.*[12] reported that the addition of magnesium ions or thiamin could overcome the oxygen sensitivity of pyruvate oxidation in *S. aureus*, and suggested that this was due to replacement of leached compounds lost during washing of the cells. In the present work, magnesium was maintained at an adequate level and washing avoided in the preparation of the cells. The effect of various concentrations of thiamin on cultures oxidizing sucrose under 1 atm of oxygen is given in Table I. It can be seen that 250 mµmoles/ml prevented the accumulation of pyruvic acid and increased oxygen uptake slightly. Thiamin pyrophosphate, at equimolar concentrations, produced quantitatively similar results. Disappearance of pyruvate from the incubation mixture due to thiamin catalysed decarboxylation of pyruvate, as shown by YATCO-

MANZO *et al.*[38], was considered unlikely as both concentrations of reactants and pH of the medium were far removed from the optima found by these authors. In control experiments with known amounts of pyruvate incubated for 3 h with 250 mμmoles of thiamin per ml under 1 atm oxygen, no losses of pyruvate were detected. Acetoin,

TABLE I

EFFECT OF THIAMIN COMPOUNDS ON OXYGEN UPTAKE AND PYRUVATE ACCUMULATION BY *A. vinelandii* CELLS INCUBATED 3 H AT 30° UNDER EITHER 0.3 ATM OR 1.0 ATM O_2

Addition	*Amount added (mμmoles)*	*Oxygen uptake (μl)*		*Pyruvate accumulation (μg)*	
		pO_2 = 0.3 atm	pO_2 = 1.0 atm	pO_2 = 0.3 atm	pO_2 = 1.0 atm
None		641	259	8	126
Thiamin	250		271		43
TPP	100		258		80
TPP	250		275		44

known to be produced from pyruvate by reactions involving thiamin pyrophosphate, was not detected in the presence or absence of thiamin under 1 atm oxygen, indicating that pyruvate is not removed in this direction. Pre-incubation of the cells with thiamin to allow time for absorption and phosphorylation did not decrease the oxygen inhibition. Another important cofactor of pyruvate oxidation, α-lipoic acid, was tested alone and in combination with thiamin and TPP, but did not exert any reversing effect on the oxygen inhibition. A similar lack of effect of these compounds was noted when these materials were tested using pyruvate as substrate. It is possible, however, that the α-lipoic acid may not penetrate the cell wall of this organism although it does so with *Streptococcus faecalis*[39].

These results suggest that, although thiamin compounds are able to redirect the oxidation of pyruvate or prevent its liberation, they are unable to prevent the toxic effect of oxygen.

Mechanism of the inhibition

In *S. aureus*, the inhibitory effects of oxygen and methylene blue were found to be similar[12]. When methylene blue was tested as an inhibitor with *A. vinelandii*, a similar result was obtained with lower concentrations ($5 \cdot 10^{-6}$ *M* compared to $4 \cdot 10^{-5}$ *M* for *S. aureus*). Methylene blue inhibited the oxidation of pyruvate and caused its accumulation when cultures were metabolizing sucrose. It seemed likely, therefore, that peroxides could be the toxic agents, arising either from autoxidation of methylene blue or from the effects of oxygen, as suggested by MANN AND QUASTEL[7] for brain tissue. This possibility in *Azotobacter* was therefore examined from three directions: (*i*) direct addition of peroxide to the cells (up to concentrations of 10^{-4} *M* in the culture); (*ii*) addition of excess catalase to decompose exogenous peroxide; and (*iii*) estimation of peroxide production in the cultures.

Method (*i*) failed to demonstrate any continued toxicity after 15 min, an apparent decrease in oxygen uptake in this period probably resulting from peroxide decomposition.

Approach (*ii*) did not reveal any beneficial effect of catalase in the presence of methylene blue at 0.3 atm oxygen or in its absence at 1.0 atm oxygen.

Estimation of peroxide showed no traces of hydrogen peroxide, although the estimation described is sensitive to 1 μg of peroxide.

From these results it is concluded that accumulation of hydrogen peroxide to toxic proportions is not responsible for the inhibition.

Pyruvate oxidation in *Azotobacter* is sensitive to arsenite[40]. This finding has been confirmed with the demonstration of an accumulation of pyruvic acid in cultures oxidizing sucrose in the presence of 10^{-3} *M* arsenite. The oxidation of glycerol, but not ethanol, is similarly affected by arsenite, with a similar accumulation of pyruvic acid. Vicinal dithiols are believed to be the system attacked, by analogy to other systems where the action of arsenite has been studied in more detail. Added dithiols, such as 2,3-dimercaptopropan-1-ol (BAL), have been shown to reactivate arsenite poisoned systems in other organisms[39]. The effect of BAL on oxygen-poisoned cultures of *A. vinelandii* was therefore studied after non-inhibitory concentrations were established under 0.3 atm oxygen. At these concentrations (up to 10^{-3} *M* for pyruvate and 10^{-4} *M* for sucrose) BAL did not restore oxygen uptake in cells oxidizing sucrose or pyruvate.

Neither sucrose oxidation[3] nor pyruvate oxidation was stimulated by the addition of glutathione or cysteine to the cells incubated under oxygen.

Oxygen effects on cell-free systems

Cell-free extracts were prepared from frozen, unwashed cells. Since *Azotobacter* incorporates acetate (and therefore presumably pyruvate) into the tricarboxylic

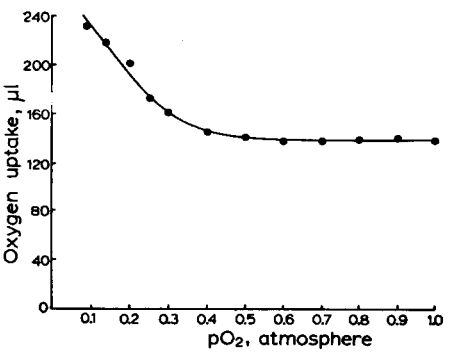

Fig. 6. Effect of oxygen partial pressure on pyruvate oxidation by cell-free extracts from *A. vinelandii*. Each flask contained: main compartment, 1 ml bacterial extract in 0.015 *M* phosphate buffer, pH 7.2, containing 10 μmoles nicotinamide; side arm, 0.5 ml solution containing 25 μmoles sodium pyruvate, 10 μmoles sodium oxaloacetate and 1.33 μmoles Mg^{2+}; centre well, 0.2 ml CO_2 buffer system for 0.005 atm CO_2. Substrate tipped after 15 min equilibration at 30°. Data for oxygen uptake, 30–60 min.

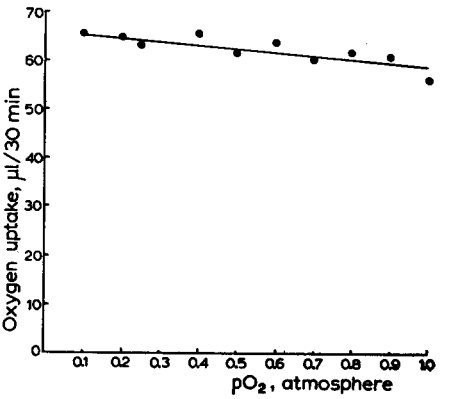

Fig. 7. The effect of oxygen partial pressure on succinate oxidation by cell-free extracts of *A. vinelandii*. Each flask contained: main compartment, 1 ml bacterial extract in 0.015 *M* phosphate, pH 7.2, and 0.1 ml 10^{-2} *M* sodium ethylenediaminetetraacetic acid, pH 7.2; side arm, 50 μmoles sodium succinate, 7.5 μmoles phosphate, pH 7.2 in final volume of 0.5 ml; centre well, 0.2 ml CO_2 buffer system for 0.005 atm CO_2. Substrate tipped after 15 min at 30°; data recorded above are for period 15–45 min.

acid cycle via a conventional condensing enzyme system[41] oxaloacetate was included in the assay for oxygen uptake with pyruvate. The response of this cell-free system to oxygen partial pressure was measured and demonstrates a very pronounced inhibition of oxygen uptake from 0.1 atm to 0.4 atm oxygen with a flattening off above this level. The flat portion of the curve is unexplained, since an increasing inactivation would be expected at higher pO_2 values. Further oxidation of citrate is an unlikely explanation of this apparently oxygen-insensitive uptake, since citrate was not rapidly oxidized by these extracts. Oxygen uptake was not followed below 0.1 atm because of the difficulty of ensuring an adequate diffusion rate of oxygen into the liquid phase with lower pressures. Experiments were restricted to 1 h or less to avoid complete destruction of added oxaloacetate by the active oxaloacetic acid decarboxylase known in *Azotobacter*[42].

The evidence from this section strongly supports the other observations presented in indicating that pyruvate oxidation is one of the stages at which normal metabolism is interrupted by oxygen inhibition.

It has been reported that succinate oxidation is considerably increased when air is replaced by oxygen in the gas phase[43]. In the present work, succinate oxidation in crude extracts was found to be decreased slightly at higher partial pressures, but the decrease is hardly significant (5 % between 0.1 atm and 1.0 atm) (Fig. 7). The site of inhibition is therefore probably not located in the cytochrome chain of the organism. Whichever view is correct, all sources are in agreement that higher partial pressures of oxygen do not depress succinoxidase activity.

DISCUSSION

The quantities of pyruvic acid accumulating in the presence of high oxygen partial pressures point to a considerable inhibition of the pyruvate oxidation system. That the inhibition is also present in cultures metabolizing acetate[3] seems to indicate that the actual site of inhibition lies in the reactions below those generating acetyl-CoA. With the operation of what appears to be a conventional condensing enzyme system in this organism[41], it is reasonable to expect that the equilibrium values of the reactants will approximate those found for the mammalian systems, where the equilibrium is strongly in favour of citrate synthesis[44]. In this case, therefore, the actual accumulation of pyruvic acid indicates a serious derangement of metabolism.

The response of pyruvate accumulation to oxygen partial pressure shown in Fig. 3 is not of the form likely to occur if pyruvate oxidation is the only system affected. The functionally similar α-ketoglutarate oxidase system seems a very likely site for further inhibition and this is supported by the finding of α-ketoglutaric acid in incubations under oxygen. The slow appearance of this acid is presumably due to the low concentrations of substrates passing the pyruvate stage as a result of the substantial inhibition at this point.

The effects of thiamin and TPP in reversing the accumulation of pyruvate under oxygen at first suggested the possibility that the steps involving thiamin pyrophosphate might be the actual reactions affected. The almost complete lack of response in terms of oxygen uptake gives no support to this idea (Table I) but indicates, as found for *S. aureus*[12], that the presence of excess thiamin may divert pyruvate into other pathways.

The finding of pyruvic acid accumulations in cultures exposed to oxygen makes it unlikely that the toxic effects of oxygen are due to poisoning of some carrier transporting substrates into the cell.

The lack of inhibitory effects of oxygen during the oxidation of glycerol has already been reported[3]. Subsequent experiments have shown that ethanol oxidation is also resistant to oxygen. Ethanol oxidation does not appear to involve pyruvate as an intermediate, since no pyruvate can be demonstrated in cultures exposed to sodium arsenite. Glycerol, however, is apparently metabolized through pyruvate, since the keto-acid can be demonstrated in cultures incubated with 10^{-3} *M* arsenite. No pyruvate can be found in cultures oxidizing glycerol as a sole source of carbon under oxygen. A possible explanation covering these contradictory results is a direct protective effect of glycerol on the enzyme(s) poisoned by oxygen. This is analogous to the case of the protective effects of glycerol against the increased toxicity of X-radiation in the presence of oxygen[45]. In this case, also, glycerol is protective, the effects being attributed to the action of glycerol as a free-radical "scavenger" as shown for hydrogen sulphide[46]. Recent results[45] indicate that this scavenging action is not necessarily the true mechanism, since the protective concentrations of glycerol do not alter with oxygen concentration or temperature. The explanation of the protective effects of glycerol in either situation is not clear at the present stage.

Oxygen inhibition in *Azotobacter* thus affects two very important processes in the metabolism of the organism: (*i*) the fixation of atmospheric nitrogen[20]; and (*ii*) the uptake of oxygen coupled with the oxidation of substrates. Of these two, nitrogen fixation is the more sensitive, as fixation is almost suppressed at 0.3 atm O_2 where oxygen uptake is near its maximum. The oxygen uptake appears to be more sensitive in the extracts than in the cell, more closely approximating the situation for nitrogen fixation. It would, however, be unwise to make positive statements about results from cell-free extracts of this organism, since the activity recovered usually represents only a few per cent of that in the whole cell (see, for example, ref. 43). A complete loss of one mechanism for pyruvate oxidation could occur undetected and results must be considered in this light.

From work already done on oxygen poisoning in different organisms, it appears that the keto-acid oxidase complexes and their component enzymes are most frequently affected by oxygen. Thus, pyruvic acid oxidation appears to have been affected in peas[9], potato tubers[10], apples[11], *S. aureus*[12] and possibly *M. vulgaris*[13]. This list can now be extended to include *A. vinelandii*. The three micro-organisms, however, show a greater sensitivity to oxygen than do the plant tissues, where concentrations of oxygen needed to produce toxicity are much higher.

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