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OXYGEN INHIBITION IN *AZOTOBACTER VINELANDII*

SOME ENZYMES CONCERNED IN ACETATE METABOLISM

M. J. DILWORTH* AND I. R. KENNEDY

Institute of Agriculture, University of Western Australia, Nedlands (Australia)

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SUMMARY

Evidence for the presence of phosphate transacetylase and malate synthase in *Azotobacter vinelandii* is presented. The latter enzyme completes the requirements for the operation of the glyoxylate cycle in this organism.

Malate synthase and phosphate transacetylase did not show a requirement for thiol activation, while EDTA substituted for cysteine as an activator for acetate kinase.

The oxygen sensitivity of acetate kinase, phosphate transacetylase, isocitrate lyase and malate synthase has been examined with crude cell-free extracts. In 6 h, these enzymes lost 45, 94, 32 and 42% of their activity, respectively, when exposed to 1.0 atm oxygen. The relation of these losses to the oxygen inhibition of acetate oxidation in *A. vinelandii* has been discussed.

INTRODUCTION

The accumulation of pyruvic and α-ketoglutaric acids by *Azotobacter vinelandii* cultures incubated in sucrose media under 1.0 atm oxygen has been described[1]. Since the oxygen inhibition of respiration accompanying sucrose oxidation at this pressure was also seen when acetate was used as the substrate[2], and acetate is known to be introduced into the tricarboxylic acid cycle *via* citrate synthase (EC 4.1.3.7)[3], it appeared possible that acetate oxidation should also be accompanied by α-ketoglutaric acid accumulation. As no such accumulation could be detected, it seemed likely that a large proportion of acetate metabolism proceeded *via* the glyoxylate pathway. The oxygen sensitivity of the enzymes synthesizing acetyl-CoA from acetate, and of isocitrate lyase (L-isocitrate glyoxylate lyase, EC 4.1.3.1) and malate synthase (L-malate glyoxylate lyase, EC 4.1.3.2), therefore merited examination.

Some of the enzymes specific for acetate metabolism in *A. vinelandii* are already known. Acetate kinase (ATP: acetate phosphotransferase, EC 2.7.2.1) has been reported in extracts of sucrose-grown cells, although phosphate transacetylase (acetyl-CoA: orthophosphate acetyltransferase, EC 2.3.1.8) was not detected[4]. It would seem that acetate metabolism proceeds *via* acetyl phosphate, making the

* Present address: Microbiology Unit, Department of Biochemistry, University of Oxford, England.

occurrence of phosphate transacetylase probable, despite the negative result reported. Further metabolism allowing growth on acetate would involve the glyoxylate cycle. Isocitrate lyase has been reported for both *A. vinelandii* and *A. agile* grown on acetate[6], although malate synthase has not been reported from these organisms. Other enzymes necessary for the operation of the glyoxylate cycle are involved in the tricarboxylic acid cycle known in *A. vinelandii*[3,6].

In this communication, evidence is presented for the occurrence of both phosphate transacetylase and malate synthase, together with some observations on their sensitivity to atmospheric oxygen. Isocitrate lyase and acetate kinase have been examined similarly.

METHODS

Bacteriological

The methods used for preservation, growth and harvesting of cells of strains O and S of *A. vinelandii* have been described[1,7]. The acetate medium used in respirometric studies contained 4.8 g Na acetate per l of the salts medium[1].

Analytical

Oxygen uptakes were determined manometrically at 30° by conventional techniques, under a p_{CO_2} of 0.005 atm[8], with correction for autoxidation[9].

Protein was measured with the Folin phenol reagent[10] with crystalline lysozyme as standard. Glyoxylic acid was measured colorimetrically as 1,5-diphenylformazan carboxylic acid[11], and acetyl phosphate as acetohydroxamic acid[12].

Malate was determined fluorimetrically as homoumbelliferone over the range 1–10 μg malic acid/30 ml final volume with a Hilger 553 fluorimeter, following the method of HUMMEL[13]. A 0.5-ml sample (5–10 μg malic acid) was heated for 10 min in a boiling-water bath with 9 ml 0.08% (w/v) orcinol (twice recrystallized from chloroform) in H_2SO_4–water (3 : 1), cooled and diluted with concentrated H_2SO_4. Fluorescence was measured against 0.05% (w/v) quinine sulphate in 0.1 N H_2SO_4. At the concentrations used, no components of the malate synthase assay system interfered seriously. The preliminary treatment with 2,4-dinitrophenylhydrazine and precipitation of calcium malate were omitted from the original method[13].

Reagents

Aloxite 600 (The Carborundum Co., Manchester) was washed once with 0.01 M EDTA (pH 8.0) and rinsed with deionized water before drying at 80°.

Biochemicals were purchased as follows: CoA, Na_2ATP and lysozyme from Nutritional Biochemicals Corporation; NADP from Sigma Chemical Co.; glyoxylic acid hydrate from L. Light and Co.; Na_3-threo-D_sL_t-isocitrate from Fluka Chemische Fabrik and *Clostridium kluyveri* phosphate transacetylase from Worthington Biochemical Corporation. Isocitrate was 45% active isomer by assay of NADP reduction with *A. vinelandii* isocitric dehydrogenase in the system of KORNBERG[14], and glyoxylic acid hydrate was 99.5% pure by titrimetric assay[11].

Lithium acetyl phosphate was synthesized from isopropenyl acetate[15] and assayed at 82% purity based on succinic anhydride.

CoA was reduced as follows for use in the phosphate transacetylase or malate synthase assays: 0.5 mg 70-75% CoA was dissolved in 3 ml of a solution of pH 6.8 containing 7.5 mg reduced glutathione, 5 μmoles EDTA and 50 μmoles Tris buffer, and incubated for 60 min at room temperature before use, following the method of BURTON AND STADTMAN[16].

Preparation of cell-free extracts

Cell-free extracts were prepared either by alumina grinding[17] or by sonic disruption under hydrogen in a Raytheon 10-kc/sec oscillator operating at 0°. The alumina extracts were taken up in the appropriate buffer and centrifuged at 3500 × g for 10 min and then at 20 000 × g for 20 min; the sonic extracts were centrifuged at 20 000 × g for 15 min. All operations with the crude extracts were performed at or below 3°.

The following buffers were used to extract the material: for acetate kinase, 0.05 M Tris (pH 7.0); for phosphate transacetylase, 0.1 M Tris (pH 7.8); for isocitrate lyase, 0.015 M phosphate (pH 7.0); and for malate synthase, 0.1 M Tris (pH 8.0).

Enzyme assays

Acetate kinase activity was measured by acetohydroxamic acid formation from acetate and ATP in the presence of excess hydroxylamine[4]. The reaction mixture contained, in addition to *A. vinelandii* extract, the following (in μmoles/2.1 ml final volume): EDTA (pH 7.4), 2; KCl, 1400; neutralized hydroxylamine, 1400; Tris buffer (pH 7.4), 97; potassium acetate, 1550; $MgCl_2$, 20; Na_2ATP, 20. When assays were performed in Warburg flasks, acetate, Tris, Mg^{2+} and ATP were in the side arm and other components in the main compartment. Reaction was initiated by addition of substrates, and stopped with 0.4 ml 100% (w/v) trichloroacetic acid. In some experiments, cysteine replaced EDTA as in the original method[4].

Phosphate transacetylase activity was assayed according to STADTMAN[18]. The reaction mixture contained, in addition to *A. vinelandii* extract, the following (in μmoles/0.6 ml final volume): Tris buffer (pH 7.8), 50; KCl, 25; potassium arsenate, 50; acetyl phosphate (Li salt), 2; CoA, 0.018. The final addition was CoA rather than arsenate, since contaminating acetyl phosphatase in the extract was readily and completely inhibited by prior addition of arsenate. Reaction time was 30 min at 30°; reaction was stopped by addition of neutralized hydroxylamine. In some experiments, cysteine or EDTA was added as activator; these were found unnecessary for optimum assay.

Isocitrate lyase activity was assayed as described previously[7]. The reaction mixture contained, in addition to *A. vinelandii* extract, the following (in μmoles/3.0 ml final volume): Tris buffer (pH 7.8), 200; $MgCl_2$, 6; EDTA (pH 7.8), 2; Na_3-DL-isocitrate, 25. After gassing with nitrogen, reaction was initiated in Warburg flasks by the addition of isocitrate, and stopped by the addition of 0.2 ml 80% (w/v) trichloroacetic acid. After centrifugation, glyoxylate was determined[11].

Malate synthase activity was measured by malic acid formation from acetyl-CoA and glyoxylate, using an excess of phosphate transacetylase and acetyl phos-

phate to generate acetyl-CoA. The assay medium contained, in addition to *A. vinelandii* extract and 0.37 mg *C. klayveri* phosphate transacetylase, the following (in μmoles/1.3 ml final volume): Tris buffer (pH 8.0), 100; $MgCl_2$, 10; KCl, 50; acetyl phosphate, 6; glyoxylic acid, 2.5 or 5; CoA, 0.064. Reaction was usually initiated by addition of glyoxylic acid and stopped with 0.1 ml 80% (w/v) trichloroacetic acid. After centrifugation, malic acid content was determined[13].

RESULTS

Acetate oxidation by whole cells

Oxygen at pressures above 0.3 atm is inhibitory to acetate oxidation by whole cells of *A. vinelandii*[2]. During acetate oxidation at 1.0 atm no keto-acids were produced, unlike the situation during sucrose oxidation[1]. In addition, there is no increase of the inhibition with time, as found for pyruvate or sucrose oxidation[1]. Table I shows some typical figures for acetate oxidation at 1.0 atm oxygen.

The pathways of acetate and pyruvate metabolism presumably intersect at the formation of acetyl-CoA, indicating that the accumulation of pyruvate and the decline in oxygen uptake rate during sucrose oxidation are probably both due to an inhibition of a step in the pyruvic oxidase complex before acetyl-CoA formation. However, the oxygen inhibition of acetate oxidation still remains unexplained.

TABLE I

OXYGEN UPTAKES (μL) FOR *A. vinelandii* CULTURES ON SUCROSE OR ACETATE

Substrate	Culture density $E^{1\,cm}_{660\,m\mu}$ (5)	P_{O_2} (atm)	Time (min) 0–30	15–45	30–60	45–75	60–90
Sucrose	0.065	0.3	94				
		1.0	70	62	51	46	39
Acetate	0.140	0.3	140				
		1.0	101	100	101	104	101

Acetate kinase

Initial acetate activation could occur *via* acetate kinase or acetyl-CoA synthetase ((EC 6.2.1.1), formerly aceto-CoA-kinase); ROSE *et al.*[4] have reported that only the former enzyme occurs in *A. vinelandii*. In the assay of acetate kinase, cysteine has commonly been used as the enzyme activator. The requirement for thiol activation makes the determination of oxygen sensitivity difficult; cysteine in the medium in which the enzyme is exposed to oxygen inevitably leads to production of cystine by oxidation, particularly at neutral or alkaline pH. There is then the danger of indirect enzyme inactivation through disulphide exchange with cystine. If the enzyme is first exposed to oxygen and then treated with cysteine, reduction of disulphides generated in the enzyme may occur, with only irreversible changes becoming apparent.

The use of a chelating agent such as EDTA avoids these problems, although other limitations are imposed as a consequence. Metal ions are extremely important in oxygen inactivation of enzymes[19,20], and such ions will be largely removed by

EDTA. However, the bound metal ions responsible for the reversible de-activation of the enzymes appear to originate from the assay reagents[7,21] or from other tissues[20]. Where metal ions are chelated to the enzyme, exposure to oxygen *in vitro* automatically predisposes it to oxidative inactivation whether or not the enzyme in its intracellular state is exposed simultaneously to oxygen and metals. Enzymes which were activated by chelating agents have therefore been examined for oxygen sensitivity in the presence of EDTA; those which responded neither to cysteine nor to EDTA were tested in the absence of chelators.

Acetate kinase responded to addition of EDTA or cysteine as shown in Table II. The activation by EDTA at low concentrations is in contrast to the effect noted by ROSE *et al.*[4] at higher concentrations, where chelation of Mg^{2+} inhibited the enzyme.

TABLE II

ACTIVATION OF ACETATE KINASE IN CELL-FREE EXTRACTS OF *A. vinelandii* S BY CYSTEINE OR EDTA

Assay system: bacterial extract, 1.3 mg protein, and activator (cysteine or EDTA), with the following (μmoles/1.1 ml final volume): neutral NH_2OH, 700; KCl, 700; Tris buffer (pH 7.4), 48; potassium acetate, 775; $MgCl_2$, 10; Na_2ATP, 10. Conditions, 30 min at 30°.

Activator			
Cysteine		EDTA	
Amount added (μmoles)	Acetyl phosphate formed (μmoles)	Amount added (μmoles)	Acetyl phosphate formed (μmoles)
0	2.1	0	2.2
1.25	2.8	0.25	2.9
2.5	2.7	0.50	3.0
5.0	2.6	1.00	2.8
7.5	2.6	1.50	3.0
10.0	2.6	2.00	2.7

Acetate kinase activity in the extracts varied between 4.5 and 7.2 μmoles acetyl phosphate formed/h/mg protein, using EDTA activation. These values compare favorably with that of 3.0 cited by ROSE *et al.*[4] for this organism. The difference may lie in the growth substrate supplied to the cells—sucrose in the earlier study and acetate in the present—in which case synthesis of acetate kinase would appear to be partly adaptive.

The oxygen sensitivity of the enzyme in crude extracts was tested by following the activity with time of exposure, using nitrogen as an inert control to correct for denaturation. The results are shown in Fig. 1. The lines were computed by the method of least-squares, and both regressions are linear (p less than 0.001 on 1, 5 d.f.). The slopes of these lines were significantly different (p less than 0.05 on 1, 6 d.f.), indicating a direct toxic action of oxygen. Inactivation under nitrogen was 16% over 6 h; that under oxygen was 45%.

During the incubation, a considerable gas evolution occurred in all flasks, but was most noticeable in the oxygen series. Distortion of results by loss of hydroxylamine trapping efficiency for acetyl phosphate was considered a possible complica-

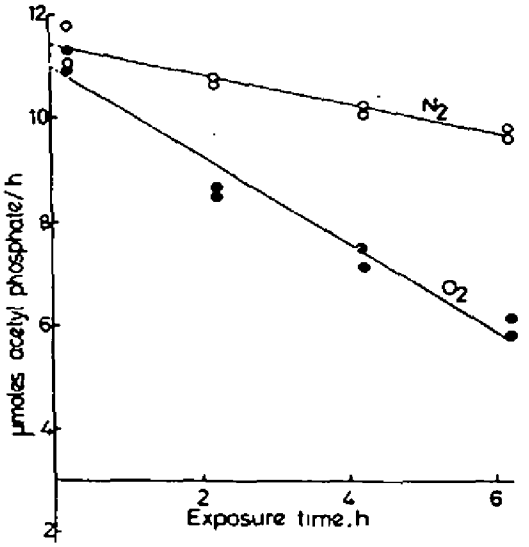

Fig. 1. The effect of oxygen or nitrogen on the acetate kinase activity of cell-free extracts of *A. vinelandii* S. Extract (1.5 mg protein) was shaken under oxygen or nitrogen in Warburg flasks at 30°. At the times indicated, activity was assayed in the flask with the standard system. Reaction time was 20 min, each point represents a separate enzyme incubation. ○—○, nitrogen; ●···●, oxygen.

tion. Assay of fresh enzyme in reaction mixtures shaken in oxygen for 5.5 h gave only slightly (−6%) different results from those in completely fresh medium, a figure clearly within experimental variability. Further, adequate hydroxylamine remained to react with levels of succinic anhydride in excess of the levels of acetyl phosphate produced. It is therefore concluded that adequate quantities of reagents remained to give satisfactory assays.

Phosphate transacetylase

Although this enzyme has been found commonly in organisms possessing acetate kinase, it was not detected in *A. vinelandii*[4]. In the present work, phosphate transacetylase was demonstrated (a) directly, by the normal assay procedure[18], and (b) indirectly, by malic acid synthesis from acetyl phosphate, glyoxylic acid, CoA and Mg^{2+} in the presence of an extract from acetate-grown *A. vinelandii* containing malate synthase (see Table V).

The reduction of CoA prior to enzyme assay[16] apparently removed the requirement for cysteine in the system (Table III), suggesting that the function of cysteine is normally that of ensuring CoA reduction.

Enzymic hydrolysis of acetyl phosphate occurred in the absence of arsenate; with arsenate but no CoA, no decomposition of added acetyl phosphate occurred with extract in 30 min, indicating that arsenate completely inhibited the interfering phosphatase. The decomposition of acetyl phosphate in the presence of arsenate was CoA-dependent, confirming the action of a phosphate transacetylase.

TABLE III

PHOSPHATE TRANSACETYLASE ACTIVITY IN CELL-FREE EXTRACTS OF *A. vinelandii* O IN THE PRESENCE OF CYSTEINE OR EDTA

Reaction mixture: standard system, with addition of either 5 μmoles cysteine or 1 μmole EDTA; extract, 1.4 mg protein. Conditions, 30 min at 30°.

Addition to standard system	*Phosphate transacetylase activity* (μmoles acetyl phosphate/h/mg protein)*
None	2.57
Cysteine, 5 μmoles	2.09
EDTA, 1 μmole	2.22

* Mean of 4 determinations.

The sensitivity of phosphate transacetylase was then examined; only the extract in buffer was exposed to oxygen, and samples assayed for activity as described. Activity fell rapidly in samples under either oxygen or nitrogen (Table IV), but oxygen was clearly responsible for a greater degree of inactivation. The enzyme was also found extremely unstable to freezing and thawing.

TABLE IV

EFFECT OF INCUBATION UNDER OXYGEN OR NITROGEN ON THE PHOSPHATE TRANSACETYLASE IN CELL-FREE EXTRACTS OF *A. vinelandii* O

The bacterial extract was shaken at 30° in Warburg flasks under oxygen or nitrogen. Activity was determined at 0, 2 and 4 h, using the standard assay system with a 0.2-ml sample of extract (1.4 mg protein).

Exposure time (h)	*Phosphate transacetylase activity* (μmoles acetyl phosphate/h/mg protein)*	
	Nitrogen	*Oxygen*
0**	2.54	2.59
2	1.07	0.04
4	0.90	0.16

* Mean of 2 determinations.

** Before assay, all 0-h samples were held 15 min under N_2 at 3° while flasks were gassed.

Isocitrate lyase

The activation of isocitrate lyase by EDTA in preference to the use of thiol compounds has been described[7]. The incubation mixture contained buffer, EDTA and an excess of Mg^{2+} under oxygen or nitrogen, and the activity was assayed at various times. Preliminary experiments showed that significant differences between enzyme exposed to oxygen and nitrogen could be detected after 30 min. The results for longer-term experiments, shown in Fig. 2, were fitted with straight lines by the least-squares method and the slopes compared. The difference was significant (p less than 0.001 on 1, 14 d.f.), indicating clearly that oxygen exerted a toxic effect on the

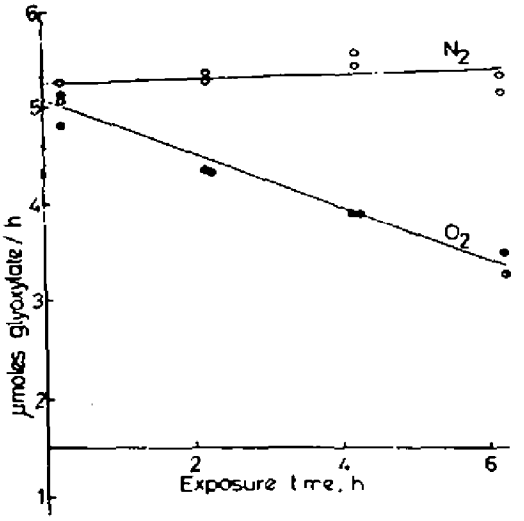

Fig. 2. The effect of oxygen or nitrogen on the isocitrate lyase activity of cell-free extracts of *A. vinelandii* S. Extract (0.64 mg protein) was shaken under oxygen or nitrogen in Warburg flasks at 30°. At the times indicated, activity was assayed in the flask by addition of isocitrate. Reaction time was 15 min; each point is the mean of 2 analyses for glyoxylate from each enzyme incubation. ○—○, nitrogen; ●—●, oxygen.

enzyme. Under oxygen, isocitrate lyase activity fell by 32% in 6 h, while a slight apparent increase (3%) occurred under nitrogen. The enzyme was, however, quite stable for several months at −20° (ref. 7).

The addition of excess isocitrate to extracts caused no change in absorbancy at 340 mμ, indicating that the NADP-isocitrate dehydrogenase (EC 1.1.1.42), which could cause the reduction of 0.58 μmole NADP/min/mg protein, was inactive as a result of a lack of NADP in unfortified extracts. The possibility that oxygen caused an apparent inhibition of isocitrate lyase by removing isocitrate through the tricarboxylic acid cycle is therefore considered unlikely.

The concentration of isocitrate used did not produce a substrate inhibition with the *A. vinelandii* enzyme[7], as reported for *Pseudomonas indigofera* isocitrate lyase[32], and is considerably higher than the K_m of $8.5 \cdot 10^{-5}$ M (ref. 7).

Malate synthase

Although the occurrence of a complete glyoxylate cycle can be inferred from the occurrence of isocitrate lyase[5], direct demonstration of a malate synthase has now been made in *A. vinelandii*.

The identification of the reaction product as malic acid can be deduced from the specificity of the chemical method used: of a wide range of substances tested, only fructose 1,6-diphosphate and glucose 6-phosphate produced a blue fluorescence after reaction with orcinol, and then only at high concentrations[13]. These compounds also produce a visible yellow colour, which was not observed in our experiments; hydrolysis of the sample in 0.2 N NaOH for 10 min at 100°, which prevents produc-

tion of fluorescence from the sugar phosphates[18], did not reduce the fluorescence observed after incubation with the *A. vinelandii* extracts.

Without added phosphate transacetylase, malic acid synthesis required the presence of Mg^{2+}, acetyl phosphate, CoA and glyoxylic acid. The lack of activity when either CoA or acetyl phosphate was omitted indicates that the actual substrate was acetyl-CoA (Table V). Malate synthase activity was linear with protein concentration, at least up to 1.5 mg protein/ml.

TABLE V

MALATE SYNTHESIS BY CELL-FREE EXTRACTS OF *A. vinelandii* S

Reaction system: *A. vinelandii* extract (4 mg protein); and the following (μmoles/3.0 ml final volume): Tris buffer (pH 8.0), 100; $MgCl_2$, 6; cysteine hydrochloride, 2; acetyl phosphate, 6; KCl, 50; CoA, approx. 0.04; glyoxylic acid, 25. Reaction was initiated by addition of glyoxylic acid in Thunberg tubes under nitrogen, and allowed to proceed 20 min at 30°.

Reaction system	*Malate synthase activity (μmoles malate/h)*
Complete system	2.55
Complete system minus CoA	0
Complete system minus *A. vinelandii* extract	0
Complete system minus acetyl phosphate	0
Complete system minus glyoxylic acid	0
Complete system minus Mg^{2+}	1.32

Excess *C. kluyveri* phosphate transacetylase was used to ensure continuous generation of acetyl-CoA, and the basic level (0.37 mg protein) was found satisfactory (Table VI). The high level of KCl used was both to activate the added enzyme[18] and to protect it from inhibition by Li^+ or Na^+ (ref. 18).

TABLE VI

THE EFFECT OF *C. kluyveri* PHOSPHATE TRANSACETYLASE ON MALATE SYNTHESIS BY CELL-FREE EXTRACTS OF *A. vinelandii* S

Reaction mixture: 1.2 mg *A. vinelandii* protein, 0.37 mg *C. kluyveri* protein (basic level); and the following (μmoles/1.3 ml final volume): Tris buffer (pH 8.0), 100; $MgCl_2$, 10; KCl, 50; CoA, approx. 0.04; acetyl phosphate, 6; glyoxylic acid, 5. Reaction was performed under N_2 in Thunberg tubes, started by addition of glyoxylic acid, and run for 20 min at 30°.

Reaction system	*Malate synthase activity (μmoles malate/h)*
Complete system	0.72
Complete system plus 0.37 mg *C. kluyveri* phosphate transacetylase	0.67
Complete system minus *C. kluyveri* phosphate transacetylase	0.19
Complete system minus *A. vinelandii* extract	0

A satisfactory concentration of CoA was found from a study of the effect of CoA concentration on malate synthesis in the presence of excess acetyl phosphate and phosphate transacetylase (Fig. 3). An approximate value for the half-maximum

acetyl-CoA concentration for malate synthase was derived, using the double-reciprocal method[23]. Assuming a minimum purity of 65% (manufacturer's estimate "about 75%"), this value corresponded to $1.6 \cdot 10^{-3}$ M acetyl-CoA for half-maximum malate synthase activity, somewhat greater than the value "less than $1 \cdot 10^{-5}$ M" for *Pseudomonas ovalis*[24]. In subsequent experiments on oxygen sensitivity, a CoA concentration four times the half-maximum value was used.

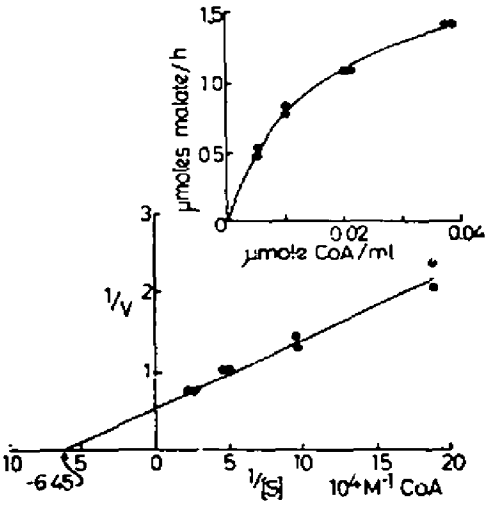

Fig. 3. The effect of CoA concentration on malate synthase activity in extracts of *A. vinelandii* S. Reactions were performed in Thunberg tubes under nitrogen, and started by the addition of 5 µmoles glyoxylic acid after temperature equilibration and incubation for 10 min to ensure complete acetylation of CoA. Reaction time was 20 min. The standard assay system was used, with the indicated amounts of CoA, and 1.13 mg protein.

The pH-activity relationship for malate synthase was not studied owing to the complication of pH effects on the phosphate transacetylase required for acetyl-CoA synthesis. The pH used (8.0) was a compromise selected from some reported values[24-26].

The stoichiometry of the reaction, as shown in Table VII, is in agreement with that for malate synthase[25]. With sucrose-grown strain O cells, direct hydrolysis of acetyl phosphate occurred, but no such complication arose with acetate-grown cells of strain S.

TABLE VII

STOICHIOMETRY OF MALATE SYNTHASE REACTION IN CELL-FREE EXTRACTS OF *A. vinelandii* S

Experiment	Changes in reaction system (µmoles)		
	Malic acid	Glyoxylic acid	Acetyl phosphate
1	+ 0.259	− 0.280	− 0.256
2	+ 0.616	− 0.598	Not determined

No requirement for EDTA or cysteine activation of malate synthase was demonstrated. A similar lack of effect of thiols has been noted with *P. ovalis* malate synthase[24], where acetyl-CoA was used as the substrate. Full reduction of CoA by glutathione before use apparently removed the need for thiols noted in previous reports[25]. Use of cysteine as an activator would be unwise in view of its non-enzymic reaction with glyoxylic acid[7,27]. The enzyme was also stable to freezing to −20° and thawing.

Neither thiols nor EDTA was included in the system for examination of the oxygen sensitivity of *A. vinelandii* malate synthase. Only the enzyme was exposed to oxygen in order to protect the *C. kluyverii* phosphate transacetylase, and to prevent reoxidation of reduced CoA. The enzyme sample was de-oxygenated by successive evacuation and gas replacement with nitrogen following introduction of the phosphate transacetylase and other reagents. Although no pre-incubation period was allowed for CoA acetylation, the activity measured for the phosphate transacetylase from *C. kluyverii* showed that the amount of CoA present would have been acetylated within the first half minute.

The results of these experiments are shown in Fig. 4. Straight lines were fitted to the data by the least-squares method, and the slopes compared. The regressions

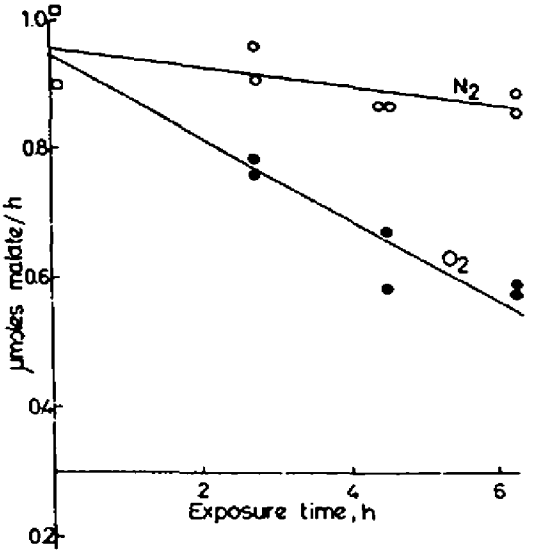

Fig. 4. The effect of oxygen or nitrogen on the malate synthase activity of *A. vinelandii* S extracts. *A. vinelandii* extract (1.43 mg protein) was incubated at 30° under nitrogen or oxygen in Thunberg tubes. After 0, 2.75, 4.5 or 6.25 h the following reagents were added to each (in µmoles/1.0 ml final volume): Tris buffer (pH 8.0), 100; $MgCl_2$, 10; KCl, 50; and 0.37 mg *C. kluyverii* phosphate transacetylase to the main tube, and CoA, approx. 0.064; acetyl phosphate, 5; and glyoxylic acid, 2.5, to the side bulb. Tubes were re-gassed with nitrogen, brought to 30°, and reaction allowed for 23 min. The points shown are means of duplicate malate estimations on each enzyme incubation. □—□, enzyme not treated with either gas; ○—○, enzyme treated with nitrogen; ●—●, enzyme treated with oxygen.

for both sets were linear (p less than 0.01 for the nitrogen set and less than 0.001 for the oxygen set) and the slopes significantly different (p less than 0.001 on 1, 14 d.f.). Malate synthase activity fell 8% in 6.25 h under nitrogen and 42% under oxygen, indicating a definite inhibition due to oxygen.

It is improbable that oxygen caused an apparent inactivation by removal of malate, since assays were always conducted under nitrogen, and the appearance of malate was stoichiometric with the disappearance of glyoxylate and acetyl phosphate under such conditions.

DISCUSSION

Although isotope data definitely involving the glyoxylate cycle in acetate metabolism in *A. vinelandii* are not available, the occurrence of the two key enzymes, isocitrate lyase and malate synthase, and the adaptive formation of isocitrate lyase[5,7], are strong presumptive evidence for its operation in this organism, by analogy with *Micrococcus denitrificans*[28]. However, the relatively low activity of malate synthase (0.7 μmole of malate formed/h/mg protein) by comparison with isocitrate lyase activity (10 μmoles of glyoxylate formed/h/mg protein)[7], suggests that sub-optimal conditions for cell growth or for enzyme assay may have been employed. Growth of *A. vinelandii* with acetate as carbon source was slow[7], and the results reported here for malate synthase were not necessarily obtained at the optimum pH. Higher specific activities of malate synthase are usually recorded for acetate-grown organisms[24,29], though one exception has been noted[30]. In the present case, even if only part of the total original activity has been assayed, the results for malate synthase were reproducible, and are acceptable for assessment of oxygen sensitivity.

The conversion of acetate to acetyl phosphate[3] indicated that phosphate transacetylase could well be present. Malate synthesis from acetyl phosphate, CoA and glyoxylate, and a direct CoA-dependent decomposition of acetyl phosphate in the presence of arsenate confirm its occurrence. No activity is quoted, since the CoA concentration for maximum activity is not known for this enzyme.

The results concerning oxygen inactivation must be interpreted cautiously, particularly in view of the difficulties mentioned earlier with regard to thiols, metal ions and chelating agents. The use of EDTA to remove chelated metal ions from the enzymes probably protected them appreciably from oxygen damage[19,20], and it is therefore possible that acetate kinase and isocitrate lyase are more sensitive than actually found. Thus, EDTA protected *A. vinelandii* triosephosphate dehydrogenase[7] (EC 1.2.1.12) from loss of activity under oxygen over a period of 5.5 h, while this enzyme in other systems has been reported as sensitive to 0.2 atm oxygen[31,32].

Examination of the literature concerning these enzymes in other organisms shows them to possess the following characteristics: acetate kinase, essential –SH groups (by inhibition studies with *p*-chloromercuribenzoate and iodosobenzoate, and their reversal by cysteine[4]); phosphate transacetylase, essential –SH groups (by inhibition studies with *p*-chloromercuribenzoate and reversal with thiols[18]); isocitrate lyase, essential –SH groups (by inhibition studies with *p*-chloromercuribenzoate or iodosobenzoate, or with Cu^{2+} and Zn^{2+}, and reversal with cysteine[30]); and malate synthase, no requirements (no inhibition by iodoacetate or *N*-ethylmaleimide[24]). Activation by thiol compounds has not been taken as necessarily involving –SH groups, in view of the similar effects of EDTA shown here and elsewhere[7,21]. Assuming that the *A. vinelandii* enzymes have the same properties, the oxygen inactivation of the first three could be attributed to effects on SH groups, although the inactivation of malate synthase remains unexplained. The *A. vinelandii*

enzyme either differs markedly from others studied, or is inactivated by a mechanism not involving -SH groups in the active centre.

The applicability of the effects of oxygen noted with the crude enzyme preparations to inhibition of respiration in the whole cell is difficult to evaluate. In the first instance, there is no evidence suggesting the accumulation of the substrates of any of these enzymes *in vivo* when whole cells are incubated under oxygen, although tests have been made for acetyl phosphate and glyoxylate in oxygen-poisoned cells. Secondly, the intracellular position of the enzymes and the access of oxygen and metal ions to them are not known. It can be inferred that the immediate development of oxygen inhibition of acetate oxidation indicates that the entire cell rapidly equilibrates with external oxygen concentration. Thirdly, although the effects observed are eventually attributable to oxygen, the possibility remains that the inactivation of cell-free enzymes in crude extracts is in fact mediated by other enzymes. Such a case is the apparent suppression of nitrate reductase (EC 1.6.6.1) by cytochrome oxidase (EC 1.9.3.1), which competes with it for electrons in the presence of oxygen[33]. While the present studies are not explained by a similar mechanism, since the enzymes concerned are not terminal oxidation systems, some other enzymic interaction may apply. On the other hand, a system where such interactions are still possible is probably closer to the intracellular one than a completely purified enzyme. Fourthly, no information is available concerning any systems responsible for "maintenance" of enzymes within the cell against the effects of oxygen. Thus, it has been shown that a protein disulphide reductase in pea seeds could reactivate oxygen-treated triosephosphate dehydrogenase (EC 1.2.1.9), and the suggestion made that aerobic glycolysis was controlled by processes regulating sulphhydryl group reduction within the cells[34].

Of the enzymes studied, the most oxygen-labile appeared to be phosphate transacetylase. The rate of inactivation of this enzyme, if operative *in vivo*, would explain the decrease in acetate oxidation. As mentioned previously, the EDTA activation of isocitrate lyase and acetate kinase may have unduly protected them. Some further work on the influence of metals is therefore indicated. The levels of inhibition by oxygen observed with the four enzymes studied seem sufficient to account for the inhibition of acetate oxidation in *A. vinelandii* cells. It is still possible, however, that the enzyme inactivations reported are not operative in the whole cells, and that the site of oxygen inhibition of acetate oxidation lies elsewhere. The results also indicate likely effects of oxygen on cell-free enzymes and emphasize the need for exclusion of oxygen from some assay systems.

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