Regulation of Growth of *Acinetobacter calcoaceticus* NCIB8250 on Benzyl Alcohol in Batch Culture

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SUMMARY

Formation of benzoate and catechol during oxidation of benzyl alcohol by washed suspensions of *Acinetobacter calcoaceticus* NCIB8250 confirmed earlier results indicating that this organism metabolizes benzyl alcohol via benzaldehyde, benzoate, and the 3-oxoadipate pathway. There was no evidence for feedback inhibition of benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase II. Examination of growth curves and patterns of substrate utilization, as well as measurement of enzyme activities, showed that benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are repressed when *A. calcoaceticus* utilizes L-mandelate or phenylglyoxylate. Growth of bacteria on L-mandelate prior to their inoculation into benzyl alcohol/salts medium leads to an exceptionally long lag period before benzyl alcohol is used at the maximum rate. Benzyl alcohol metabolism is also suppressed during growth on benzoate.

INTRODUCTION

Bacteria can initiate the metabolism of benzyl alcohol either by oxidizing the carbinol group or by oxidizing the benzene nucleus (Gibson, 1971). The following evidence suggests that *Acinetobacter calcoaceticus* NCIB8250 uses the first method and oxidizes benzyl alcohol to benzaldehyde and benzoate, which are then metabolized via the 3-oxoadipate pathway: (i) there are the appropriate patterns of simultaneous adaptation and enzyme induction (Kennedy & Fewson, 1968*a*, *b*); (ii) mutant strains lacking either benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase II, or both these enzymes, fail to grow on benzyl alcohol (Livingstone *et al.*, 1972); (iii) both benzyl alcohol and benzaldehyde co-ordinately induce benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II (Livingstone *et al.*, 1972); (iv) bacteria can form benzoate and catechol from benzyl alcohol (this paper). A good deal of work has been done with Pseudomonas species and *A. calcoaceticus* on the regulation of the mandelate pathway, which converges with the oxidation of benzyl alcohol at the level of benzaldehyde (e.g. Hegeman, 1966; Stevenson & Mandelstam, 1965; Higgins & Mandelstam, 1972; Cook, Beggs & Fewson, 1975), but there has been little work on the regulation of benzyl alcohol metabolism. This paper characterizes growth of *A. calcoaceticus* on benzyl alcohol and benzaldehyde and describes experiments which indicate aspects of control, especially repression, which are important during batch culture on these compounds.

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METHODS

Materials, growth of bacteria and analytical methods. These were described by Cook *et al.*
(1975), except that the toluene and ethanol (99·7 to 100 %) used to prepare bacteria for
enzyme assay were AnalaR grade from BDH; [*carbinol-*^{14}C]benzyl alcohol was from The
Radiochemical Centre, Amersham, Buckinghamshire; and dithiothreitol was from Cal-
biochem.

Measurement of enzyme activities. Reactions were carried out at 27° C in 10 mm light-path
cuvettes in a recording spectrophotometer. Four volumes (usually 25, 50, 75 and 100 μl) of
bacteria which had been rendered permeable to substrates with toluene were assayed simul-
taneously and the enzyme activity/ml bacterial suspension was calculated by the method of
least squares for a plot of enzyme activity against volume. The intercept on the activity axis
and the correlation coefficient were also calculated, and these values were used as an indica-
tion of how far the results deviated from a straight line through the origin. Correlation
coefficients were usually greater than 0·99. Enzyme units (u.) are defined as μmol of substrate
converted/min.

We previously measured enzyme activities in extracts prepared by ultrasonication (e.g.
Livingstone & Fewson, 1972) but have used toluene-treated bacteria in the present work
because of the greater speed and convenience of this method, and because smaller amounts
of bacteria were required. Preliminary experiments showed that benzyl alcohol dehydro-
genase and L-mandelate dehydrogenase are 'unmasked' by toluene at different rates; for
both enzymes, longer times of treatment are needed than those commonly used for other
enzymes from different organisms. The conditions described below are optimal for each
enzyme (Beggs, 1974) and gave reproducible activities which were similar to the activities
measured in extracts. The addition of the small amounts of toluene used to prepare bacteria
for assay of benzyl alcohol dehydrogenase was facilitated by dissolving it in ethanol (Holms
& Robertson, 1974); ethanol is not a substrate, inhibitor or inducer for benzyl alcohol
dehydrogenase (Fewson, 1966; Beggs, 1974). Unfortunately ethanol could not be used to
prepare bacteria for measurement of L-mandelate dehydrogenase because it lowered the final
specific activity of the enzyme.

For the determination of benzyl alcohol dehydrogenase, harvested bacteria were resus-
pended in ice-cold salts medium, containing 2 mM-dithiothreitol, to a turbidity of 0·5 to 2
at 500 nm. Portions of the suspension (1·9 or 3·8 ml) were added to 0·1 or 0·2 ml, respectively,
of 4 % (v/v) toluene in ethanol in 6 × 0·75 in test tubes and mixed for 30 s on a vortex mixer.
The tubes were covered with metal caps and incubated at 27 °C for 4 h. Bacteria were then
assayed in reaction mixtures containing: 200 μmol sodium pyrophosphate buffer, pH 9·0;
6 μmol NAD$^+$; 600 nmol benzyl alcohol to initiate the reaction; total volume 3·0 ml. The
rate of NAD$^+$ reduction was followed at 340 nm; the oxidation of 1 μmol benzyl alcohol/
assay gives a ΔE_{340} of 2·07. Oxidation of benzaldehyde, a product of this reaction, contributes
to the reduction of NAD$^+$; however, benzyl alcohol dehydrogenase and benzaldehyde
dehydrogenase II are co-ordinately controlled, so this leads to a constant proportional
error. The contribution of benzaldehyde dehydrogenase I is almost always insignificant
(Beggs, 1974) because its specific activity is so low, even in bacteria grown on L-mandelate
(Livingston & Fewson, 1972).

For the determination of L-mandelate dehydrogenase, bacteria were resuspended in ice-
cold salts medium to a turbidity of 0·5 to 2 at 500 nm. Portions of the suspension (2 or 4 ml)
were added to 5 or 10 μl, respectively, of toluene in 6 × 0·75 in test tubes and mixed for 30 s.
The tubes were covered with metal caps and incubated at 27 °C for 90 min. Bacteria were

then assayed in reaction mixtures containing: 200 μmol KH_2PO_4/K_2HPO_4 buffer, pH 7·0; 200 nmol 2,6-dichlorophenolindophenol; 1·5 μmol L-mandelate (adjusted to pH 7·0) to initiate the reaction; total volume 3·0 ml. The reduction of the dye was followed at 600 nm; the oxidation of 1 μmol L-mandelate/assay gives a ΔE_{600} of −6·87.

In experiments to test for possible feedback inhibition in extracts, the bacteria were grown, harvested, disrupted by ultrasonication, and assayed as described by Livingstone *et al.* (1972).

Chromatography. Compounds were separated and identified by ascending thin-layer chromatography using the following freshly prepared solvents: A, n-hexane/glacial acetic acid (49:1, v/v; modified from Nozaka & Kusunose, 1968); B, butan-1-ol saturated with ammonium carbonate buffer, pH 9·7 (Fewster & Hall, 1951); C, toluene/chloroform/ acetone (7:2:1, by vol; Murphy & Lynen, 1975) saturated with ammonium carbonate buffer, pH 9·7 (Fewster & Hall, 1951). Unknowns and standards were applied as solutions in diethyl ether to Serva Feinbiochemica Silufol sheets, UV 254 (Micro-Bio Laboratories, London SW6 3DX). The chromatograms were developed at approx. 20 °C and spots were located under ultraviolet light.

Identification of benzoate and catechol as products of benzyl alcohol metabolism. Bacteria were grown in benzyl alcohol/salts medium, harvested, washed and resuspended (4 mg wet wt ml^{-1}) in 50 mM-phosphate buffer, pH 7·0, with 2·5 mM-benzyl alcohol. The suspensions were incubated at 30 °C with slow shaking to limit aeration and to increase the accumulation of intermediates. Samples taken after 30 to 90 min, from which bacteria were removed by centrifuging, showed a positive Arnow (1937) test for 'catechol'. The media, adjusted to pH 6·5, were extracted three times with 0·1 vol. diethyl ether. The extracts were bulked, washed once with 0·01 M-phosphate buffer, pH 6·5 (Goldman, Milne & Pignataro, 1967) and concentrated by drying at room temperature. Chromatography in solvent C gave brown spots at R_F 0·14, the same as authentic catechol. Dried extracts redissolved in 0·1 M-HCl had the same absorption spectrum as authentic catechol (λ_{max} 275 nm), and gave the same red colour ($\lambda_{max}^{\prime}$ 504 nm) in the Arnow (1937) test.

HCl (final concentration 0·1 M) was added to samples from which catechol had been extracted. These acidified fractions were then extracted three times with 0·1 vol. chloroform. The chloroform extracts were bulked and extracted three times with 0·1 vol. 0·1 M-NaOH. When these samples were adjusted to pH 2 with HCl, their u.v. absorption spectra were almost identical to that of benzoic acid (λ_{max} 230, 273 nm). They were then extracted with diethyl ether and chromatographed; in all cases there was a major spot, well separated from residual benzyl alcohol, with the same mobility as authentic benzoic acid (R_F 0·32 in solvent B; moved approx. 2 cm in 30 min in solvent A).

RESULTS

Growth on benzyl alcohol or benzaldehyde

The growth of *A. calcoaceticus* in benzyl alcohol/salts medium varied with the inoculum (Fig. 1). When the inoculum had been grown in homologous medium, there was immediate exponential growth at a rate, μ, of 0·88 h^{-1}. Utilization of benzyl alcohol was followed by (i) direct spectrophotometric assay, and (ii) measuring the loss of ^{14}C from [*carbinol*-^{14}C]-benzyl alcohol. The carbinol-C is lost by decarboxylation of benzoate so that theoretically the rate-limiting step could involve benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase, benzoate oxygenase or cyclohexadienediolcarboxylate dehydrogenase. However, the inset in Fig. 1 shows that benzyl alcohol disappearance and loss of carbinol-^{14}C were coincident, so no intermediates before the decarboxylation step could have accumulated. In a few
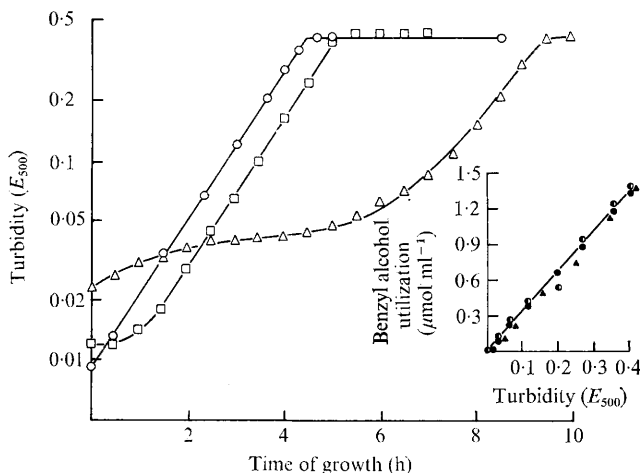
Fig. 1. Effects of different pre-induction on the growth of *A. calcoaceticus* in benzyl alcohol/salts medium. The bacteria used for inoculation were grown in 5 mM-benzyl alcohol/salts medium (○), 2 mM-benzoate/salts medium (□) or 10 mM DL-mandelate/salts medium (△). They were harvested at turbidities (E_{500}; Cook *et al.*, 1975) of 0·4, 0·2 and 0·1 respectively, resuspended in ice-cold basal medium and added to 1·4 mM-[*carbinol*-^{14}C]benzyl alcohol/salts medium (300 ml; 15 μCi). The cultures were grown at 30 °C with aeration and samples were taken at intervals for measurement of growth (E_{500}) and benzyl alcohol utilization. The inset shows the disappearance of benzyl alcohol as a function of growth: benzyl alcohol was measured spectrophotometrically (◑) or radiochemically (see Cook *et al.*, 1975) by the loss of carbinol-^{14}C (●) in the culture inoculated with bacteria grown on the homologous carbon source, or radiochemically in the culture inoculated with bacteria grown on mandelate (▲).
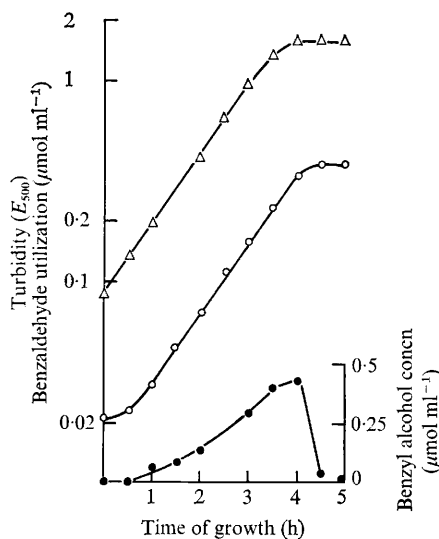

Fig. 2. Growth of *A. calcoaceticus* on benzaldehyde as carbon source. The bacteria used for inoculation were grown in 5 mM-benzyl alcohol/salts medium, harvested and resuspended in ice-cold basal medium, and then added to 1·5 mM-benzaldehyde/salts medium (600 ml). The culture was grown at 30 °C with aeration and samples were taken at intervals for measurement of growth (E_{500}; ○), and the spectrophotometric determination of benzaldehyde (△) and benzyl alcohol (●).

experiments there was a very small (less than o·1 mM) and transitory accumulation of benzoate just before the end of growth. Throughout growth there was a constant linear relation between substrate utilization and growth; this was equivalent to a yield of 81 μg dry wt/μmol benzyl alcohol utilized.

Growth of bacteria on benzoate before inoculation into benzyl alcohol/salts medium introduced a lag into the growth curve; presumably this was caused by the necessity for induction of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II.

The pattern of growth when the inoculum had been grown on mandelate was quite different. There was a period, usually extending to several hours, of slow and irregular growth before the bacteria finally achieved an exponential phase. Despite the unusual nature of the growth curve, substrate utilization paralleled growth (Fig. 1, inset).

Bacteria grew exponentially (μ o·80 h^{-1}) on benzaldehyde as carbon source (Fig. 2) if the inoculum had been grown on benzaldehyde or benzyl alcohol. Benzyl alcohol accumulated in the medium and was then utilized after exhaustion of benzaldehyde. The formation of benzyl alcohol by reduction of benzaldehyde in well-aerated cultures is consistent with our explanation for the accumulation of these two compounds during growth on L-mandelate (Cook *et al.*, 1975).

Growth on pairs of substrates

Benzyl alcohol and mandelate. Adding mandelate to bacteria growing on benzyl alcohol decreased the growth rate (Fig. 3a). Growth was now little more than arithmetical and μ fell progressively from o·88 h^{-1} to o·38 h^{-1}. Decarboxylation of L-[*carboxy*-^{14}C]mandelate was observed after about 200 min, which corresponded to the time when growth started to deviate from the control pattern. From the results of the spectrophotometric assay (Fig. 3b), utilization of benzyl alcohol in the culture containing mandelate was apparently considerably slower after about 200 min. This decrease in the rate of utilization was complicated by the formation of benzyl alcohol in the metabolism of mandelate (Cook *et al.*, 1975) as was seen from the steadily decreasing specific radioactivity of benzyl alcohol (Fig. 3b). Benzaldehyde and benzoate could not be detected, so the specific activity of the benzyl alcohol in conjunction with the amount of benzyl alcohol present (Fig. 3b) enabled us to compensate for this formation of unlabelled benzyl alcohol. The rate of benzyl alcohol oxidation in the control culture was calculated from the gradient of the graph of benzyl alcohol utilization. The rate of benzyl alcohol utilization in the cultures to which mandelate was added was calculated by measuring the rate of utilization of [*carbinol*-^{14}C]benzyl alcohol from a graph of disintegrations min^{-1} versus time, and dividing by the specific activity of the benzyl alcohol at the corresponding times. The rate of benzyl alcohol utilization in the culture containing mandelate was slower than in the control culture from 180 min onwards. After 200 min, when both cultures had the same turbidity, the rates of utilization in the control culture and the culture containing mandelate were 10·4 and 8·2 nmol (ml culture)$^{-1}$ min^{-1} respectively. At a turbidity of o·34 at 500 nm (i.e. after 230 and 240 min for the two cultures respectively) the rates were 15·3 and 9·9 nmol (ml culture)$^{-1}$ min^{-1}. These measurements suggest that benzyl alcohol dehydrogenase synthesis is repressed during mandelate metabolism.

Phenylglyoxylate, L-mandelate and DL-mandelate, but not D-mandelate, decreased the rate of growth when added to bacteria growing on benzyl alcohol, or when present in the inoculated medium.

Experiments were carried out to examine directly the repression of benzyl alcohol dehydrogenase (Fig. 4). After adding mandelate, synthesis of benzyl alcohol dehydrogenase slowed to an arithmetical rate and the total activity levelled off after about 180 min. Benzyl alcohol
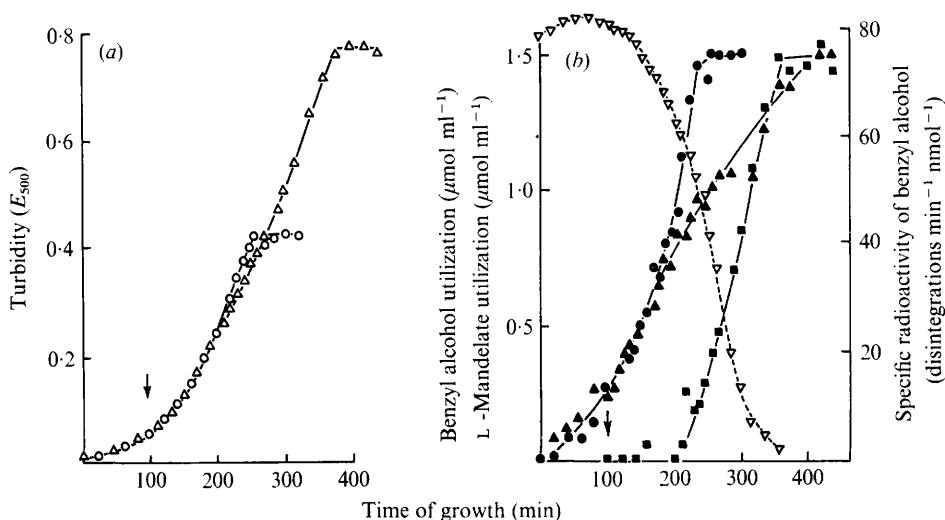
Fig. 3. Effect of mandelate on (a) growth and (b) substrate utilization by A. calcoaceticus growing on benzyl alcohol. The bacteria used for inoculation were grown in 5 mM-benzyl alcohol/salts medium, harvested and resuspended in ice-cold basal medium, and then added to three flasks containing 1·5 mM-benzyl alcohol/salts medium (600 ml). Two of the flasks contained [carbinol-^{14}C]benzyl alcohol (30 μCi). After 100 min growth (indicated by the arrows), DL-mandelate (final concn 3 mM) was added to one of the flasks containing ^{14}C-labelled benzyl alcohol; DL-[carboxy-^{14}C]mandelate (3 mM;30 μCi) was added to the third flask. Samples were taken at intervals for measurement of turbidity (E_{500}), radiochemical measurement of substrate, and spectrophotometric determination of benzyl alcohol. The results of turbidity and spectrophotometric benzyl alcohol measurement for the duplicate flasks to which mandelate was added were similar, and are averaged in this Figure. Growth (E_{500}): control culture (○); cultures with mandelate added (△). Utilization of benzyl alcohol (spectro- photometric assay): control culture (●); cultures with mandelate added (▲). Utilization of L-[carboxy-^{14}C]mandelate (■). The specific radioactivity of [carbinol-^{14}C]benzyl alcohol in the culture to which unlabelled mandelate was added (▽) was calculated by dividing disintegrations/min by the benzyl alcohol concn as determined spectrophotometrically. The specific radioactivity of benzyl alcohol in the control culture remained about constant. Note: growth is presented as an arithmetic plot to aid comparison with the rate of substrate utilization.

was almost certainly still present in the medium (see e.g. Fig. 3b), and was sufficient to cause the small renewal in the synthesis of benzyl alcohol dehydrogenase after exhaustion of the added mandelate, which was in turn indicated by cessation of synthesis of L-mandelate dehydrogenase at about 300 min (Fig. 4).

Benzyl alcohol was apparently used only after exhaustion of mandelate in cultures containing both substrates and inoculated with bacteria previously grown on mandelate (Fig. 5). The only effect of benzyl alcohol was to extend the final part of the growth curve in which growth in the control culture proceeds at the expense of benzyl alcohol formed earlier from mandelate (see Cook et al., 1975). The rate of mandelate metabolism and the early stages of the growth curve were not altered by the presence of benzyl alcohol. This strengthens the evidence for repression of benzyl alcohol utilization during mandelate oxidation.

Benzyl alcohol and benzoate. Diauxic growth, in which benzoate was decarboxylated first to the exclusion of benzyl alcohol, was observed if the inoculum had been grown on benzoate (Fig. 6). The evidence for biphasic growth depended on one point (at 4·5 h) in this experiment, but the effect was reproducible and was confirmed by the pattern of substrate disappearance (Fig. 6b). The rate of metabolism of [carboxy-^{14}C]benzoate was not affected
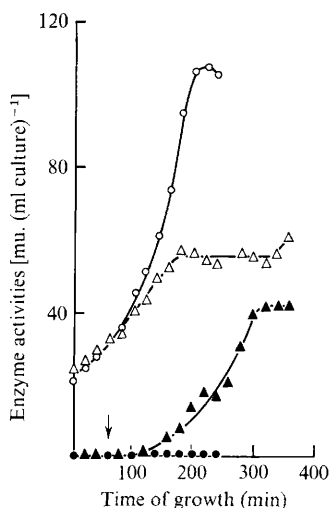
Fig. 4. Effect of mandelate on the synthesis of benzyl alcohol dehydrogenase in the presence of benzyl alcohol. The bacteria used for inoculation were grown in 5 mM-benzyl alcohol/salts medium, harvested and resuspended in ice-cold basal medium, and then added to two flasks containing 3 mM-benzyl alcohol/salts medium (600 ml). The cultures were grown at 30 °C with aeration. After 60 min (indicated by the arrow) DL-mandelate (final concn 6 mM) was added to one culture (△, ▲) and an equal volume of distilled water to the control culture (○, ●). Samples were taken at intervals until growth ceased, and benzyl alcohol dehydrogenase (△, ○) and L-mandelate dehydrogenase (▲, ●) were measured.

by the presence of benzyl alcohol, and loss of $^{14}CO_2$ from [*carbinol*-^{14}C]benzyl alcohol started only a few minutes before exhaustion of benzoate.

Results of a similar experiment in which the inoculum had been grown on benzyl alcohol are more difficult to interpret because it is possible that [*carboxy*-^{14}C]benzoate formed from [*carbinol*-^{14}C]benzyl alcohol, by the enzymes already present in the inoculum, equilibrated with exogenous unlabelled benzoate. There was no evidence for diauxic growth in this case and the decarboxylation of [*carbinol*-^{14}C]benzyl alcohol accelerated throughout growth on the pair of substrates. Our previous suggestion (Cook & Fewson, 1972b) that these results might indicate an inducible transport system for benzyl alcohol is still possible; however, at first sight it is strange that benzyl alcohol should not be able to cross the bacterial membrane even without a specific transport system. In any case, apparent differences between the responses to mixtures of substrates depending on whether the inoculum had been grown on benzoate or benzyl alcohol, which we previously thought were analogous to the initial evidence for glucose transport in *Pseudomonas aeruginosa* (Hamilton & Dawes, 1959), might be explained by equilibration of benzoate from different sources. The results in this paper cannot resolve this point, but they do show unambiguously that benzyl alcohol utilization was suppressed by benzoate if the inoculum had previously been grown in benzoate/salts medium (Fig. 6). Experiments in which the inoculum was grown in nutrient broth (i.e. not induced for any of the enzymes of benzoate or benzyl alcohol metabolism) also indicated substantial repression of benzyl alcohol dehydrogenase during benzoate metabolism.

Possible feedback inhibition tested in bacterial extracts

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were assayed in bacterial extracts in the presence of L-mandelate (0·1 or 1·0 mM), phenylglyoxylate (0·1 or
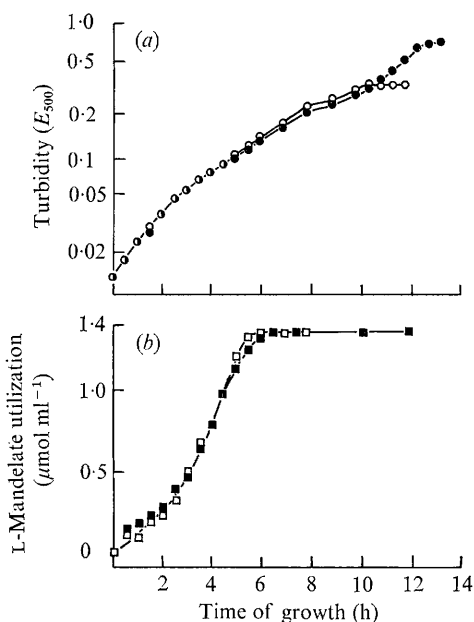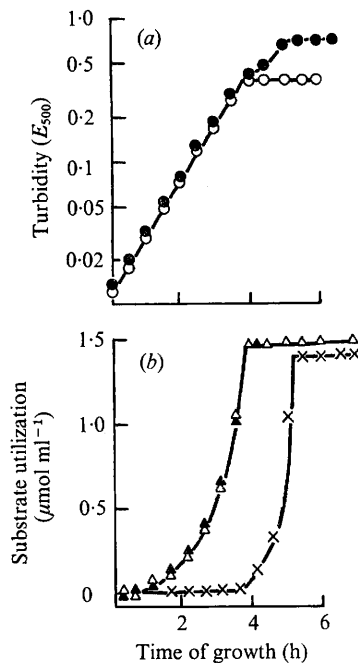
Fig. 5   Fig. 6

Fig. 5. The effect of benzyl alcohol on growth and substrate utilization by *A. calcoaceticus* in mandelate/salts medium. The bacteria used for inoculation were grown in 10 mM DL-mandelate/salts medium, harvested and resuspended in ice-cold basal medium. (*a*) Growth was followed turbidimetrically at 500 nm in salts media containing 2·8 mM DL-[*carboxy*-¹⁴C]mandelate with (●) or without (○) 1·3 mM-benzyl alcohol. (*b*) L-Mandelate utilization was measured in the cultures with (■) or without (□) benzyl alcohol.

Fig. 6. The effect of benzoate on growth and substrate utilization by *A. calcoaceticus* in benzyl alcohol/salts medium. The bacteria used for inoculation were grown in 2 mM-benzoate/salts medium, harvested at a turbidity of 0·2 at 500 nm, resuspended in ice-cold basal medium and used immediately. (*a*) Growth was followed turbidimetrically in salts media containing 1·4 mM-benzoate with (●) or without (○) 1·4 mM-benzyl alcohol. (*b*) Substrate utilization was measured in duplicate cultures by loss of ¹⁴C from [*carbinol*-¹⁴C]benzyl alcohol or [*carboxy*-¹⁴C]benzoate: benzyl alcohol utilization measured in the presence of benzoate (×); benzoate utilization measured in the absence (△) or presence (▲) of benzyl alcohol.

1·0 mM), benzoate (0·1 mM) or succinate (0·1 or 1·0 mM). The additions were at an equal or higher concentration than the enzyme substrates. In no case was significant inhibition or activation observed.

Metabolism of benzyl alcohol by washed bacterial suspensions

Formation of benzoate and catechol. Washed suspensions of bacteria formed benzoate and catechol from benzyl alcohol (see Methods). Occasional samples gave a positive Rothera test (Wilson, 1947); this probably indicated the presence of 3-oxoadipate. The formation of benzoate, catechol, and possibly 3-oxoadipate, confirms previous observations (see Introduction) indicating that *A. calcoaceticus* NCIB8250 oxidizes the carbinol group of benzyl alcohol to give benzoate which is then metabolized via the 3-oxoadipate pathway.

Effects of various metabolic intermediates on the oxidation of benzyl alcohol. Benzyl alcohol utilization was followed spectrophotometrically (Cook *et al.*, 1975). The rate of oxidation of 1·5 mM-benzyl alcohol [approx. 200 nmol min⁻¹ (mg protein)⁻¹] was not significantly

affected by 3 mM DL-mandelate, 1·5 mM-phenylglyoxylate or 1·5 mM-benzoate. The results suggest that these compounds do not inhibit the enzymes oxidizing benzyl alcohol (see also the previous section on feedback inhibition) or the passage of benzyl alcohol into the bacteria, and therefore the repression of benzyl alcohol dehydrogenase probably occurs at the level of transcription or translation. With 1·5 mM-succinate in the reaction mixture, benzyl alcohol utilization was at first totally inhibited and then occurred at a gradually increasing rate until the uninhibited control rate was reached just before exhaustion of the benzyl alcohol.

<h2 style="text-align:center">DISCUSSION</h2>

There is repression of benzyl alcohol dehydrogenase (and presumably also of benzaldehyde dehydrogenase II since they are co-ordinately controlled; Livingstone *et al.*, 1972) during the metabolism of L-mandelate or phenylglyoxylate. This has the unusual effect of lowering the growth rate (Fig. 3) since benzyl alcohol supports a growth rate (μ 0·88 h^{-1}) much higher than the maximum rate supported by L-mandelate (μ 0·66 h^{-1}; Cook *et al.*, 1975). The effect was also unexpected because bacteria, which had been grown so that they contained benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II and then inoculated into salts medium containing L-mandelate, grew rapidly and exponentially without any of the complications (caused by accumulation of benzaldehyde and benzyl alcohol) which are observed in bacteria growing on L-mandelate without pre-induction of the benzyl alcohol enzymes (Cook *et al.*, 1975). The molar growth yield on benzyl alcohol is also greater than on mandelate. This is another example of bacteria not controlling their metabolism so as to grow at the fastest possible rate (see also Higgins & Mandelstam, 1972); but presumably in soil these organisms encounter the ring-substituted derivatives whose growth characteristics are largely unknown. The repression of benzyl alcohol metabolism contrasts with the situation in *Pseudomonas putida* where benzaldehyde metabolism is dominant over mandelate metabolism (Stevenson & Mandelstam, 1965). The mechanism is not known but there is preliminary evidence that in *A. calcoaceticus* the repression is directly caused by one of the enzymes of the mandelate pathway rather than by a metabolite (Beggs & Fewson, 1974). The long lag (over 1 h) before mandelate represses benzyl alcohol dehydrogenase (Figs. 3 and 4) may be caused by inability of mandelate to enter bacteria previously grown on benzyl alcohol (Cook & Fewson, 1972*a*), or by the time needed to synthesize some repressing molecule.

The results of experiments on growth on benzoate and benzyl alcohol (Fig. 6) indicate repression of benzyl alcohol dehydrogenase during growth on benzoate. This repression may not be caused by benzoate itself (Beggs & Fewson, 1974) but rather by a subsequent intermediate of the pathway. Repression of benzyl alcohol dehydrogenase during growth on benzoate or mandelate are thus quite different phenomena; this is indirectly illustrated in Fig. 1 by the very long lag before growth occurred on benzyl alcohol if the inoculum had been grown on mandelate but not if it had been grown on benzoate.

There is no evidence for feedback inhibition of the benzyl alcohol pathway. The inhibition by succinate of benzyl alcohol oxidation by intact bacteria contrasted with the failure of succinate to inhibit either of the dehydrogenases in bacterial extracts; this is similar to the effects of succinate on mandelate metabolism (Cook *et al.*, 1975).

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