Regulation of Growth of *Acinetobacter calcoaceticus* NCIB8250 on L-Mandelate in Batch Culture

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SUMMARY

Batch culture of *Acinetobacter calcoaceticus* in L-mandelate- or phenylglyoxylate-salts medium showed an unusual non-exponential pattern unless the inoculum had been grown on benzyl alcohol. There were transient accumulations of benzaldehyde and benzyl alcohol caused by the limitation of L-mandelate oxidation by low activities of benzaldehyde dehydrogenase and the diversion of reducing power to the formation of benzyl alcohol. *In vivo* enzymic activities were estimated from patterns of substrate utilization in batch cultures containing pairs of substrates. When bacteria previously grown in L-mandelate-salts medium were inoculated into media containing L-mandelate and a second carbon source, metabolism of L-mandelate was arithmetical in the presence of benzoate, catechol or succinate, but accelerated on exhaustion of the second substrate. This indicated repression of the enzymes involved in L-mandelate oxidation. Inoculation of bacteria grown in benzoate-salts medium into medium containing L-mandelate and benzoate gave diauxie with initial utilization of benzoate. Similar experiments showed that benzoate oxidation was not repressed by catechol and only partially repressed by succinate. Measurement of L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I in bacterial extracts showed no evidence for feedback inhibition by intermediates of the pathway. The rates of L-mandelate and benzoate utilization by bacterial suspensions were inhibited by succinate and catechol but not by other intermediates of the pathway.

INTRODUCTION

Acinetobacter calcoaceticus, NCIB8250, oxidizes L-mandelate to catechol (Kennedy & Fewson, 1968*a, b*; Livingstone *et al.* 1972; Livingstone & Fewson, 1972) and this is then metabolized by the β -oxoadipate pathway (see review by Stanier & Ornston, 1973). The intermediates of this inducible catabolic sequence appear to be the same in all bacteria that can metabolize mandelate (Stanier, 1948; Kennedy & Fewson, 1968*a, b*; Rosenberg, 1971; Stanier & Ornston, 1973), but the induction patterns for the enzymes show considerable differences between species (e.g. Hegeman, 1966*a, b, c*; Rosenberg, 1971; Livingstone *et al.* 1972; Livingstone & Fewson, 1972; Stanier & Ornston, 1973). The mandelate pathway of *Pseudomonas* species is controlled not only by induction but also by a complex system of multi-sensitive feedback repression (Mandelstam & Jacoby, 1965; Stevenson & Mandelstam, 1965; Farr & Cain, 1968; Higgins & Mandelstam, 1972*a*). Furthermore, in both *P. putida* (Higgins & Mandelstam, 1972*a, b*) and *A. calcoaceticus* (Cook & Fewson, 1972*a*) there is evidence for transport of aromatic compounds by carrier systems which present possible

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loci of control. We have examined in more detail the regulation of the mandelate pathway in *A. calcoaceticus* NCIB8250, and have studied patterns of growth and substrate utilization in batch culture on pairs of carbon sources in order to assess the various contributions to regulation. This paper describes experiments which indicate the existence of feedback repression and which characterize and explain the unusual non-exponential pattern of growth in L-mandelate- and phenylglyoxylate (benzoylformate)-salts media. Some of the results described below have been presented in preliminary form (Cook & Fewson, 1972*b*).

METHODS

Organisms and growth media. *Acinetobacter calcoaceticus* NCIB8250 (Véron, 1966; Fewson, 1967; Baumann, Doudoroff & Stanier, 1968) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. Stock cultures, subcultures and primary nutrient broth cultures were as described by Kennedy & Fewson (1968*b*). The basal medium, salts medium and filter sterilization of carbon sources have been described previously (Livingstone *et al.* 1972). Radiochemicals used in growth experiments were dissolved without added carrier in distilled water and sterilized by filtration in Nalgene disposable, sterile filter units (120; Nalge Sybron Corp., Rochester, New York, U.S.A.) before aseptic addition to sterile medium.

Measurement of growth. Growth was measured turbidimetrically at 500 nm in a Unicam SP 800 spectrophotometer (Pye Unicam Instruments Ltd, Cambridge) connected to a Servoscribe chart recorder (Smiths Industries, Wembley, Middlesex). Turbidities were measured against air with individual 1 cm light-path cuvettes for each culture. The recorded turbidity for a given sample was corrected by subtraction of the E_{500} of the uninoculated medium in the same cuvette and sample position, and then further corrected for linearity by reference to a standard graph of E_{500} against bacterial density. Bacterial growth as indicated by turbidity is usually given directly as E_{500} in order to avoid introducing ambiguities arising from conversion factors which may not be applicable to all conditions. Occasionally turbidities were converted to μg of bacterial protein/ml, using the relationship that an E_{500} of 1 is equivalent to 143 μg protein/ml; this was derived from protein determinations (Kennedy & Fewson, 1968*b*) on bacterial suspensions. Rates of bacterial growth are quoted as μ , the specific growth rate.

Inocula for experiments on growth and substrate utilization. Preliminary experiments employed inocula grown in nutrient broth, as in previous work (e.g. Kennedy & Fewson, 1968*a, b*). The nutrient broth carried over in the inocula, however, allowed one generation of growth before adaptation to the new medium. To avoid this and to use inocula with a more appropriate and defined phenotype in order to simplify the interpretation of results, inocula for later experiments were pre-induced by growth in salts medium with appropriate carbon sources. Pre-induced inocula were usually prepared by growth in 1 l salts medium with DL-mandelate, benzyl alcohol or benzoate (as detailed in the legends to the appropriate Figures), in 2 l flat-bottomed flasks, and magnetically stirred at 30 °C on the apparatus of Harvey, Fewson & Holms (1968). A 24 h nutrient broth culture was used as the inoculum; unless otherwise stated, the volumes of inocula were 0.5, 1.0 and 4.0 ml with benzoate-, benzyl alcohol- or DL-mandelate-salts media respectively. The cultures were grown for about 9 h and used for inoculation when they reached the required turbidity. Portions (40 ml) of the culture were centrifuged in polycarbonate bottles (50 ml, 59416; Measuring and Scientific Equipment Ltd, Crawley, Sussex) at 12000 *g* for 10 min at 4 °C. A uniform suspension was prepared by blowing chilled basal medium from a pipette on to the pellet,

with brief agitation on a vortex mixer. The volume of basal medium was chosen to give an initial turbidity (E_{500}) of about 0.02 with a 1 to 2 % inoculum. The resuspended organisms were used for inoculation within 5 min.

Experiments to follow growth and substrate utilization simultaneously. Experiments where gas exchange was not measured were done at 30 °C with 300 ml batches of medium in 1 l flasks fitted with side-arms using the apparatus described by Harvey *et al.* (1968). To avoid possible loss of volatile intermediates, no airline was connected to the flasks; measurement of pO_2 and examination of the growth curves showed that diffusion of air through closures was sufficient to prevent oxygen limitation of growth at the low bacterial densities used in these experiments. To basal medium was added 6.0 ml of sterile 2 % (w/v) $MgSO_4 \cdot 7H_2O$, 4.5 ml of 100 mM-carbon source (200 mM in the cases of DL-mandelate and succinate), and water to make the final volume 300 ml in each flask. At this point a sample (4.5 ml) was taken from each flask to provide pre-inoculation blanks for the various assay procedures. Sterile, 'carrier free', radiochemically labelled (3 $\mu Ci/ml$) carbon source (5 ml) was then added to each growth flask. In dual substrate experiments two flasks were used, each having one substrate radiochemically labelled; equal turbidities were achieved within 10 min periods, even at the end of growth in the slow-growing mandelate cultures. Cultures were inoculated at 30 s intervals, 1 min was allowed for temperature equilibration, and then a sample (4.5 ml) was taken for turbidity measurement and assay of appropriate compounds. Cultures were sampled at intervals throughout growth.

Cultures monitored for oxygen consumption and carbon dioxide production were grown under similar conditions except that the total volume of medium was 800 ml and all the additions were correspondingly greater. The neck of the flask was fitted with a condenser and air-inflow tube, and air supplied at 150 ml/min. Dr I. D. Hamilton helped to measure oxygen consumption and carbon dioxide production by paramagnetism and infrared absorption respectively, using the apparatus described by Hamilton & Holms (1970). Samples of the culture were obtained using a disposable syringe fitted into a 6 in dispensing canulus which passed through a silicone rubber bung in the side-arm of the flask.

Assays for mandelate pathway intermediates in culture media. (i) Radiochemical measurements. A portion (1.0 ml) of culture was mixed with 1.0 ml of 0.2 M-HCl in a Polytube (15 × 41 mm; Metal Box Co. Ltd, Plastics Group, Portslade, Sussex) and left unstoppered for about 90 min, with occasional agitation. Portions (250 μl) of the sample were added within 48 h to scintillation fluid and counted as described by Cook & Fewson (1972a). Most of the $^{14}CO_2$ formed by decarboxylation of substrate was lost from the culture since, at the end of growth in [*carboxy*- ^{14}C]benzoate-salts medium, only 2 % of the original radioactivity remained in the culture and at least 75 % of this could not be washed out of cells harvested on membrane filters. Substrate utilization at time t was calculated as:

$$U = \frac{(R_0 - R_t) \times C}{R_0 - R_{ss}},$$

where U is the substrate utilization (nmol/ml culture), R_0 the radioactivity at zero time (pCi/ml culture), R_{ss} the radioactivity in stationary state (pCi/ml culture), R_t the radioactivity at time t (pCi/ml culture), and C is the concentration of radioactively-labelled carbon source at zero time (nmol/ml culture). Substrate utilization was sometimes most conveniently presented as a semi-logarithmic plot against time; for these cases, the zero-time values were obtained by calculating the amount of labelled substrate required to produce the inoculum, using the molar growth yield found in that experiment.

(ii) Chemical determinations. All compounds except benzaldehyde were measured after

precipitation of bacteria. Samples (4 ml) of culture were added to 1 ml of ice-cold 30 % (w/v) perchloric acid, mixed and left on ice for at least 10 min. After neutralization by 3 ml of approximately 1 M-KOH (the precise concentration was determined by preliminary titration) and centrifugation at 12000 g for 10 min, the supernatant liquids were decanted and stored at -20°C . Standard solutions of the appropriate compounds and control samples of salts medium were treated similarly and assayed in duplicate to provide a standard curve for each experiment. All mixing was done with a Whirlimixer (Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire).

To measure benzoate and phenylglyoxylate, samples (1 ml) were added to 3 ml of ice-cold 13.4 % (w/v) perchloric acid and left on ice for 10 min before centrifuging at 12000 g for 10 min. Supernatant liquids (3.5 ml) were added to 5 ml of chloroform and mixed for 15 s. The aqueous phase was removed by suction and 3 ml of the chloroform phase added to 5 ml of 0.2 M-NaOH and mixed for 15 s. A sample (3 ml) of the aqueous phase was added to 1 ml of 1 M-HCl and mixed. Measurements of E_{230} and E_{255} were then made. If $K_{B,230}$, $K_{B,255}$ and $K_{P,230}$, $K_{P,255}$ represent the gradients of graphs of E_{230} and E_{255} against standard benzoate concentrations and against standard phenylglyoxylate concentrations respectively, and $E_{0,230}$ and $E_{0,255}$ are absorbances of the zero-concentration standard, then benzoate (B) and phenylglyoxylate (P) concentrations are given by

$$B = \frac{(E_{230} - E_{0,230})K_{P,255} - (E_{255} - E_{0,255})K_{P,230}}{(K_{B,230} - K_{P,255}) - (K_{B,255} - K_{P,230})},$$

$$P = \frac{(E_{255} - E_{0,255})K_{B,230} - (E_{230} - E_{0,230})K_{B,255}}{(K_{B,230} - K_{P,255}) - (K_{B,255} - K_{P,230})}.$$

Benzyl alcohol was measured by following NAD^+ reduction in the presence of horse-liver alcohol dehydrogenase. The reaction mixtures contained: 2 ml buffer (this was prepared immediately before use and contained, in 600 ml, 20 ml of 2 M-NaOH, 20 g $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 5 g semicarbazide hydrochloride, and 1 g glycine, adjusted to pH 11.1 with 2 M-NaOH); $3 \mu\text{mol}$ NAD^+ ; and $100 \mu\text{g}$ enzyme (prepared immediately before use as a 1 mg/ml suspension in ice-cold distilled water). The mixtures were placed in a 27°C water bath and 1 ml samples added at intervals, followed by gentle agitation. After 2 h incubation the E_{340} values were measured.

To measure benzaldehyde, samples of culture (1 ml) were added to 0.1 ml of 1 M-NaOH and 10 ml of *n*-hexane (Cummins & Perry, 1969), mixed for 30 s, and the E_{240} of the top phase read against *n*-hexane blanks.

Metabolism by washed bacterial suspensions. *Acinetobacter calcoaceticus* was grown in 10 mM DL-mandelate- or 2 mM-benzoate-salts media, harvested at turbidities (E_{500}) of 0.1 and 0.2 respectively, washed in ice-cold 0.05 M- KH_2PO_4 buffer, pH 7.0, resuspended to 20 mg wet wt/ml in phosphate buffer and stored on ice. Experiments were done in Erlenmeyer flasks (50 ml) shaken in a 30°C water bath and containing final volumes of 10 ml. Incomplete reaction mixtures (7.5 ml) containing substrates, and portions of the stock bacterial suspensions were pre-incubated separately and the reaction initiated by the addition of bacteria (2.5 ml) that has been grown on the homologous substrate. Samples ($100 \mu\text{l}$) were taken at intervals and assayed radiochemically for substrate utilization or incorporation. For substrate utilization, samples containing [*carboxy*- ^{14}C]benzoate or [*carboxy*- ^{14}C]mandelate were mixed with 1.0 ml of 0.1 M-HCl to remove $^{14}\text{CO}_2$ and then counted (Cook & Fewson, 1972*a*). For substrate incorporation, samples were harvested on filter mem-

branes, washed twice with 1 ml portions of chilled distilled water, and the filter immediately immersed in scintillation fluid (Cook & Fewson, 1972*a*).

Measurement of the mandelate pathway enzymes. The organisms used for the preparation of extracts were grown, harvested and sonicated as described by Livingstone *et al.* (1972). Phenylglyoxylate carboxy-lyase (benzoylformate decarboxylase, EC 4.1.1.7) was assayed by following the disappearance of substrate (Hegeman, 1966*a*). The other enzymes were assayed as described by Livingstone *et al.* (1972) and Livingstone & Fewson (1972).

Bacteria growing in mandelate-salts medium were assayed for L-mandelate dehydrogenase and benzyl alcohol dehydrogenase after treatment with toluene to allow permeation of substrates (Beggs, 1974).

Dissolved oxygen. pO_2 was measured with an LKB Biotec LP 100-8 meter.

Carbon content of media. Residual medium after removal of bacteria by centrifugation was slightly acidified and dried at 30 °C. Residue was sent for carbon analysis to Dr F. B. Strauss, 10 Carlton Road, Oxford.

Materials. All reagents were of the highest grade that could be obtained commercially. With the exception of the compounds listed below, reagents were obtained from the sources indicated by Kennedy & Fewson (1968*a, b*), Cook & Fewson (1972*a*) and Livingstone *et al.* (1972). Alcohol dehydrogenase (horse liver) was from Sigma, chloroform from May & Baker Ltd, Dagenham, Essex, *n*-hexane (product No. 28488, low u.v. absorption) from BDH, and [*ring*- ^{14}C (U)]benzoic acid and [*ring*- ^3H (G)]catechol were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Many experiments were done with DL-[*carboxy*- ^{14}C]mandelic acid synthesized in this laboratory (Vogel, 1948; Quickfit & Quartz, 1962); others were done with material obtained from Mallinckrodt Nuclear, St Louis, Missouri, U.S.A. *Acinetobacter calcoaceticus* utilizes L- but not D-mandelate (Kennedy & Fewson, 1968*b*) and in many growth experiments DL-mandelate was used as carbon source after confirming that the D-isomer had no effect on the results.

RESULTS

Batch culture on L-mandelate

Batch culture of *A. calcoaceticus* on L-mandelate or phenylglyoxylate gave a non-exponential pattern of growth. Figure 1 shows growth on 1.5 mM L-mandelate (3 mM DL-mandelate in the medium) from an inoculum grown in homologous medium. There was an initial phase of rapid growth ($\mu = 0.66 \text{ h}^{-1}$) lasting 90 min, followed by slower growth ($\mu = 0.27 \text{ h}^{-1}$) for 300 min, then there was a shoulder in the growth curve when growth almost stopped, and finally a phase of slow growth (accelerating to $\mu = 0.13 \text{ h}^{-1}$) lasting 120 min. Substrate utilization did not follow the same complex pattern; L-mandelate was decarboxylated exponentially (specific rate of utilization 0.55 h^{-1}) and was exhausted 240 min before the end of growth (see also inset to Fig. 2). There were transient accumulations of benzaldehyde and benzyl alcohol. The peak of benzaldehyde accumulation occurred at 300 min, just before L-mandelate exhaustion. Maximum benzyl alcohol accumulation occurred at 390 min, and corresponded to the shoulder in the growth curve and to exhaustion of benzaldehyde. Benzyl alcohol disappeared during the last, slow phase of growth. No benzoate or phenylglyoxylate was detected at any stage. The patterns of gas exchange were also complex but corresponded to the various phases of growth. The initially high rates decreased at 90 min, at the same time as the slower rate of growth was established, and there was a concomitant increase in the respiratory quotient from about 0.53 to 0.85. There were sharp falls in carbon dioxide production and oxygen consumption at the shoulder

Fig. 1

Fig. 2

Fig. 1. Growth of *A. calcoaceticus* in DL-mandelate-salts medium. The culture (2 l) used for inoculation was grown from a nutrient broth inoculum (9 ml) in 10 mM DL-mandelate-salts medium for 9 h at 30 °C. The bacteria were harvested, resuspended in ice-cold basal medium and added to 3 mM DL-[carboxy- ^{14}C]mandelate-salts medium (800 ml, 40 μCi). The culture was grown at 30 °C with aeration as described by Hamilton & Holms (1970) and rates of oxygen consumption (∇) and carbon dioxide production ($\square$) were measured. At intervals samples were assayed, as described in Methods, for turbidity E_{500} ; $\circ$, utilization of L-[carboxy- ^{14}C]mandelate (Δ), and accumulation of benzaldehyde ($\bullet$) and benzyl alcohol ($\blacktriangle$).

Fig. 2. Effect of inoculum on the growth of *A. calcoaceticus* in DL-mandelate-salts medium. The bacteria used for inoculation were grown in 10 mM DL-mandelate-salts medium ($\circ$) or 5 mM-benzyl alcohol-salts medium (Δ). The bacteria were harvested at turbidities (E_{500}) of 0.1 and 0.4 respectively, resuspended in ice-cold basal medium and added to 2.8 mM DL-[carboxy- ^{14}C]mandelate-salts medium (300 ml, 15 μCi). The cultures were grown at 30 °C with aeration (Harvey *et al.* 1968) and samples taken at intervals for measurement of growth (E_{500}) and ^{14}C . The inset shows the decarboxylation of L-[carboxy- ^{14}C]mandelate as a function of growth for the cultures grown from inocula prepared in DL-mandelate-salts medium ($\bullet$) or benzyl alcohol-salts medium ($\blacktriangle$).

in the growth curve but both then rose again as benzyl alcohol was utilized. Measurement of dissolved oxygen in parallel experiments showed that it never fell below 90 % of saturation. In other experiments a Vigreux column containing *n*-hexane was attached to the gas train. Approximately 8 % of the original L-mandelate was trapped as benzaldehyde in this way. The residual medium after growth contained less than 2 % of the carbon originally present in the growth substrate. Although these experiments were not designed to measure the carbon balance, within the overall limits of accuracy ($\pm$ about 5 % for the final carbon recovery) there was no reason to think that any compound was unaccounted for.

Reliable enzyme assays were not possible at all stages of growth with 1.5 mM-substrate. However, benzyl alcohol dehydrogenase and L-mandelate dehydrogenase activities were measured during growth on 6 mM DL-mandelate, where the same pattern of growth was observed but more bacteria were available for assay. The differential rate of synthesis of L-mandelate dehydrogenase rose from an original value of 200 mu/mg of protein to 365 mu/mg

Fig. 3. The effects of benzoate on growth and substrate utilization by *A. calcoaceticus* in DL-mandelate-salts medium. The culture used for inoculation was grown in 10 mM DL-mandelate-salts medium, harvested at a turbidity (E_{500}) of 0.1, resuspended, and used immediately. Growth (a) was followed turbidimetrically (E_{500}) in salts media containing 2.8 mM DL-mandelate + 1.4 mM-benzoate (●) or 2.8 mM DL-mandelate alone (○). Substrate utilization (b) was measured radiochemically as described in Methods. L-Mandelate utilization was measured in the culture where it was the only carbon source (□), or in the presence of benzoate (■). Benzoate decarboxylation was also measured in the appropriate culture (▲).

of protein, and then fell to zero at the shoulder in the growth curve. The differential rate of synthesis of benzyl alcohol dehydrogenase was 130 mu/mg protein up to the shoulder, and then rose to 260 mu/mg of protein during the last phase of growth.

With 1.5 mM-phenylglyoxylate as sole carbon source, the same patterns of growth, substrate utilization and accumulation of benzaldehyde and benzyl alcohol were observed as for growth on L-mandelate.

The complex growth on L-mandelate or phenylglyoxylate, which was very reproducible, was observed if the inoculum had been grown on L-mandelate (Fig. 1) or phenylglyoxylate. Growth of the inoculum on benzoate or L-glutamate introduced an initial lag, presumably caused by the need to induce the mandelate enzymes, into an otherwise identical pattern. With an inoculum grown in nutrient broth the pattern was very similar; however, if the inoculum was large enough and if there was carry-over of nutrient broth, the details of the curves were less clear and there was only slight deviation from exponential growth, which is why the significance of these observations was not clear from our original work (Kennedy & Fewson, 1968a). An entirely different result was obtained if the inoculum was grown on benzyl alcohol (Fig. 2). After a slight lag, growth was exponential and relatively fast ($\mu = 0.57 \text{ h}^{-1}$). No benzaldehyde or benzyl alcohol accumulated, and unlike an inoculum grown in homologous medium, substrate utilization paralleled growth (inset to Fig. 2). The slightly higher yield from an inoculum grown on benzyl alcohol (ΔE_{500} of 0.27/mM compared with ΔE_{500} of 0.23/mM) may be due to the smaller proportional contribution of maintenance energy in the culture growing more rapidly; in this experiment there was no stream of air over the culture so loss of volatile compounds would not occur.

Utilization of pairs of substrates

Inocula pre-induced by growth in mandelate-salts medium. The growth curve characteristic of mandelate was markedly altered by the presence of benzoate (Fig. 3a). Growth accelerated in the presence of benzoate to a rate ($\mu = 0.83 \text{ h}^{-1}$) which was maintained till benzoate was exhausted at about 260 min (see Fig. 3b), and there followed a phase of growth with mandelate as sole carbon source. During benzoate utilization, the rate of mandelate decarboxylation was arithmetical. Thus the total activity of the mandelate-oxidizing enzymes

Fig. 4. The effects of mandelate on growth and substrate utilization by *A. calcoaceticus* in benzoate-salts medium. The culture used for inoculation was grown in 2 mM-benzoate-salts medium, harvested at a turbidity (E_{500}) of 0.2, resuspended and used immediately. Growth (a) was followed turbidimetrically (E_{500}) in salts media containing 1.4 mM-benzoate (○) or 1.4 mM-benzoate + 2.8 mM DL-mandelate (●). Substrate utilization (b) was measured radiochemically as described in Methods. Benzoate utilization was measured in the culture where it was the only carbon source (△) or in the presence of mandelate (▲). L-Mandelate utilization was measured in the appropriate culture (■).

remained constant despite a 27-fold increase in the bacterial mass over this period. To determine whether benzoate altered the activity of pre-existing mandelate-oxidizing enzymes, benzoate was added to a culture growing in mandelate-salts medium when the turbidity was 0.08. Growth accelerated to $\mu = 0.74 \text{ h}^{-1}$ (control: $\mu = 0.27 \text{ h}^{-1}$) on addition of benzoate, but reverted to a pattern characteristic of growth on mandelate alone on the exhaustion of benzoate. During the period of benzoate utilization, mandelate utilization was arithmetical at the rate observed immediately before the addition of benzoate, whereas the rate of mandelate utilization in the control culture continued to rise. Benzoate thus does not inhibit mandelate utilization, and the arithmetical rate of mandelate utilization under these conditions must indicate repression of the enzymes for mandelate oxidation or transport.

When a pre-induced mandelate inoculum grew in 1.4 mM-catechol + 2.8 mM DL-mandelate-salts medium, initial growth at a rate $\mu = 0.66 \text{ h}^{-1}$, which is typical of growth on catechol, was accompanied by a slow arithmetical utilization of mandelate. After a turbidity increase of 0.34 there was a sudden drop in the growth rate accompanied by a sharp increase in mandelate utilization. Growth of a nutrient broth inoculum in 1.4 mM-catechol + 2.8 mM DL-mandelate-salts medium accelerated to a rate of $\mu = 0.69 \text{ h}^{-1}$ and mandelate utilization was very slow; after a turbidity increase of 0.35 a sharp drop in growth rate coincided with the onset of significant mandelate utilization. Catechol thus led to repression of the mandelate-oxidizing regulon.

A pre-induced mandelate inoculum grew in 2.8 mM-succinate + 2.8 mM DL-mandelate-salts medium and attained a rate of $\mu = 0.88 \text{ h}^{-1}$. Diauxie occurred after a turbidity increase of 0.43. The period of rapid growth was accompanied by a very low but accelerating rate of mandelate utilization, indicating that suppression of mandelate utilization was considerable but not quite complete in the presence of succinate.

Inocula pre-induced by growth in benzoate-salts medium. The control cultures grew exponentially ($\mu = 0.92 \text{ h}^{-1}$) in benzoate-salts medium (Fig. 4a). Diauxic growth, indicating repression, was observed in benzoate + mandelate-salts medium (Fig. 4a); the first phase was identical with benzoate alone and the second phase accelerated to $\mu = 0.22 \text{ h}^{-1}$. Benzoate decarboxylation occurred first at a rate unaffected by the presence of mandelate (Fig. 4b). Mandelate oxidation started about 45 min after benzoate exhaustion.

Growth of organisms pre-induced by growth in benzoate-salts medium and inoculated into 1.5 mM-[carboxy- ^{14}C]benzoate + 1.3 mM-catechol-salts medium was initially exponential (turbidity 0.005 to 0.4; stationary state in control culture growing in benzoate alone was at $E_{500} = 0.38$) but with a lower growth rate ($\mu = 0.66 \text{ h}^{-1}$) than the control ($\mu = 0.92 \text{ h}^{-1}$). There was no diauxie but the growth rate decreased steadily throughout the last generation, during which time there was no benzoate left in the medium. When benzoate decarboxylation was plotted as a function of the turbidities of the control culture and the culture in this dual substrate mixture, the lines were identical ($4.1 \text{ mM}/\Delta E_{500}$ of 1.0) till benzoate exhaustion. The phenomenon was repeated when organisms grown in nutrient broth (i.e. not induced to utilize any aromatic compound) were used as inoculum for benzoate + catechol-salts medium. Thus the induction of 'benzoate oxidase' was not detectably affected by the presence of equimolar catechol.

Initially, *A. calcoaceticus* grew exponentially ($\mu = 0.88 \text{ h}^{-1}$) in 1.5 mM-benzoate + 2.6 mM-succinate-salts medium. At $E_{500} = 0.5$ there was a lag, corresponding in time to the exhaustion of benzoate, followed by a small ($\Delta E_{500} = 0.14$) and slow ($\mu = 0.06 \text{ h}^{-1}$) period of further growth. There was an acceleration in benzoate decarboxylation when expressed as a function of growth; for the period during which turbidity rose from 0.005 to 0.3, benzoate utilization was $2.3 \text{ mM}/\Delta E_{500}$ of 1.0 (i.e. about $16 \mu\text{mol}/\text{mg}$ protein) and as the turbidity increased to $E_{500} = 0.5$, benzoate utilization rose to $4.1 \text{ mM}/\Delta E_{500}$ of 1.0 (i.e. about $29 \mu\text{mol}/\text{mg}$ protein). Co-utilization of benzoate and succinate was also observed when the inoculum had been grown in nutrient broth. There was thus partial repression of benzoate utilization by succinate.

Possible feedback inhibition tested in bacterial extracts

L-Mandelate dehydrogenase and phenylglyoxylate carboxy-lyase were assayed in the presence of benzyl alcohol (0.5 and 1.0 mM), benzaldehyde (2.5 and 5 mM), benzoate (0.5 and 1 mM) or succinate (0.5 and 1 mM); benzaldehyde dehydrogenase I was assayed in the presence of benzyl alcohol (1 mM), benzoate (0.1 mM) or succinate (1 mM). The added intermediates were equimolar with, or at a higher concentration than, the enzyme substrates. In no case was significant inhibition or activation observed, so that at least under the conditions tested there was no evidence for feedback inhibition.

Substrate utilization by bacterial suspensions and the effects of other intermediates

These experiments, in conjunction with the results from enzyme assays in extracts, were designed partly to test for control of the transport of L-mandelate and benzoate by other intermediates of the pathway. L-Mandelate (1 mM) decarboxylation was linear with time ($84 \text{ nmol}/\text{min}/\text{mg}$ dry wt) and neither 1 mM-benzyl alcohol nor 1 mM-benzoate significantly affected this rate. In the presence of 1 mM-catechol, L-mandelate decarboxylation accelerated from a very low rate to one 67 % of the control. The inhibition by catechol may be non-specific. Succinate (1 mM) caused 27 % inhibition of the rate of L-mandelate disappearance. Benzoate (1 mM) decarboxylation was not significantly affected by 2 mM DL-mandelate, 1 mM-phenylglyoxylate or 1 mM-benzyl alcohol. Succinate (1 mM) decreased the rate by about 50 %. Catechol (1 mM) also inhibited benzoate decarboxylation, and the pattern of catechol and benzoate utilization was examined further (Fig. 5). Benzoate decarboxylation was decreased by about 40 % in the presence of equimolar catechol (Fig. 5a). In contrast, using [ring- ^{14}C (U)]benzoate the incorporation of carbon from benzoate into macromolecules was reduced by 90 % in the presence of catechol (Fig. 5b), while material from [ring- ^3H (G)] catechol was incorporated into macromolecules (Fig. 5c), presumably in place of benzoate.

Fig. 5. The effect of catechol on the decarboxylation and incorporation of benzoate by washed suspensions of *A. calcoaceticus*. Washed suspensions of bacteria which had been grown on benzoate were prepared as described in Methods. Reaction mixtures contained [*carboxy*- ^{14}C]- or [*ring*- ^{14}C (U)]-benzoate (10 μmol ; 0.1 and 0.3 $\mu\text{Ci}/\mu\text{mol}$ respectively) with or without [*ring*- ^3H (G)]catechol (10 μmol ; 2.5 $\mu\text{Ci}/\mu\text{mol}$) and were sampled at intervals as described in Methods or by Cook & Fewson (1972a). (a) Decarboxylation of [*carboxy*- ^{14}C]benzoate in the absence (○) or presence (△) of added catechol; (b) incorporation of [*ring*- ^{14}C (U)]benzoate into macromolecules in the absence (○) or presence (△) of added catechol; (c) incorporation of [*ring*- ^3H (G)]catechol (□). The considerable scatter in (c) is caused by the large amount of catechol, or a compound derived from it, which binds to the filter and thus gives rise to high 'blank' values.

Thus benzoate is probably decarboxylated and the catechol formed equilibrates with any exogenous catechol. This clarifies the results described above for growth on benzoate + catechol which might otherwise have been explained as preferential utilization of benzoate, which would have been surprising since catechol lies lower down the pathway.

DISCUSSION

Growth on L-mandelate or phenylglyoxylate. Most features of the complex pattern of growth of *A. calcoaceticus* on L-mandelate or phenylglyoxylate can be explained by the rate-limitation of benzaldehyde dehydrogenase, the reduction of benzaldehyde to benzyl alcohol, and the repression of enzyme synthesis. There is no evidence that the unusual shape of the growth curve is a trivial effect due to factors such as oxygen limitation. Figure 1 shows that L-mandelate utilization was complete with only 58 % of the final yield achieved. This discrepancy arose from the transient accumulation of benzaldehyde and benzyl alcohol. The accumulation of benzaldehyde but not of phenylglyoxylate, confirms results from measurements of enzyme activities in extracts (Livingston & Fewson, 1972) indicating that the rate-limiting step in the conversion of L-mandelate to benzoate is benzaldehyde oxidation. Benzaldehyde is generally considered to be a relatively unstable compound (see, e.g. Hegeman, 1966a) but sterile salts medium containing 1 mM-benzaldehyde did not change significantly in concentration during 26 h aeration under the same conditions as were used for growth; this indicates that benzyl alcohol formation must be enzyme-catalysed. The low activity of benzyl alcohol dehydrogenase in the early stages of batch culture on L-mandelate (see also Livingstone & Fewson, 1972) could account for the reduction of benzaldehyde to benzyl alcohol; once benzaldehyde accumulated, the K_{eq} of the reaction and the plentiful supply of reducing power would ensure benzyl alcohol formation. A dismutation of benzaldehyde into benzyl alcohol and benzoate has been inferred from patterns of NAD^+ reduction in bacterial extracts (Kennedy & Fewson, 1968b). This diversion of reducing power could also explain the decrease in growth rate between 90 and 120 min (Fig. 1) as well as the corresponding increase in respiratory quotient. During the last, slow phase of growth at the expense of benzyl alcohol the respiratory quotient decreased as more reducing

power was made available for growth. During the period of benzaldehyde accumulation, benzyl alcohol dehydrogenase (and presumably benzaldehyde dehydrogenase II which is synthesized co-ordinately; Livingstone *et al.* 1972) was synthesized at a low differential rate. Even after exhaustion of L-mandelate, when there were high concentrations of benzaldehyde and benzyl alcohol in the medium, the differential rate of synthesis was much less than for a culture growing on benzyl alcohol alone (260 compared with 720 $\mu\text{u}/\text{mg}$ protein; J. D. Beggs, unpublished observations) which confirms that synthesis of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II is repressed in bacteria pre-induced for the mandelate enzymes (Beggs & Fewson, 1974). On this basis, the exponential and much more rapid growth on L-mandelate if the inoculum had been grown on benzyl alcohol (Fig. 2) may be explained by the fact that these bacteria contain considerably higher activities of benzaldehyde dehydrogenase II (Livingstone *et al.* 1972); benzaldehyde therefore does not accumulate, and so it is irrelevant that these bacteria also contain high activities of benzyl alcohol dehydrogenase.

We have confirmed the observation that *P. putida* NCIB9494 grows exponentially on L-mandelate ($\mu = 0.6 \text{ h}^{-1}$; Hegeman, 1966*a*) and found no accumulation of benzaldehyde (C. A. Fewson, unpublished observations). This organism contains two benzaldehyde dehydrogenases when grown on mandelate (Gunsalus, Stanier & Gunsalus, 1953) and this probably prevents such a drastic limitation of the rate of benzaldehyde oxidation. Furthermore, one of the dehydrogenases is NADP⁺-linked and this, together with the very low activity of benzyl alcohol dehydrogenase, presumably diminishes the probability of benzyl alcohol formation.

Repression of enzymes in the mandelate pathway. Repressive control of the mandelate enzymes was observed in the presence of benzoate (Figs. 3 and 4). The minor inhibitory effects of catechol and succinate on the decarboxylation of L-mandelate in suspensions of non-growing bacteria are not sufficient to explain the degree of repression observed with these two compounds, so they must also cause repression. The repression mechanism probably operates at some stage in messenger transcription or translation, rather than for example at the process of transport, because repression occurs in organisms pre-induced to growth in L-mandelate-salts medium without detectable alteration of the activity of pre-existing enzymes. This system of repression has similarities to that in *P. putida* (Mandelstam & Jacoby, 1965; Stevenson & Mandelstam, 1965) where benzoate, catechol and succinate cause repression. Our results show that the effects on enzyme synthesis have the expected consequences for substrate utilization by growing bacteria.

The results described for growth on mixtures of substrates including benzoate indicate that 'benzoate oxidase' (benzoate oxygenase + cyclohexadienecarboxylate dehydrogenase; Reiner, 1971), is not repressed by catechol, and there is only partial repression by succinate which cannot be quantified by this growth technique. This contrasts with control in *P. putida* where succinate and especially catechol repress the synthesis of benzoate oxidase (Mandelstam & Jacoby, 1965; Stevenson & Mandelstam, 1965; Higgins & Mandelstam, 1972*a*). It is not clear what role benzoate transport (Cook & Fewson, 1972*a*) plays in control.

No catabolite inhibition (McGinnis & Paigen, 1969) has been observed in this work, and enzyme inhibition by pathway intermediates appears to play no part in control, a finding made by Hegeman (1966*c*) in the mandelate pathway of *P. putida*. The inhibition of substrate metabolism by succinate in whole organisms, contrasted with the lack of effect observed when succinate was added to extracts of the mandelate enzymes, may indicate some type of control. This could be at the level of permeation or due to a balance of cofactors. Modulation of phenylglyoxylate carboxy-lyase by adenine nucleotides has been observed (Jamaluddin &

Fewson, 1973) but the physiological significance is uncertain; other enzymes might also be regulated in this way.

This paper is chiefly concerned with repression, but it is clear that a balance occurs between induction and repression. This balance seems common and is observed in the mandelate pathway of *P. putida* (Higgins & Mandelstam, 1972*a*), and in the glucose-citrate system (Ng & Dawes, 1973) and the amidase system (Clarke, 1970) of *P. aeruginosa*. The molecular mechanisms by which these systems operate are as yet largely unknown although they are being investigated.

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