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EVIDENCE FOR SPECIFIC TRANSPORT MECHANISMS FOR AROMATIC COMPOUNDS IN BACTERIUM N.C.I.B. 8250

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SUMMARY

A membrane filtration assay has been developed to measure pools of mandelate and benzoate in cells of bacterium N.C.I.B. 8250. Intracellular concentrations were calculated after correction for interstitial and filter-bound material. Cells grown on various non-inducing substrates showed different degrees of permeability to mandelate. In these cases the rate of entry of mandelate was very much less than the rate of mandelate utilization by induced cells. No permeability barrier to benzoate could be detected. In cells induced for the metabolism of benzoate and preloaded with salicylate, benzoate caused the exit of salicylate against a concentration gradient.

Higgins and Mandelstam[1,2] have recently presented several lines of evidence which implicate an inducible factor in the active transport of low concentrations of extracellular mandelate into *Pseudomonas putida*. We now report that under some conditions it is possible to demonstrate a permeability barrier against some aromatic compounds in bacterium N.C.I.B. 8250, a member of the *Acinetobacter–Moraxella* group of bacteria[3], and that presumably there must be inducible transport systems for these compounds. A preliminary account of part of this work has been published[4].

Bacterium N.C.I.B. 8250 and a mutant strain lacking L-mandelate dehydrogenase[5] (NF 1408) were maintained, grown and harvested as already described[6,7]. Cells were washed in 0.05 M KH_2PO_4–Na_2HPO_4 buffer (pH 7.0) at 4 °C, resuspended to 20 mg wet wt per ml in the same buffer and stored on ice. The amount of bacterial dry weight was determined turbidimetrically by reference to a calibration curve in which $A_{500\ nm}$, measured in 10-mm light-path cuvettes using a Unicam SP800 Spectrophotometer (Pye Unicam Instruments Ltd., Cambridge, U. K.) connected to a Servoscribe Chart Recorder (Smiths Industries Ltd., Wembley, Middlesex, U.K.), was plotted against dry weight of bacteria. Bacterial cell water was measured by a modification of the method of Winkler and Wilson[8] using [5-^{3}H]glucose as an indication of the space outside the cell membrane (A. M. Cook and C. A. Fewson, unpublished results). This gave a value of 1.65 μl cell water per

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mg of dry wt. Experiments with inulin gave a value of 3.09 μl cell water per mg of dry wt, but this included the space of the cell walls. Liquid scintillation fluid was prepared by mixing 1 l of toluene, 300 ml of ethanol and 5 g of 2,5-diphenyloxazole. Liquid scintillation spectrometry was done in Phillips Liquid Scintillation Analysers, each vial containing 13 ml of liquid scintillation fluid. Conventional methods were used for calculation of the absolute amounts of ^{3}H and ^{14}C present.

Pool sizes were determined by a modification of the membrane filtration technique[8,9,10]. A simple and compact filtration apparatus was constructed with Millipore XX1002500 assemblies (Millipore (U.K.) Ltd., Abbey Road, London, U.K.) individually connected to a manifold of gas taps in a vacuum line. To allow easy, reproducible streaking of the samples on filters, the Millipore funnels were replaced by short (1.2 cm) perspex funnels machined to the dimensions of the base of the original component. The assay mixtures (10 ml) contained in 50-ml erlenmeyer flasks were shaken through approximately 4.5 cm at 110 strokes/min in a water bath at 30 °C. Complete assay mixtures contained 500 μmoles of KH_2PO_4–Na_2HPO_4 buffer (pH 7.0), 50 mg wet wt of cells, 1 μmole of [5-^{3}H]glucose (25 Ci/mole, as a measure of interstitial material) and aromatic compounds labelled with ^{14}C in the carboxyl group as indicated in the legends to the appropriate figures. "Incomplete assay mixtures" (7.5 ml), which contained all components except cells and that portion of buffer in which those cells were suspended, and stock cell suspensions were preincubated separately for 5 min in 50-ml erlenmeyer flasks in the shaking water bath at 30 °C. Filter membranes (type 11307025, Sartorius Membranfilter GmbH, Göttingen, Germany) were mounted eccentrically, fractionally over the edge of the filter bases to allow rapid removal. They were wetted thoroughly with the appropriate wash fluid, the funnels were clamped in position and the membranes left under vacuum. Incomplete assay mixtures were sampled (2 times 20 μl) to estimate the radioactivity. Assays were initiated by the addition of stock cell suspension (2.5 ml). Samples (100 μl) were taken at intervals with Eppendorf pipettes (Eppendorf Marburg Mikropipet; Netheler and Hinz GmbH, Hamburg, Germany) and streaked on the filters. When the sample liquid had disappeared, the filter and cells were washed by the addition of 1 ml of solution of the same composition as the assay mixture but without cells or radioactivity. The wash solution was at 0 °C. The filter was removed into the scintillation fluid as soon as the wash fluid had disappeared. Each experiment was repeated without cells to find the radioactive "reagent blank" for material bound to the filter membrane.

D,L-[*carboxy*-^{14}C]Mandelic acid was supplied by Mallinckrodt Nuclear, St. Louis, Mo., U.S.A. [*carboxy*-^{14}C]Benzoic acid, [*carboxy*-^{14}C]salicylic acid, [5-^{3}H]glucose and *n*-[1,2-$^{3}H_2$]hexadecane (radioactive reference material) were obtained from Radiochemical Centre, Amersham, Bucks., U.K. [G-^{14}C]Toluene (radioactive reference material) was from Packard Instrument Co., Downers Grove, Ill., U.S.A., 2,5-diphenyloxazole (puriss) from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K and ethanol (fermented, absolute) from James Burrough Ltd., London, U.K. The sources of other reagents are listed elsewhere[6,7] or were from British Drug Houses, Ltd, Poole, Dorset, U.K., generally as AnalaR grade.

The reagent blanks for [*carboxy*-^{14}C]benzoate, D,L-[*carboxy*-^{14}C]mandelate and [5-^{3}H]glucose were equivalent to 0.14, 0.02 and 0.02 μl of the respective assay mixtures. Previous filtration of the radioactive solutions did not affect these blanks,

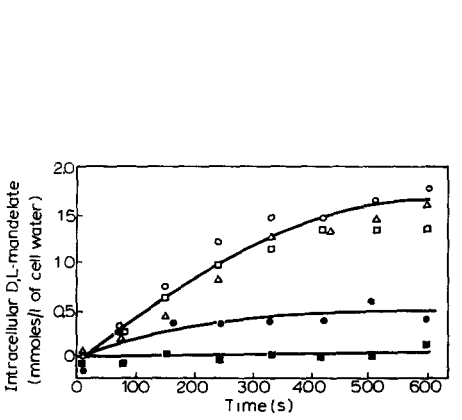
nor did latex beads, present to simulate a bacterial suspension. Interstitial fluid varied from 0 to 0.3 μl for 100-μl aliquots of assay mixtures containing different types of cells. The intracellular water space was about 0.14 μl per 100 μl sample. The use of [5-^{3}H]glucose as an extracellular marker for the measurement of both intracellular water space and the determination of interstitial material on the filters is based on the failure of this compound to penetrate the membrane of bacterium N.C.I.B. 8250 (A. M. Cook and C A Fewson, unpublished results).

The use of *carboxy*-^{14}C-labelled substrates simplified the identification of intracellular material since all the substrates were decarboxylated by the first or second metabolic enzymes There was no significant incorporation of ^{14}C into cell material. One wash with chilled assay mixture (but containing no ^{14}C or ^{3}H) appeared to leave the intracellular pools intact as a second wash had little effect on the recorded pool, while subsequent washes, especially if at 30 °C rather than 0 °C, decreased the pool. The presence of 5 % (w/v) NaCl in the wash did not have an appreciable effect on the apparent size of the pool and decreased the filtration rate The pools could be entirely removed by washing with water or 2 M $HClO_4$, each at 0 °C

Varying degrees of permeability to mandelate were observed in cell suspensions prepared from samples of bacterium N.C.I.B 8250 that had been grown without induction of the mandelate enzymes (Fig. 1). Cells harvested from benzoate–salts medium showed no permeation by mandelate in 10 min. There was slow entry into cells harvested from benzyl alcohol–salts medium The rate of entry into cells grown in nutrient broth was faster but was still less than 0.6 % of the rate of mandelate decarboxylation by cells that had been grown in mandelate–salts medium (84 nmoles/mg dry wt per min). The presence of benzoate or benzyl alcohol did not significantly affect the rate of entry into cells grown in nutrient broth. No pool of mandelate could be detected in cells grown on, and metabolising, mandelate. Presumably this was because the steady state pool size was low or because of the inevitable delay of 1 to 2 s between disappearance of reaction medium from the filter membrane and addition of wash Even if the intracellular concentration was equal to the external level as the reaction medium disappeared, the pool would be totally decarboxylated during this delay In the non-induced cells the intracellular concentration of mandelate was never found to exceed the extracellular concentration Mutant strain NF 1408 was grown in phenylglyoxylate–salts medium so that it lacked L-mandelate dehydrogenase but contained the other mandelate enzymes[5]. Mandelate entered these mutant cells at about 4 % of the maximum rate of mandelate decarboxylation by the wild-type strain The intracellular concentration attained the extracellular concentration within 2 min and subsequently maintained this concentration

In contrast to the results with mandelate, no permeability barrier to benzoate could be detected in cells in which benzoate oxidase had not been induced. Using cells grown in succinate–salts medium, the intracellular concentration of benzoate achieved, and occasionally slightly exceeded, the extracellular level by the time that the first sample could be taken for filtration (approx 10 s) This rapid equilibration occurred over the range 0.5–5.0 mM benzoate and was confirmed by experiments in which centrifugation rather than filtration was used to separate cells from the assay mixture. In further centrifugation experiments it was shown that no significant benzoate accumulation occurred even at extracellular concentrations as low as

4.6 μM. The presence of succinate had no effect on the equilibration. No pool of benzoate could be detected when using suspensions prepared from cells grown in benzoate–salts medium, even in the presence of 1 mM α,α-bipyridyl to inhibit benzoate oxidase[11]. However, there was some evidence for possible facilitated diffusion of benzoate. Cells in suspension, harvested from benzoate–salts medium, were penetrated by salicylate which attained and maintained the extracellular concentration. The addition of benzoate to these cells caused an exit of salicylate against a concentration gradient (Fig. 2a). Salicylate had little effect on the rate of benzoate decarboxylation and was not itself decarboxylated in the presence or absence of benzoate. Simultaneous addition of benzoate and salicylate caused an apparent overshoot in the salicylate concentration inside the cells (Fig. 2b) followed by exit of salicylate against a concentration gradient.



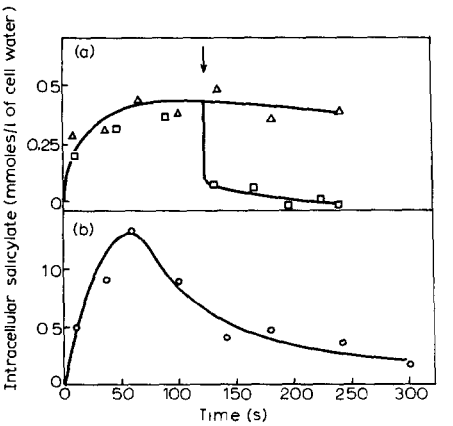


Fig 1 Entry of mandelate into cells of bacterium N C I B 8250 non-induced for the mandelate enzymes Assay mixtures (10 ml) contained 20 μmoles of D,L-[*carboxy*-^{14}C]mandelate (0 5 Ci/mole) Samples (100 μl) were taken at intervals for filtration as described in the text Suspensions were prepared from cells grown in nutrient broth and the uptake of mandelate was measured alone (○), or in the presence of 1 mM benzoate (△), or of 1 mM benzyl alcohol (□) Suspensions were also prepared from cells grown in benzyl alcohol–salts medium (●) or benzoate–salts medium (■) and in these cases mandelate was the only aromatic compound present in the assay mixture Each point is the mean of duplicate determinations

Fig 2 Effect of benzoate on the entry of salicylate into cells of bacterium N C I B 8250 harvested from benzoate–salts medium Uptake of salicylate was measured by taking samples (100 μl) for filtration as described in the text The aromatic compounds present in the assay mixtures (10 ml) were (a) 5 μmoles of [*carboxy*-^{14}C]salicylate (2 Ci/mole) from zero time (△) or 5 μmoles of [*carboxy*-^{14}C]salicylate (2 Ci/mole) from zero time and 50 μmoles of benzoate (0 5 ml) added at the time indicated by the arrow (□); (b) 10 μmoles of [*carboxy*-^{14}C]salicylate (1 Ci/mole) and 10 μmoles of benzoate from zero time (○) Each point is the mean of duplicate determinations

The existence of a permeability barrier to mandelate in non-induced cells of bacterium N.C.I.B. 8250 implies the existence of an inducible system to allow an adequate rate of passage of mandelate to support the observed rates of mandelate metabolism by intact induced cells. The permeability barrier was most pronounced in cells grown in benzoate–salts medium, these are conditions under which the mandelate enzymes are known to be repressed[12]. The failure of Hegeman[13] and Higgins and Mandelstam[2] to observe a permeability barrier to mandelate in uninduced

cells of *P. putida* may have been due either to a difference between the two organisms or to the fact that their cells possessed low levels of the transport factor. The transport factor could be present at considerably less than maximum activity and still give equilibration within the time necessary to take the first sample for filtration, let alone the longer time necessary when the centrifugation method is used. We have never observed accumulation of mandelate above the external concentration; this is in agreement with the results of Higgins and Mandelstam[2] who found accumulation in *P. putida* only with external concentrations lower than those we used in this work. Both Hegeman[13] and Higgins and Mandelstam[2] used strains of *P. putida* lacking L-mandelate dehydrogenase. In the present work, mutant strain NF 1408 lacking L-mandelate dehydrogenase showed a relatively low rate of mandelate permeation, even when induced for the rest of the mandelate enzymes. The reason for this is not clear but the possibility of a fairly close association between mandelate transport and oxidation cannot be ruled out, especially as L-mandelate dehydrogenase appears to be associated with the cell membrane[6]. In any case L-mandelate dehydrogenase may well aid mandelate transport in the wild type cell by lowering its intracellular concentration.

The exit of salicylate against a concentration gradient, in response to the addition of benzoate (Fig. 2a), closely resembles the exit counterflow studies of Wong and Wilson[14] Experiments in which benzoate and salicylate were added together (Fig. 2b) were not of the type normally designed to demonstrate counterflow, but the results indicated that entry is not by passive diffusion. More work will have to be done to reveal the full significance of these observations, but it appears probable that benzoate transport is by a system of facilitated diffusion.

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