Carbon-13 Nuclear Magnetic Resonance Studies of Mandelate Metabolism in Whole Bacterial Cells and in Isolated, in Vivo Cross-Linked Enzyme Complexes†

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ABSTRACT: Physical association among cellular enzymes in the bacterium *Pseudomonas putida* A.3.12 (ATCC 12633) was investigated by treating whole cells with the bifunctional cross-linking agent, dimethyl suberimidate (DMSI), followed by isolation of an enzymatically active high molecular weight complex. DL-Mandelic acid can serve as sole source of carbon and energy for growth of *P. putida*. Its use is initiated by the concerted action of a suite of five inducible enzymes (*the mandelate group*) that act to convert D-mandelate to benzoate. L-Mandelate dehydrogenase, the second enzyme of this group, is membrane bound. The other four enzymes as well as those enzymes functional in the further conversion of benzoate are found in the cytosol following conventional extraction procedures. The crude extract obtained by sonic disruption of DMSI-treated cells and a control extract from cells not treated with DMSI were subjected to gel permeation chromatography. Two peaks of activity were observed in the extract of cells treated prior to extraction, but only one set was observed in the control extract. The extra peak of activities from treated cells, presumably a cross-linked complex, appeared in the excluded fractions of the column effluent, but only L-mandelate dehydrogenase appeared in these fractions when the control extract was fractionated. Both kinds of extracts exhibited larger peaks for pathway activities in fractions predictable from the known molecular weights of the enzymes. The in vivo complex of mandelate-metabolizing enzymes was studied with ^{13}C NMR spectroscopy. Reference experiments to determine which metabolites would be observable by NMR were carried out with whole cells of *P. putida* in the presence of 90% enriched DL-[α-^{13}C]mandelic acid. Following periods of oxygenation, the signals of the labeled α carbons of benzaldehyde, benzoic acid, and benzyl alcohol (a previously unknown metabolite in mandelate metabolism) appeared and increased. [^{13}C]Bicarbonate, a product of the decarboxylation of benzoate, was also observed. Oxygenation of the isolated in vivo cross-linked enzyme complex, as with whole cells, resulted in the conversion of DL-[α-^{13}C]mandelate to [α-^{13}C]benzoate. Other metabolites observed included [α-^{13}C]benzoylformate, [α-^{13}C]benzaldehyde, and [α-^{13}C]benzoate. ^{13}C NMR spectra of high molecular weight material isolated from untreated cells showed ~50% conversion of DL-[α-^{13}C]mandelate, and [α-^{13}C]benzoylformate was the only product observed. This was due to the presence of membrane-bound L-mandelate dehydrogenase in high molecular weight material from extracts of both untreated and DMSI-treated cells. From these observations it is clear that all five enzymes of the *mandelate group* are present in the isolated complex resulting from in vivo cross-linking with DMSI. From the data at hand, however, it cannot be determined whether these enzymes are complexed with each other or are individually cross-linked to cellular components of high molecular weight.

Biochemical reactions follow an orderly sequence of chemical events. The enzymes involved in these sequential reactions may by organized or clustered in a particular manner to facilitate the biosynthesis or the dissimilation of compounds essential for cellular vitality. A number of multienzyme complexes have already been isolated. But the question that still remains to be answered unequivocally is whether the enzymes of a given metabolic pathway can associate by using relatively weak interactions to form a catalytically efficient unit. Herein, we examine an approach to explore this interesting question in the pathway dedicated to mandelic acid metabolism in the bacterial strain, *Pseudomonas putida*.

P. putida biotype A, strain 90 [ATCC 12633; also designated as *P. putida* (or *fluorescens*) A.3.12 and as PRS-1 (Chakrabarty et al., 1968; Chakrabarty & Gunsalus, 1969)], is capable of utilizing DL-mandelic acid as its sole source of carbon and energy by the strictly inducible pathways shown in Figure 1. The metabolic sequences had been proposed by Stanier and co-workers (Stanier, 1947, 1948, 1950; Sleeper & Stanier, 1950; Sleeper et al., 1950), and the enzymes involved in the conversion of mandelate to benzoate were isolated by Gunsalus and Stanier (Gunsalus et al., 1953a,b; Stanier et al., 1953). These same enzymes also accept DL-4-hydroxymandelate as a substrate, producing 4-hydroxybenzoic acid. This latter product is converted to succinate and acetyl-CoA by the protocatechuate branch of the β-ketoadipate pathway which converges with the catechol branch at the level of β-ketoadipate enol lactone.

Results of a series of studies investigating the regulation of enzyme synthesis and the transduction of genes designating this synthesis lend strength to an argument for the intracellular organization of the enzymes involved in mandelate metabolism. Synthesis of the five enzymes of the mandelate group in the wild type and in blocked mutants of *P. putida* was found to be coordinately induced by the first three compounds of the

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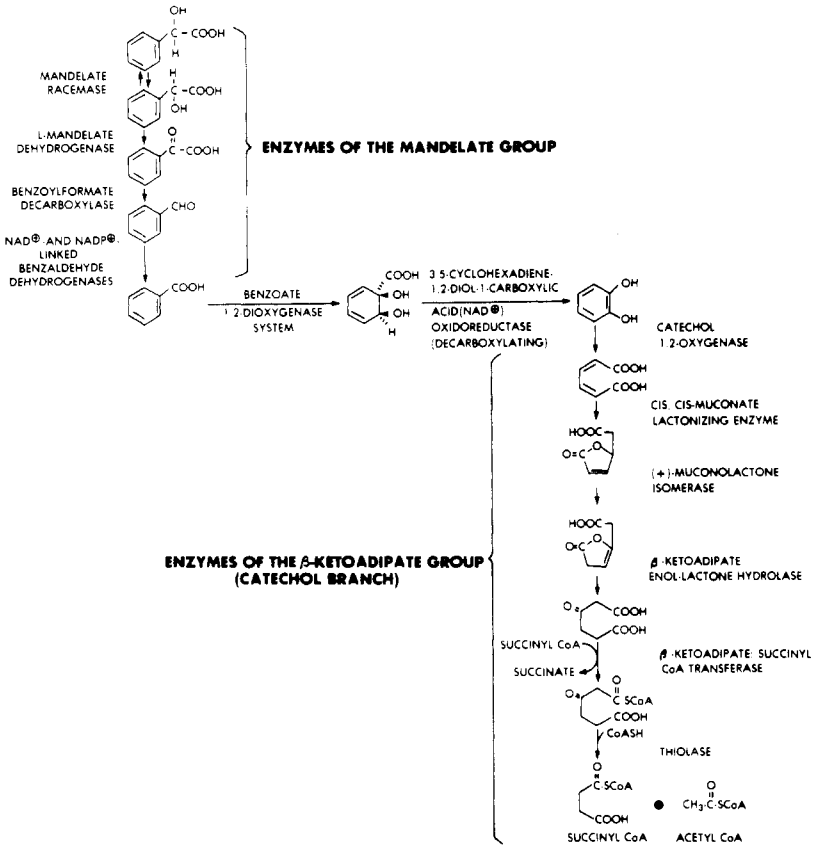


FIGURE 1: The pathway for the dissimilation of DL-mandelic acid in *P. putida*.

pathway (Hegeman, 1966a,b), and apparent operator constitutive mutants, capable of constitutive, coordinate synthesis of the same five enzymes in the absence of an inducer, were isolated (Hegeman, 1966c).

Genetic experiments by Gunsalus and co-workers, using the host-range mutant pf16h2, demonstrated the intra- and interstrain transduction of a large segment of DNA[1] containing at least four genes functional in the conversion of mandelate to benzoate (Chakrabarty et al., 1968) and the transfer of the structural gene for the mandelate group plus a regulatory locus between the constitutive mutant isolated by Hegeman (1966c) and a camphor-degrading, mandelate-deleted strain of *P. putida* (Chakrabarty & Gunsalus, 1969). Thus the genes specifying the synthesis of the mandelate enzymes are closely linked, if not contiguous, on the bacterial chromosome and therefore constitute an operon.

Moreover, Ornston and co-workers (Meagher et al., 1972) have demonstrated that the enzymes of the β-ketoadipate pathway (Figure 1) form an aggregate in a crude extract that does not become resolved on a DEAE-cellulose column until treated with 0.4 M ammonium sulfate.

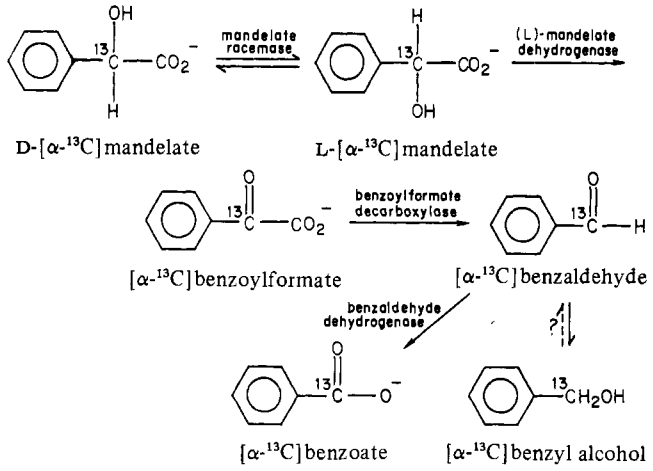
Bifunctional bis(imido esters), which are readily available and reactive under mild conditions, have been used extensively as cross-linking reagents in studies of the protein–protein interactions in ribosomes (Kurland, 1974) and the subunit structure of oligomeric proteins (Davies & Stark, 1970). Therefore, it was anticipated that one of these bis(imido esters) would provide sufficient stabilization of a putative complex, permitting its isolation and characterization. As a probe of in vivo macromolecular structure, we have therefore employed imido esters as relatively gentle protein cross-linking reagents.

In the course of this project we have turned to the use of ^{13}C NMR spectroscopy for the partial characterization of our isolated, cross-linked enzyme complex. The effectiveness of ^{13}C NMR as a probe of the in vivo metabolism of a specifically labeled substrate was first demonstrated by Eakin et al. (1972) in yeast cells. Shulman and co-workers have subsequently used both ^{31}P and ^{13}C NMR to study glycolysis and bioenergetics in a variety of whole-cell systems such as *Escherichia coli*, yeast cells, rat liver cells, and purified rat liver mitochondria [reviewed in Shulman et al. (1979)]. Scott and co-workers were able to observe directly the biosynthesis of porphyrinogen in two bacterial strains from ^{13}C-enriched substrates by using ^{13}C NMR (Scott et al., 1979). Further NMR studies demonstrated the presence and elucidated the structure of a transient intermediate in the formation of uroporphyrinogens I and III (Burton et al., 1979a,b).

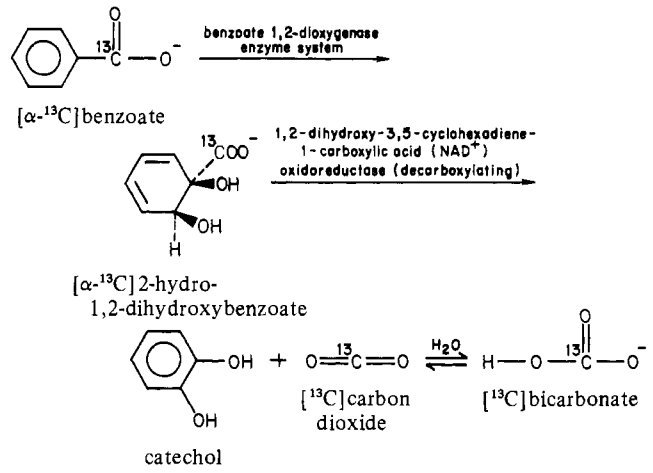
^{13}C NMR spectroscopy of the crude complex, therefore, in the presence of an appropriately enriched mandelic acid, seemed to us to be a highly promising technique for providing conclusive evidence for the presence of any or all of the enzymes functional in the metabolism of the primary substrate. The metabolism of DL-mandelic acid, 90% enriched with ^{13}C on the α carbon, could be followed through all the intermediate steps until the label was lost as [^{13}C]carbon dioxide in the conversion of benzoate to catechol as is shown in Schemes I and II. The sequence of reactions would be limited to the mandelate group of enzymes [plus the benzoate oxidase system if benzoate were metabolized (Scheme II)], which could greatly simplify the number of intermediates to be observed in the NMR spectrum. In order to determine which metabolites would be observable by NMR and also to optimize NMR parameters, we carried out initial ^{13}C NMR experi-

[1] Abbreviations used: DNA, deoxyribonucleic acid; DMSI, dimethyl suberimidate dihydrochloride; TNM buffer, 0.05 M triethanolamine hydrochloride (pH 8.0), 0.1 M NaCl, and 0.01 M $MgCl_2$ buffer; NOE, nuclear Overhauser enhancement; Me_4Si, tetramethylsilane; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; $NADP^+$, NAD phosphate; FMN, flavin mononucleotide; DEAE, diethylaminoethyl.

Scheme I



Scheme II



ments with whole cells from *P. putida*.

Materials and Methods

Materials were obtained from the following sources: all commonly used inorganic salts and organic solvents were analytical grade and obtained from either Mallinckrodt or J. T. Baker Chemical Co.; yeast extract and agar were both products of Difco Laboratories; Ultrogel AcA22 was from LKB; dimethyl suberimidate dihydrochloride (DMSI)[1] was from Pierce; NAD^+, FMN, bovine serum albumin, bovine γ-globulin, glutamine, ADP, potassium arsenate, γ-glutamylhydroxamate, and brilliant Coomassie Blue G250 were all from Sigma Chemical Co.; DL-mandelic acid, D(−)-mandelic acid, L(+)-mandelic acid, benzoylformic acid, benzaldehyde, benzoic acid, benzyl alcohol, imidazole, and deuterium oxide, 99.8 atom %, were purchased from Aldrich Chemical Co.; thiamine pyrophosphate chloride was from Boehringer Mannheim; DL-[α-^{13}C]mandelic acid (90% enriched) was synthesized by Dr. E. T. Maggio (Maggio et al., 1975); *cis,cis*-muconic acid was synthesized by the method of Elvidge et al. (1950). L-Mandelate dehydrogenase containing vesicles were prepared as described by Hegeman et al. (1970). Water used for all solutions was distilled and deionized. Spectrophotometric assays were performed on a Gilford 2220A spectrophotometer.

Conditions of Cultivation. Stock cultures of *P. putida* A.3.12 were maintained on slants of solid medium containing 1% (w/v) yeast extract and 2% (w/v) agar in 0.01 M NaK-HPO_4 buffer (pH 6.8). The cultures were transferred at monthly intervals, grown at 30 °C for 8–12 h, and stored at 4 °C. Cells of *P. putida* were grown in 500 mL of mineral medium (Hegeman, 1966a) containing DL-mandelate as the ammonium salt at a final concentration of 10 mM. The cultures were incubated with vigorous shaking at 30 °C. Growth was measured turbidimetrically in a Klett–Summerson colorimeter with a No. 54 filter. The cells were harvested in the late-log phase by centrifugation at 10000*g* for 10 min. The sedimented cells were suspended in 0.05 M triethanolamine hydrochloride buffer (pH 8.0), containing 0.1 M NaCl and 10 mM $MgCl_2$ (hereafter designated as TNM buffer), and centrifugation was repeated. Two pellets of packed cells, each of 0.7–0.9 g wet weight, were obtained and stored overnight at 4 °C or at −20 °C for longer periods of storage.

Dimethyl Suberimidate Treatment of Whole Cells. Freshly harvested whole cells (0.7–0.9 g wet weight) were suspended in 3 mL of TNM buffer, and 3.0 mL of the cell suspension was transferred to a small Erlenmeyer flask. Immediately prior to use, the dimethyl suberimidate solution, 0.1 M, was prepared in TNM buffer, and the pH was adjusted to pH 8.0 with NaOH. The solution was added to the whole cells to a final concentration of 6 mM. Control cells were treated in the same manner except that TNM buffer was added instead of DMSI. The mixture was incubated at 25 °C with gentle swirling for 1 h. The reaction was terminated by dilution of the suspension with 30 mL of cold TNM buffer, followed by centrifugation at 10000*g* for 10 min. The packed cells were resuspended in TNM buffer, and centrifugation was repeated. Crude extracts from control and DMSI-treated cells were prepared as described below.

Preparation of Crude Extracts. The packed cells of untreated and DMSI-treated whole cells were each suspended in 3.5–4.0 mL of TNM buffer (pH 8.0) and disrupted by sonic oscillation for 1 min in a salt–ice bath at 180 W using a probe-type oscillator (Branson Sonifier Cell Disruptor Model W350). The cellular debris was removed by centrifugation at 10000*g* for 10 min, and the supernatant (crude extract) was kept at 4 °C for further experiments.

Isolation of High Molecular Weight Material by Ultracentrifugation. Crude extracts from freshly prepared control and DMSI-treated whole cells were diluted to 10 mL with TNM buffer and centrifuged at 100000*g* for 1 h at 4 °C. The high molecular weight material was suspended by homogenization in TNM buffer, diluted to 10 mL, and centrifuged again at 100000*g* for 1 h. The resuspension–centrifugation step was repeated twice more. After the third wash, the material was suspended by homogenization in TNM buffer to a final volume of 2 mL and assayed without further dilution for enzyme activities described below.

Gel Permeation Chromatography. The crude extract (20 mg of protein) or the suspension of high molecular weight material (10 mg of protein) was layered on Ultrogel AcA 22 (2% acrylamide, 2% agarose; 1.6 × 85–90 cm) through an adaptor and eluted with TNM buffer containing 0.02% NaN_3 (used as a bacteriostatic agent) at a flow rate of 1 drop/10–15 s. Each fraction contained 40 drops (~1 mL), and a total of 200 fractions was collected. Each fraction was assayed for absorbances at 260 and 280 nm, for protein concentration, and for the four enzyme activities described below.

Dimethyl Suberimidate Treatment of Crude Extract. The crude extract of freshly harvested, untreated cells was prepared as described above. To 3.0 mL of the extract was added freshly prepared DMSI to a final concentration of 6 mM. A control sample was prepared in the same manner except that TNM buffer was added instead of DMSI. The suspension was gently swirled at 25 °C for 1 h. The reaction was terminated by

dilution of the sample with cold TNM buffer to a final volume of 10 mL, followed by centrifugation at 100000*g* for 1 h at 4 °C. The high molecular weight material was suspended by homogenization in TNM buffer and centrifuged at 100000*g* for 1 h. The suspension–centrifugation step was repeated twice more. The washed material was suspended by homogenization to a final volume of 2.0 mL with TNM buffer and assayed for enzyme activities without further dilution.

Enzyme Assays and Other Assays. *cis,cis*-Muconate lactonizing enzyme [EC 5.5.1.1, (+)-4-carboxymethyl-4-hydroxyisocrotonolactone lyase (decyclizing)] activity was measured as described by Hegeman (1966a). L-Mandelate dehydrogenase [no EC number, L(+)-mandelate:(acceptor) oxidoreductase] and mandelate racemase (EC 5.1.2.2, mandelate racemase) assays employed modifications of the methods of Hegeman (1966a) described by Halpin (1980). Benzoylformate decarboxylase (EC 4.1.1.7, benzoylformate carboxy-lyase) activity was measured by a modification of the method of Jamaluddin et al. (1970) described by Halpin (1980). Glutamine synthetase (EC 6.3.1.2, L-glutamate:ammonia ligase (ADP)) activity was measured as described by Shapiro & Stadtman (1970). Protein was measured by either the biuret method (Gornall et al., 1949) using bovine serum albumin as the standard or the Coomassie Blue dye-binding procedure of Bradford (1976) using bovine γ-globulin as the standard.

Preparation of Whole Cells for ^{13}C NMR Studies. Cells were harvested in the mid-log phase when turbidity measured 100 Klett units. The harvested cells were washed 3 times with 0.1 M $NaKHPO_4$ buffer (pH 6.8) and 10 mM $MgCl_2$ with centrifugation at 10000*g* for 10 min. From a 500-mL culture, one pellet of packed cells, 0.5–0.7 g wet weight, was obtained and stored at 4 °C. The NMR experiments were carried out within 24 h of cell harvesting. The washed cells, both untreated and DMSI-treated, were suspended to a final volume of 2.5 mL with 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, and 20% D_2O and placed in a 12-mm NMR tube. DL-[α-^{13}C]Mandelate, sodium salt (pH 7), was added to a final concentration of 50 or 12.5 mM as indicated in the text. The tube was fitted with a vortex plug.

Preparation of High Molecular Weight Material from Untreated and DMSI-Treated Cells for ^{13}C NMR Studies. Stock solutions of NAD^+, 0.15 M, and thiamine pyrophosphate, 0.1 M, were prepared prior to use in 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, and 20% D_2O solution, and the pH was adjusted to 6.8, if necessary. A stock solution of FMN, 0.1 M, which was not completely soluble in the phosphate buffer, was prepared prior to use in 20% D_2O. A solution containing metal ions, anions, and other small molecules that might be necessary for the functioning of the complex was prepared in the following manner. A pellet of packed cells (grown to mid-log phase) was suspended in 4 mL of 0.1 M $NaKHPO_4$ buffer (pH 6.8) and 10 mM $MgCl_2$. The cells were disrupted by sonic oscillation for 1 min, and the cellular debris was removed by centrifugation at 10000*g* for 10 min. The crude extract (supernatant) was dialyzed against 1 L of distilled, deionized water in the cold for 24 h. The extract was discarded, and the dialyzate was evaporated to dryness on a rotary evaporator with a bath temperature of no more than 60 °C. The residue was dissolved in 1 mL of 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, and 20% D_2O.

The high molecular weight material was isolated at 100000*g* and washed as described above, except that 0.1 M $NaKHPO_4$ buffer (pH 6.8) and 10 mM $MgCl_2$ were used. The packed material was suspended by homogenization in 1 mL of 0.15 M NAD^+. To this suspension were added 0.25 mL each of 0.1 M thiamine pyrophosphate and 0.1 M FMN and 0.5 mL of dialyzate solution. The total volume was made to 2.5 mL with 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, and 20% D_2O solution. The final concentrations of cofactors were 60 mM NAD^+, 10 mM FMN, and 10 mM thiamine pyrophosphate. DL-[α-^{13}C]Mandelate was added to the suspension to a final concentration of 50 mM. The sample was placed in a 12-mm NMR tube fitted with a vortex plug.

Oxygenation of Samples. After each NMR spectrum was obtained, the whole cells or the suspensions of high molecular weight material were removed from the NMR tube, placed in a 10-mL round-bottom flask containing a small stirring bar, and allowed to come to room temperature. A rubber septum equipped with an oxygen inlet, an air vent, and a needle to which a balloon had been attached was securely wired to the flask. The sample was stirred vigorously but without foaming. Air was flushed from the system with oxygen for 10 s, and the air vent was closed. The balloon was allowed to fill with oxygen, and the system remained under oxygen tension for the times indicated in the text. The timing period began with the initial flow of oxygen. The sample was transferred to the NMR tube, cooled to 5 °C, and sealed with the vortex plug.

^{13}C T_1 (Spin–Lattice) Relaxation Time Determination of the α-Keto Carbon of Benzoylformate. In order to provide optimum conditions for spectral observation of the labeled carbons of all the products of mandelate metabolism, it was necessary to determine the T_1 value of the carbon with the longest relaxation time. Since the relaxation times of the α carbon of mandelic acid and the aldehyde carbon of benzaldehyde were facilitated predominately by ^1H–^{13}C dipole–dipole relaxation (James, 1975) and the peak of the carboxylate carbon of benzoate was readily observable under rapid-pulse conditions, it appeared that the α-keto carbon of benzoylformate would require the longest time for relaxation.

Benzoylformate, 1 M (pH 6.0), was prepared in D_2O, poured through a small column containing Chelex-100 to remove any contaminating paramagnetic metal ions, and oxygenated for ~15 min. This latter step was included since the biological samples under investigation were to be oxygenated. The T_1 value was measured by progressive saturation with homospoil recovery (James, 1975) using the following sequence: 90° pulse, homospoil sequence, delay time τ, 90° pulse, homospoil, where τ = 20, 60, 90, 200, 300, and 500 s.

The peak heights for the α-keto carbon of benzoylformate at each time were measured. These values plus the τ values in seconds were used to calculate the relaxation time from the equation

$$M_\tau = M_0(1 - e^{-\tau/T_1})$$

which is integrated to

$$\ln (M_\tau - M_0) = \ln M_0 - \tau/T_1$$

where M_τ is the magnetization along the z axis (or the peak height) at time τ and M_0 is the magnetization along the z axis at $\tau = \infty$ (or $5T_1$). A plot of $\ln (M_\tau - M_0)$ vs. τ gives a slope of $1/T_1$. The T_1 value obtained for the α-keto carbon of benzoylformate (in the presence of oxygen) was calculated to be 56.3 s.

^{13}C NMR Spectra. ^{13}C NMR spectra were obtained at a frequency of 25.158 MHz with a Varian XL-100 spectrometer, interfaced with a Nicolet Instrument Corporation Model NIC-80 data processor and modified with a Nicolet Multi Observe Nuclei Accessory (MONA). Deuterium, 20% in the solvent, was used as the internal lock signal. Free induction decays with a 45° pulse angle (12-μs radio frequency pulse)

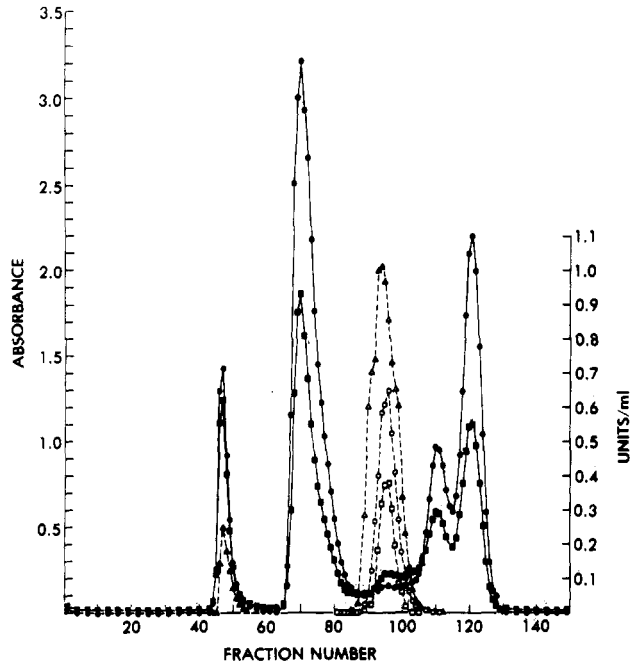


FIGURE 2: Gel permeation chromatography of crude extract (20 mg of protein) from untreated cells was applied to the Ultrogel AcA22 column using 0.05 M triethanolamine hydrochloride buffer (pH 8.0), 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.02% NaN_3 as eluant. Elution conditions are described under Materials and Methods. Each fraction was assayed for absorbances at 260 nm (●) and 280 nm (■), for protein concentration (not shown), and for the enzyme activities of benzoylformate decarboxylase (□), *cis,cis*-muconate lactonizing enzyme (○), mandelate racemase (△), and L-mandelate dehydrogenase (▲). In all cases dashed lines represent enzymic activities.

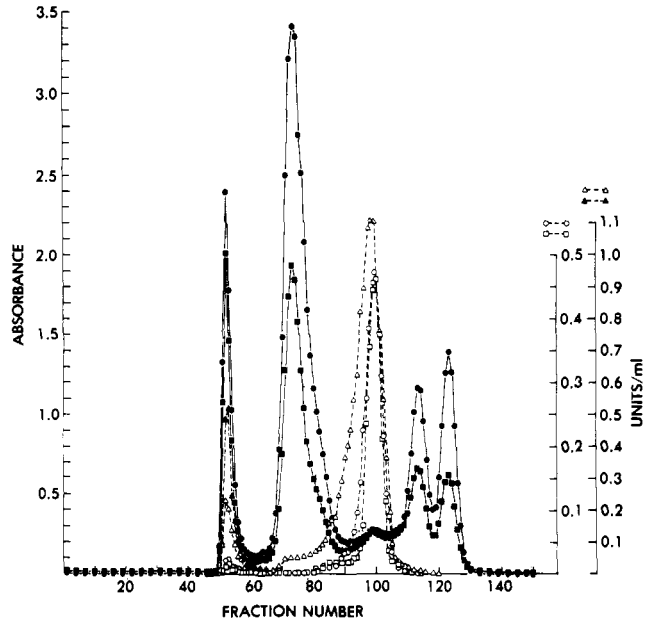


FIGURE 3: Gel permeation chromatography of crude extract from DMSI-treated cells on Ultrogel AcA22. The crude extract (20 mg of protein) and eluted fractions were treated as described in Figure 2.

and a 20.68-s time between pulses (giving equilibrium intensities) were accumulated as 8K data points. A total of 200 scans was accumulated per spectrum in a 1-h period. Broad-band proton-decoupling was employed for the spectra indicated, but the decoupling power was gated for obtaining spectra without nuclear Overhauser enhancement (NOE). All chemical shifts were measured with respect to internal dioxane, but the reported chemical shifts are relative to tetramethylsilane (Me_4Si). The dioxane carbon chemical shift in H_2O is 67.4 ppm downfield from Me_4Si (Johnson & Jankowski, 1972). The sample temperature was maintained at 5 °C during data acquisition.

Results

Chromatography of Crude Extract from Untreated Whole Cells. The elution pattern of the crude extract from untreated bacterial cells is shown in Figure 2. The solid lines denote the absorbance values at 260 and 280 nm, while the dashed lines correspond to the four enzyme activities measured. Of the four major absorbance peaks, three had $A_{260/280}$ ratios in the range between 1.5:1 and 2:1. Presumably, these peaks correspond to high molecular weight (second peak) and low molecular weight (third and fourth peaks) nucleic acids. The $A_{260/280}$ ratio of the first peak, which appeared at the void volume, was very nearly 1:1, and the fractions in this region had a slightly turbid, opalescent appearance. This material probably contained ribosomes and membrane vesicles along with other excluded materials of high molecular weight. In the region between the second and third absorbance peaks a small peak was observed with an $A_{260/280}$ ratio of ~0.7, indicating the presence of proteins. Protein assays of each fraction (results not plotted) indicated high protein content in the fractions of the first peak (probably due to ribosomal and membrane-bound proteins), a smaller amount of protein in the fractions of the second peak, and a high, broad band of protein in the fractions of the region between the second and third peaks. The protein concentration rapidly fell to 0 in the beginning region of the third peak. It was in this region of high protein concentration that the activities of benzoylformate decarboxylase, *cis,cis*-muconate lactonizing enzyme, and mandelate racemase—all enzymes normally found in the cytosol—were observed. The activity of L-mandelate dehydrogenase, a membrane-bound enzyme, appeared solely in the fractions of the exclusion volume.

Chromatography of Crude Extract from Dimethyl Suberimidate Treated Whole Cells. The elution pattern of the crude extract from DMSI-treated whole cells is shown in Figure 3. The overall absorption pattern and the location of the un-cross-linked enzymes were nearly identical with those observed in Figure 2. The activity of *cis,cis*-muconate lactonizing enzyme, however, appeared to be inhibited to some degree by the DMSI treatment. The major difference between the elution pattern of the DMSI-treated sample and that of the control was the appearance in the exclusion volume of low levels of activities corresponding to benzoylformate decarboxylase, *cis,cis*-muconate lactonizing enzyme, and mandelate racemase. There was also a low plateau of mandelate racemase activity through the fractions of the second peak, which was not observed in the control fractions. The activities of the other two enzymes were not detected in this region.

Isolation of High Molecular Weight Multienzyme Complex by Ultracentrifugation. The high molecular weight material isolated by centrifugation at 100000*g* for 1 h was washed 3 times by resuspension in buffer to remove extraneous, loosely bound enzymes. The suspension of the washed material was assayed for enzyme activities, and the results from untreated and DMSI-treated samples are summarized in Table I. Barely detectible levels of the soluble enzymes were observed in the control, whereas a 9- to 25-fold increase in enzyme activities was observed in the DMSI-treated sample. The difference in L-mandelate dehydrogenase activity in the untreated and DMSI-treated materials showed only a small decrease following DMSI treatment.

Table I: Specific Activities of Enzymes in High Molecular Weight Material from Untreated and DMSI-Treated Whole Cells[a]

enzymes	specific activity [μmol·min^{-1}·(mg of protein)$^{-1}$] untreated	DMSI-treated
benzoylformate decarboxylase	0.002 ± 0.0002	0.018 ± 0.0006
cis,cis-muconate lactonizing enzyme	0.002 ± 0.002	0.023 ± 0.006
mandelate racemase	0.008 ± 0.0008	0.178 ± 0.018
L-mandelate dehydrogenase	0.746 ± 0.032	0.629 ± 0.045
glutamine synthetase	0.000	0.032 ± 0.004

[a] Specific activity values are the mean ± standard deviation for four to six measurements.

Dimethyl Suberimidate Treatment of Crude Extract. The crude extract, rather than intact bacterial cells, was subjected to DMSI treatment followed by isolation of material sedimenting at 100000*g*. In contrast to the case when whole cells were treated with DMSI, the specific activities of the four enzymes in this material were not increased above the control levels shown in Table I.

Chromatography of High Molecular Weight Suspension Isolated from Dimethyl Suberimidate Treated Whole Cells. The elution pattern of the high molecular weight material from DMSI-treated cells was similar to that of Figure 3 except that the peaks corresponding to the un-cross-linked enzymes and low molecular weight nucleic acids were not observed. The $A_{260/280}$ ratio values were essentially the same as those observed for the first two peaks of eluted crude extracts. The protein concentration of the fractions, as was observed for the crude extracts, was much higher in the void volume peak than in the second peak. Benzoylformate decarboxylase activity was not detected in the exclusion volume fraction. Only *cis,cis*-muconate lactonizing enzyme and mandelate racemase, along with L-mandelate dehydrogenase, were observed in the high molecular weight protein peak. As in Figure 3, low mandelate racemase activity appeared in the second peak, but the activity tapered off to an undetectible level.

Metabolism of DL-[2-^{13}C]*Mandelate by Cells of P. putida.* ^{13}C NMR spectra of whole cells of *P. putida* initially containing 50 mM DL-[α-^{13}C]mandelate are shown in Figure 4. The upper spectrum, obtained prior to oxygenation, contained a single proton-decoupled ^{13}C signal for the α carbon of DL-mandelate at 75.8 ppm. In the lower spectrum following 30 min of oxygenation a number of signals were observed corresponding to the α carbons of several metabolites. The signal for the α carbon of mandelate was reduced by more than 50%. After longer oxygenation periods this peak disappeared. The other signals were due to the aldehydic carbon of benzaldehyde at 198.5 ppm, the carboxylate carbon of benzoate at 176.6 ppm, and the α carbon of benzyl alcohol at 64.8 ppm.

Identification of each new peak was made by comparison of the observed chemical shift values with those of authentic samples of each metabolite. Peak assignments were then confirmed by obtaining a proton-coupled spectrum of the oxygenated cell suspension. As is shown in Figure 5, the benzaldehyde peak was a doublet; the benzoate peak remained a sharp singlet; the mandelate peak became a doublet; the benzyl alcohol peak was split into a triplet. When the coupled spectrum was obtained, the decoupling power was gated so as to enhance the NOE effect on the methine and methylene resonances. Under these conditions, a new peak, identified as bicarbonate, appeared at 161.2 ppm.

A time-lapse plot of ^{13}C NMR spectra, showing the metabolism of DL-[α-^{13}C]mandelate as a function of oxygenation time, is shown in Figure 6. At t = 0 only the single peak for mandelate was observed. Already at t = 2 min, small peaks for benzyl alcohol and benzoate appeared. With increasing periods of oxygenation the mandelate peak decreased in intensity by more than 50%, and the intensities of the benzyl alcohol and benzoate peaks increased. A small peak corresponding to benzaldehyde was observed at t = 30 min.

Chemical oxidation of mandelate and/or its metabolites was ruled out by obtaining a ^{13}C NMR spectrum of DL-mandelate and of benzaldehyde in $NaKHPO_4$ buffer (pH 6.8) following 1 h of oxygenation. In each case only a single peak of the appropriate α carbon was observed in each spectrum with no peak seen for benzoate.

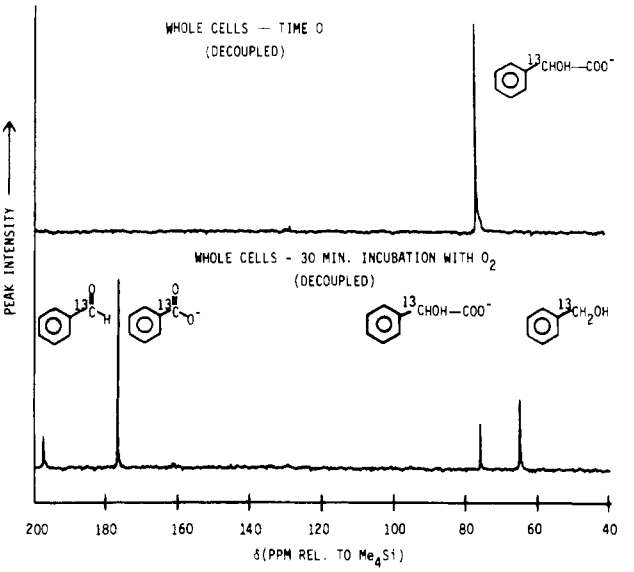


FIGURE 4: Proton-decoupled, ^{13}C NMR spectra (25.2 MHz) of *P. putida* cells prior to (upper) and following aeration with oxygen (lower). The cells were suspended in 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, and 20% D_2O. DL-[α-^{13}C]Mandelic acid, sodium salt (pH 7.0), was added to a final concentration of 50 mM at time 0. The cells were oxygenated at 25 °C as described under Materials and Methods. The time given in the lower spectrum indicates the oxygenation period. Each spectrum represents a total of 200 free induction decays accumulated in 1 h at 5 °C.

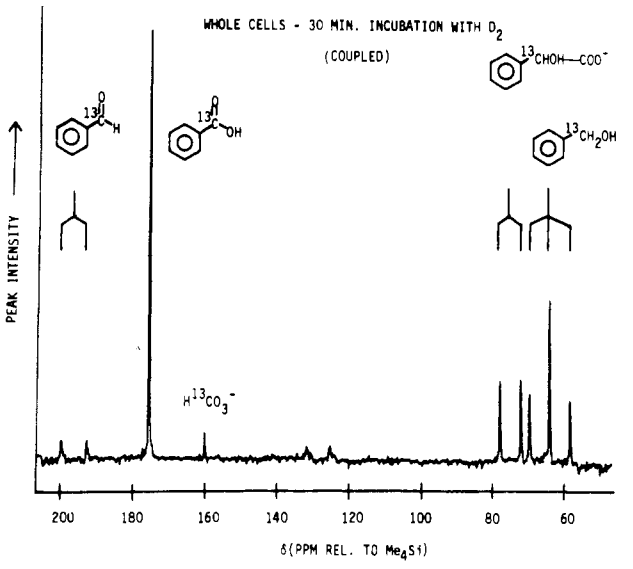


FIGURE 5: Coupled ^{13}C NMR (25.2 MHz) spectrum of *P. putida* cells following 30 min of oxygenation. The sample and other details are the same as described in Figure 4. The decoupling power was gated to provide NOE enhancement methine and methylene resonances.

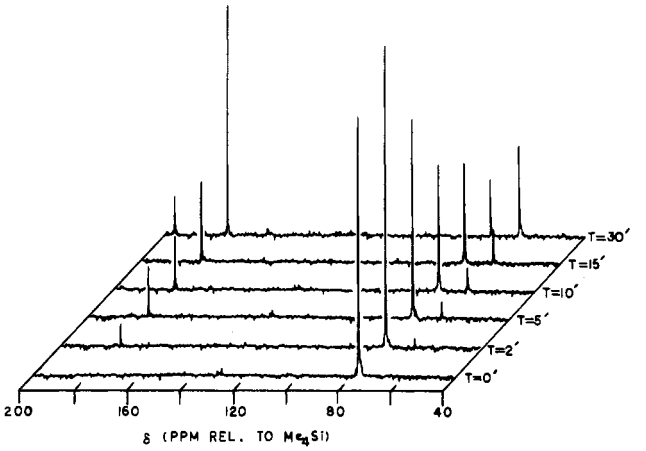


FIGURE 6: Time-lapse plot of proton-decoupled ^{13}C NMR (25.2 MHz) spectra of *P. putida* cells as a function of time of oxygenation. The sample is the same as in Figure 4. The spectra were obtained following oxygenation periods of 0, 2, 5, 10, 15, and 30 min. The carbons corresponding to the various chemical shifts are described in the text.

Metabolism of DL-[2-^{13}C]*Mandelate by Suspensions of High Molecular Weight Material from Untreated and DMSI-Treated Cells.* Addition of [^{13}C]mandelate (to either 50 or 12.5 mM final concentration) to the isolated cross-linked complex followed by oxygenation resulted in fairly rapid formation of both benzoylformate and benzaldehyde. Under the experimental conditions used here, i.e., in aqueous solutions, the aldehydic carbon of benzaldehyde and the α-keto carbon of benzoylformate had the same chemical shift value at 198 ppm. It was necessary, therefore, to obtain a coupled spectrum to demonstrate the presence of both compounds. After 1 h of oxygenation, mandelate and benzoylformate concentrations were reduced, benzaldehyde concentration was increased, and a sharp peak for benzoate appeared. A proton-coupled ^{13}C NMR spectrum of the cross-linked complex after 1 h of oxygenation is shown in Figure 7. The mandelate peak was a doublet; the benzoate was a sharp singlet; the singlet of the benzoylformate peak was surrounded by the doublet of benzaldehyde. [^{13}C]Bicarbonate had also appeared in another spectrum but was not observed here.

In a suspension of high molecular weight material from cells not treated with DMSI, signals for both [α-^{13}C]mandelate and [α-^{13}C]benzoylformate appeared in a 50:50 mixture (Figure 8), but no other metabolites were observed. Proton-coupled spectra were obtained, but in this case no benzaldehyde doublet emerged from the singlet at 198 ppm, as was observed in Figure 7.

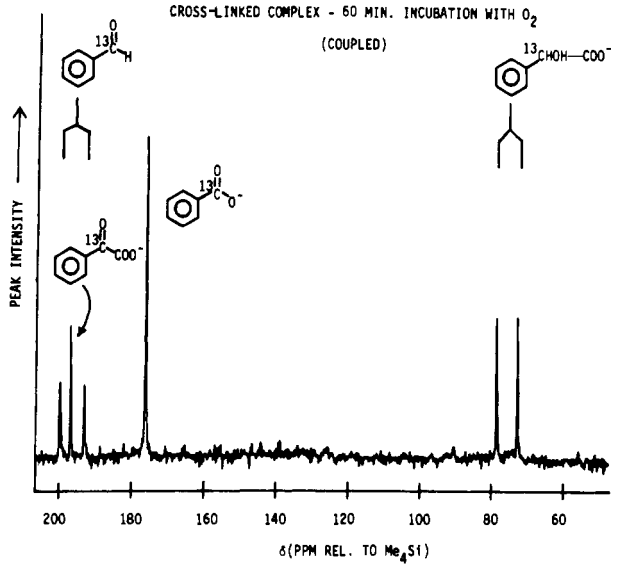


FIGURE 7: Coupled ^{13}C NMR (25.2 MHz) spectrum of the suspension of high molecular weight material from DMSI-treated cells of *P. putida* following 1 h of oxygenation. The sample was suspended in 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, and 20% D_2O containing 60 mM NAD^+, 10 mM FMN, 10 mM thiamine pyrophosphate, and 0.5 mL of dialyzate solution. DL-[α-^{13}C]Mandelic acid, sodium salt (pH 7.0), was added to a final concentration of 50 mM at time 0. The suspension was oxygenated as described under Materials and Methods. The spectrum represents a total of 200 free induction decays accumulated in 1 h at 5 °C. The decoupling power was gated to provide NOE enhancement of the methine and methylene resonances.

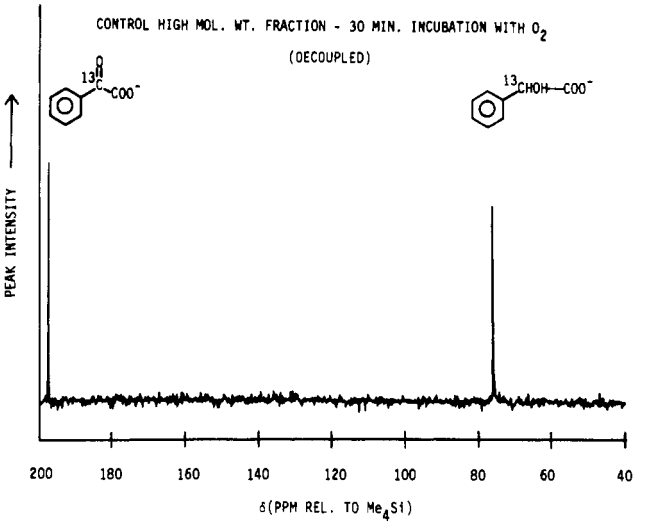


FIGURE 8: Proton-decoupled ^{13}C NMR (25.2 MHz) spectrum of the suspension of high molecular weight material from untreated cells of *P. putida* following 30 min of oxygenation. The sample was suspended in the buffer solution described in Figure 7. Other details are the same as described in Figure 7 except that the decoupling power was gated to obtain the spectrum without NOE enhancement.

Discussion

Comparison of the elution profiles of crude extracts from untreated (Figure 2) and DMSI-treated (Figure 3) cells showed that treatment of intact *P. putida* whole cells with dimethyl suberimidate permitted isolation of an enzymatically active, high molecular weight complex of proteins. DMSI treatment of the crude extract from cells disrupted by sonic oscillation, on the other hand, did not yield this high molecular weight complex. Both the cell-disruption process and the concomitant dilution by the buffer solution in which the cells were suspended apparently served to perturb the internal organization of the enzymes in the crude extract, thus reducing the likelihood that DMSI would covalently link a putative complex of enzymes.

Larger quantities of this complex, which has a molecular weight estimated to be $\sim 2.5 \times 10^6$ (Kenyon et al., 1976), were isolated by ultracentrifugation of the crude extract at 100000*g* for 1 h. The enzymatically active complex obtained by chromatography on a given column contained ~2 mg of protein, whereas ~20 mg of protein was typically present in the material isolated in a single ultracentrifugation experiment. Gel permeation chromatography of this high molecular weight material from DMSI-treated cells demonstrated the effectiveness of ultracentrifugation in the separation of cross-linked and un-cross-linked enzymes. Benzoylformate decarboxylase activity was not observed in the fractions of the void volume peak undoubtedly due to loss of activity during the course of the experiment. Its presence in the high molecular weight

material, however, was demonstrated by detection of decarboxylase activity in the material itself prior to gel chromatography and in the excluded fractions obtained from gel chromatography of the crude extract from DMSI-treated cells. Further evidence for the presence of this enzyme in the "complex" was observed in the ^{13}C NMR experiments (see below).

The appearance of low levels of mandelate racemase activity in the second peak of the eluted crude extract (Figure 3) and the eluted suspension of high molecular weight material from DMSI-treated cells may be due either to random cross-linking of mandelate racemase or to some cross-linking of the racemase and other enzymes. The activities of these other enzymes were not detected either because the enzymes were not present, they had become inactive, or they were sufficiently diluted during elution from the column to preclude their detection. However, the fact that Figure 3 shows essentially an "all or nothing" trapping of the high molecular weight complex of proteins, with little or no dimers, trimers, tetramers, etc. of the individual enzymes, indicates that a completely random cross-linking is not occurring.

A number of questions may be raised as to the composition of the cross-linked complex. Are the enzymes involved in mandelate metabolism entirely cross-linked to each other, or are only a few enzymes cross-linked, but in sufficient number to stabilize an already existing complex? Is it possible that the enzymes, instead of being cross-linked to each other, are cross-linked to some large macromolecular component of the cell? Are enzymes other than those of the mandelate pathway also incorporated in the cross-linked "complex"? Answers to these questions must await further experimentation.

From the results of these experiments, we conclude that dimethyl suberimidate can penetrate the gram-negative cellular membranes and that it covalently links those enzymes of the mandelate pathway that may be "clustered" about, or loosely associated with, the membrane-bound L-mandelate dehydrogenase. Moreover, it was necessary for the dimethyl suberimidate treatment to be performed in vivo, rather than in a crude extract, for this high molecular weight complex to be produced. Finally, this group of enzymes is sufficiently stable to survive the disruption and isolation procedures, as judged by retention of its catalytic activities characteristic of the enzymes assayed.

Although the isolated, in vivo cross-linked enzyme complex has not been purified and completely characterized, it has been possible to identify conclusively some of the enzymes present in the crude complex by using ^{13}C NMR spectroscopy. In the ^{13}C NMR experiments with whole cells, all of the known metabolities, with the exception of benzoylformate, were observed in the conversion of DL-[α-^{13}C]mandelate to [α-^{13}C]-benzoate (Scheme I). Benzoylformate was not detected, either because its conversion to benzaldehyde was too rapid to be observed on the NMR time scale or because it remained bound as an enzyme–substrate or an enzyme–product complex. Since the bacterium contains an L-specific mandelate dehydrogenase, the complete disappearance of the DL-mandelate peak following oxygenation provided evidence that mandelate racemase was functioning. The appearance of the bicarbonate peak demonstrated the presence of the benzoate oxidase system operating in the conversion of [α-^{13}C]benzoate to catechol with the concomitant loss of [^{13}C]carbon dioxide (Scheme II).

The observation of benzyl alcohol as a major metabolite in the dissimilation of DL-mandelate was unexpected in that it had not been previously reported in the literature. The cells of *P. putida* were grown in a culture medium containing 10 mM mandelate, but the concentration of the substrate in the cell suspension was 50 mM in the NMR studies. This higher concentration was chosen so that possible low levels of intermediates would be detected by NMR. Experiments in which the cell suspension contained only 12.5 mM DL-[α-^{13}C]mandelate showed that the amount of benzyl alcohol which appeared was either significantly reduced or that it was not observed. A reasonable explanation for these results is that in the presence of 50 mM mandelate sufficient benzaldehyde is produced to saturate the benzaldehyde dehydrogenase. The "spillover" benzaldehyde is then reduced to benzyl alcohol by constitutive, nonspecific dehydrogenase(s) known to be present in the cell (J. Collins and G. D. Hegeman, unpublished results). At lower mandelate concentrations little or no such saturation of benzaldehyde dehydrogenase occurs.

It is also noteworthy that complete metabolism of intermediates, even with oxygenation periods of up to 2 h, was not observed in cells containing 50 mM mandelate. In most cases significant quantities of benzyl alcohol and benzoate remained. With the initial reduction of the mandelate concentration to 12.5 mM, on the other hand, metabolism of all intermediates was complete in approximately 15–20 min. Benzaldehyde and benzyl alcohol are both cytotoxic. Therefore, it appeared that the presence of 50 mM mandelate overloaded the capacity of the enzymes to maintain low concentrations of such toxic intermediates or that oxygen absorption rates at high mandelate concentrations were insufficient to reoxidize the NADH produced and maintain normal metabolism.

^{13}C NMR spectroscopy was particularly valuable for the characterization of the crude, in vivo cross-linked enzyme complex. Preparation of the samples of high molecular weight material from control and DMSI-treated cells, however, involved a complex addition of cofactors and other small intracellular molecules that had been removed during the isolation and washing procedures. Each of the enzymes of the mandelate group, with the exception of mandelate racemase, requires a cofactor for activity: L-mandelate dehydrogenase probably requires a flavin; benzoylformate decarboxylase requires thiamine pyrophosphate; one of the two benzaldehyde dehydrogenases requires NAD^+ and the other $NADP^+$. For the experiments described here $NADP^+$ was omitted. In addition to the cofactors, a concentrated dialyzate of the crude extract (obtained as described under Materials and Methods) was added to provide the preparation with other necessary small molecules.

Spectra of the high molecular weight complex from DMSI-treated cells showed, after periods of oxygenation, all the intermediates produced in the metabolism of DL-[α-^{13}C]mandelate to [α-^{13}C]benzoate (Scheme I). [^{13}C]Bicarbonate was observed in one experiment, providing tentative evidence for the presence as well of benzoate oxidase in the complex (Scheme II). It is perhaps significant, though, that no detectible benzyl alcohol was formed in the cross-linked complex. This suggests that enzymes other than those normally functional in the pathway may be absent. We should expect that common, constitutive enzymes will be found in the isolated complex, however, since no purification has yet been attempted. For example, glutamine synthetase, an enzyme clearly not in the mandelate pathway, has been shown (Table I) to be present at $\sim$15% of its specific activity in crude extracts.

The ^{13}C NMR spectra of the high molecular weight material isolated from untreated cells consistently showed a 50% decrease of the mandelate peak with the concomitant formation of benzoylformate. This 50:50 ratio of the two peaks did not

change with longer periods of oxygenation, nor were other metabolites detected. Therefore, mandelate racemase and benzoylformate decarboxylase (and presumably the other soluble enzymes of the mandelate group) were apparently not present in significant amounts, but the sample did contain the membrane-bound, stereospecific L-mandelate dehydrogenase which converted the L isomer of mandelate to benzoylformate. Since a racemate of [α-^{13}C]mandelate had initially been added and no racemase was present, the 50% that remained was the unchanged D enantiomer, as was confirmed by a strong negative Cotton effect at 216 nm when a circular dichroic spectrum of the sample was obtained. Low-speed centrifugation of the crude extract following sonic disruption of the cells removed a large amount of the particulate fraction that contained the L-mandelate dehydrogenase. Assays of the remaining extract, however, demonstrated that the enzyme had not been completely eliminated, and it was this portion of the dehydrogenase remaining in the extract that appeared in the high molecular weight samples following centrifugation at 100000*g* (Table I).

These NMR experiments provide conclusive evidence that this high molecular weight material, isolated from cells treated with dimethyl suberimidate, contained all the enzymes necessary to convert mandelate to benzoate. The results support the idea that the enzymes of the mandelate group may be in close association with each other and, in turn, with the membrane-bound L-mandelate dehydrogenase and that treatment of whole cells of *P. putida* with dimethyl suberimidate provides sufficient covalent linkages to entrap these enzymes. The data, however, do not rule out the possibility that the enzymes may be cross-linked to cellular components of high molecular weight, i.e., cell wall fragments, rather than complexed to each other.

Experiments are in progress to determine which enzymes of the β-ketoadipate group are present in the complex by monitoring the metabolism of benzoic acid labeled with ^{13}C on the aromatic ring carbon attached to the carboxylate function.

It perhaps is appropriate to speculate about the function of the putative "mandelate complex" in *P. putida*. One may propose that a cluster of the mandelate enzymes would rapidly turn over substrate (mandelate) to product (benzoate) by utilizing a channeling mechanism not so much to localize high concentrations of substrates or to separate competing pathways but to localize toxic intermediates, thus minimizing their detrimental effects on the cell. However, until the isolated, crude complex is more completely characterized and a purified "mandelate complex" isolated with further analysis as to its structure, composition, kinetic properties, etc., such statements must remain conjecture.

Added in Proof

E. coli induced to use glucose as its energy source was similarly cross-linked with DMSI, and a high molecular weight material containing at least two enzymic activities of the glycolytic pathway was isolated (S. Mowbray, R. A. Halpin, G. Petsko, and G. L. Kenyon, unpublished results).

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