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Isolation of an in vivo cross-linked multienzyme complex of mandelate metabolism in pseudomonas putida and its characterization by Carbon-13 NMR spectroscopy

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ISOLATION OF AN *in vivo* CROSS-LINKED MULTIENZYME COMPLEX OF MANDELATE METABOLISM IN *PSEUDOMONAS PUTIDA* AND ITS CHARACTERIZATION BY CARBON-13 NMR SPECTROSCOPY

by

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DISSERTATION

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TO MY PARENTS

MY SISTER

AND MY BROTHERS

ABSTRACT

The physical association of intracellular enzymes has been investigated by treatment of whole bacterial cells with a cross-linking reagent followed by isolation of an enzymatically active, high molecular weight complex. Pseudomonas putida, a soil bacterium, is capable of utilizing mandelic acid as a sole source of carbon and energy. Five inducible enzymes (called the mandelate group) metabolize mandelate to benzoate. The benzoate oxidase system and the β-ketoadipate group of enzymes function in the further breakdown of benzoate to succinate and acetyl CoA, compounds readily able to enter general metabolism.

The whole cells of P. putida were treated with the bifunctional bisimidoester, dimethyl suberimidate (DMSI), which forms cross-links between enzymes by reacting with free amino groups on the enzyme surface. The crude extract, obtained following sonic disruption of the cells and low-speed centrifugation to remove cellular debris, was subjected to gel permeation chromatography using LKB Ultrogel AcA 22. Two peaks of enzyme activities were observed in the eluted fractions: a large peak of activity, which also appeared in control samples, corresponding to uncross-linked enzymes, and a second, smaller peak which appeared in the fractions of the void volume. This latter peak of enzyme activities was not observed in eluted extracts of untreated

cells. Larger quantities of the high molecular weight complex were isolated by ultracentrifugation of the crude extract at 100,000 x g for 1 h. Again appreciable levels of enzyme activities were observed in the 100,000 x g pellet from DMSI-treated cells, whereas little or no activities appeared in the control samples.

The composition of the isolated, *in vivo* cross-linked complex of enzymes was characterized by ^{13}C NMR spectroscopy. Initial experiments to determine which metabolites would be observable by NMR were carried out using whole cells of *P. putida* in the presence of 90%-enriched [2-^{13}C]mandelic acid. Following periods of oxygenation the signal for the labeled α-carbon of mandelate gradually decreased while signals corresponding to the α-carbons of benzaldehyde, benzoic acid, and benzyl alcohol, a previously unreported metabolite of mandelate metabolism, appeared and increased. [^{13}C]Bicarbonate, a product of the decarboxylation of benzoate, was also observed. When the starting concentration of [α-^{13}C]mandelate was reduced from 50 mM to 12.5 mM, the amount of benzyl alcohol produced (which is cytotoxic at the higher concentrations) was significantly reduced.

Oxygenation of the isolated *in vivo* cross-linked enzyme complex in the presence of 50 mM or 12.5 mM [α-^{13}C]mandelate also resulted in metabolism of mandelate to benzoate when followed by ^{13}C NMR spectroscopy. The metabolites observed

in addition to the substrate [α-^{13}C]mandelate, were [α-^{13}C]benzoylformate, [α-^{13}C]benzaldehyde, and [α-^{13}C]benzoate. ^{13}C NMR spectra of isolated, untreated, high molecular weight material showed no greater than 50% reduction of peak intensity of the mandelate signal, and [α-^{13}C]benzoylformate was the only product observed. This result was due to the presence of the membrane-bound, stereospecific L-mandelate dehydrogenase. Biochemical assays showed this enzyme to be present in crude extracts of untreated and DMSI-treated cells, even after low-speed centrifugation to remove cell membrane fragments.

The biochemical and ^{13}C NMR results are consistent with the idea that the enzymes of the mandelate group are all associated with the membrane-bound L-mandelate dehydrogenase. In vivo treatment of this group of associated enzymes with dimethyl suberimidate either entirely cross-links these enzymes, or only partially cross-links them entrapping other enzymes in a loosely entwined net.

DMSI-treatment of crude extract (instead of whole cells) followed by isolation by ultracentrifugation of the high molecular weight material showed no increase of enzyme activity over untreated samples. Ribonuclease treatment and lipase treatment of isolated in vivo cross-linked enzyme complex resulted in no loss of enzyme activity compared to untreated enzyme complex.

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LIST OF ABBREVIATIONS

DCPIP	2,6-Dichlorophenolindophenol
DMSI	Dimethyl suberimidate
DNP	2,4-Dinitrophenylhydrazine
EDTA	(Ethylenedinitrilo)tetraacetic acid
Hepes	4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid
NOE	Nuclear Overhauser enhancement
PRS	Pseudomonas, Roger Stanier collection (bacterial strain designation)
PUG	Pseudomonas, I. C. Gunsalus collection
TMS	Tetramethylsilane
TNM	Buffer: 0.05 M triethanolamine·HCl (pH 8.0), 0.1 M NaCl, 0.01 M $MgCl_2$
YAP	Yeast-agar-phosphate agar

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I. INTRODUCTION

Enzymes traditionally have been studied as individual parts, the sum of which makes up the whole - the cell. Each enzyme is isolated, purified of "contaminating" material, and subjected to a broad array of experiments to discern its structure, its mechanism of action, the nature of its active site, and so forth. Such "multienzyme complexes" as the fatty acid synthetase and pyruvate dehydrogenase complexes were considered to be the exception rather than the rule. Results from two diverse areas of research - genetics and physicochemical studies - now indicate, however, that most likely the converse is true. As isolation procedures improve, more examples of the association or compartmentation of enzymes are being observed. Current methods of cell lysis (osmotic shock, sonic disruption, compression) work against success in the separation of enzyme clusters held together by weak, noncovalent forces.

If a means could be devised by which these loosely associated enzymes would be covalently linked, a stable complex of enzymes could be isolated for detailed studies. Bifunctional imidoesters had been used to determine the quaternary structure of enzymes and to examine the nearest-neighbor relationship of proteins in ribosomes. Therefore, it appeared that these reagents, which are sufficiently reactive under mild conditions, would function as connecting

links between enzymes which were closely associated in the cell.

Results of preliminary experiments by George Kenyon, in collaboration with George Hegeman, showed that it was possible to isolate an enzymatically active, high molecular weight complex from whole bacterial cells (*Pseudomonas putida*, induced to grow on D,L-mandelic acid) which had been treated with a bisimidoester, dimethyl suberimidate.

These initial studies provided a firm base from which to begin the research project described in this thesis. Isolation of an *in vivo* cross-linked enzyme complex by gel permeation chromatography and ultracentrifugation, and its characterization by ^{13}C NMR strongly suggests that these enzymes exist in close proximity with each other, and that the entire complex is loosely associated with a membrane-bound enzyme.

II. HISTORICAL BACKGROUND

A. Metabolism of Mandelic Acid: Regulation and Genetic Control.

1. Nutritional Diversity of Fluorescent Pseudomonads.

Aerobic pseudomonads, isolated predominantly from soil and water, can be distinguished from other generic species of bacteria, particularly the coliform bacteria, by their ability to use a wide variety of organic compounds as carbon and energy sources for aerobic growth. This fact was first established by den Dooren de Jong (1926) in a thesis which unfortunately was published only in Dutch. A brief summary of this work was provided by Stanier *et al.* (1966) in an interesting report describing a detailed taxonomic study of these versatile bacteria. Stanier and his colleagues tested a total of 146 unrelated organic compounds for their ability to serve as carbon and energy sources on a collection of 267 strains representative of the principal biotypes of aerobic pseudomonads. The universally negative nutritional characters consisted solely of the inability to utilize methanol, methylamine, or oxalate, the inability to grow chemolithotrophically at the expense of H_2, and the inability to fix nitrogen. The majority of strains examined were each capable of utilizing 60 - 90 (or more) compounds for growth.

Of particular interest was the large number of strains

capable of carrying out the oxidation of aromatic compounds. p-Hydroxybenzoate was an unusual universal substrate for the majority of the strains tested; whereas benzoic acid appeared to be a species-specific substrate for the fluorescent subgroup of pseudomonads. Mandelic acid was an example of those compounds utilized only occasionally as sources of carbon and energy.

2. Biological Occurrance of Mandelate Metabolism.

The microorganisms capable of growth at the expense of mandelic acid include, in addition to the fluorescent pseudomonads, P. cepacia (multivorans), P. convexa, the fungus Aspergillus niger, an unidentified strain of yeast, and other groups of bacteria. Most of these organisms metabolize solely the L-isomer or the D-isomer, while the ability to utilize both isomers is relatively rare [Sharp, 1977, (Table I)]. Of the 267 strains studied by the Stanier group, only forty-four could metabolize either D- or L-mandelate. All twenty-nine strains of P. aeruginosa examined grew only on L-mandelate. The remaining fifteen strains - three of P. fluorescens, eight of P. putida, four of P. cepacia (multivorans) - were capable of growth on one or the other of both isomers. Only three of the eight strains of P. putida (biotype A, strains 89 and 90, biotype B, strain 53) were capable of utilizing both D- and L-isomers. Such stereospecificity indicates the presence in

these strains of a stereospecific mandelate dehydrogenase. The activities of both D- and/or L-mandelate dehydrogenase have been demonstrated in the different strains. In those strains of pseudomonads which can metabolize both enantiomers of mandelate, a single stereospecific dehydrogenase has been accompanied by a mandelate racemase [Sharp, 1977, (references in Table I)].

Mandelic acid is infrequently observed in the environment. Therefore, the question remains as to why these strains contain a complete suite of enzymes capable of metabolizing this compound. It has been observed that the mandelate group enzymes accept a broad range of mandelate derivatives substituted in the 3-, 4-, and 5- positions of the aromatic ring (Hegeman et al., 1970). Kenyon and Hegeman (1979) postulate that the principal substrates in nature are the hydroxy- and methoxy-mandelates derived from lignin and other related natural products.

3. Simultaneous Adaptation and Sequential Induction.

P. putida biotype A, strain 90 [ATCC 12633; also designated as P. fluorescens A.3.12, PRS-1[1] (Chakrabarty et al., 1968, 1969, 1970, Hegeman & Root, 1976) and PRS-90 (Sharp, 1977)] is capable of utilizing D,L-mandelic acid as its sole source of carbon and energy by the strictly inducible path-

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[1]PRS: Pseudomonas, Roger Stanier collection.

way shown in Figure II.1. This metabolic sequence has been elucidated in the laboratories of Stanier and Gunsalus (Stanier, 1947, 1948, 1950; Sleeper & Stanier, 1950; Sleeper et al., 1950; Gunsalus, I. C. et al., 1953; Stanier et al., 1953; Gunsalus, C. F. et al., 1953).

Early studies by Stanier and colleagues of the oxidation of mandelate and other aromatic compounds showed that metabolism of these compounds involved a number of complex, inducible enzyme systems. It appeared that cells, when exposed to a primary substrate, "adapted" to dissimilate that compound. These adapted cells were then capable of immediate attack if exposed again to this substrate or to any of the intermediates of that metabolic pathway. If another substrate which was not an intermediate was added to the adapted cells, it was not metabolized until new "adaptive" enzymes developed. This "simultaneous adaptation" by enzymes was developed by Stanier (1947) as a technique for studying the pathways for metabolism of aromatic compounds in bacteria. The synthesis of the enzymes catalyzing these reactions was proposed to occur by a process termed "sequential induction"; i.e. each enzyme was specifically induced by the metabolite which acted as its substrate when this substrate was added to the growth medium or appeared following the action of the previous enzyme in the pathway (Stanier, 1951). With the development of the operon concept (Jacob & Monod, 1961) to explain the induction and repress-

Figure II.1.

The convergent pathways for the oxidation of D,L-mandelic acid and D,L-p-hydroxymandelic acid in Pseudomonas putida.

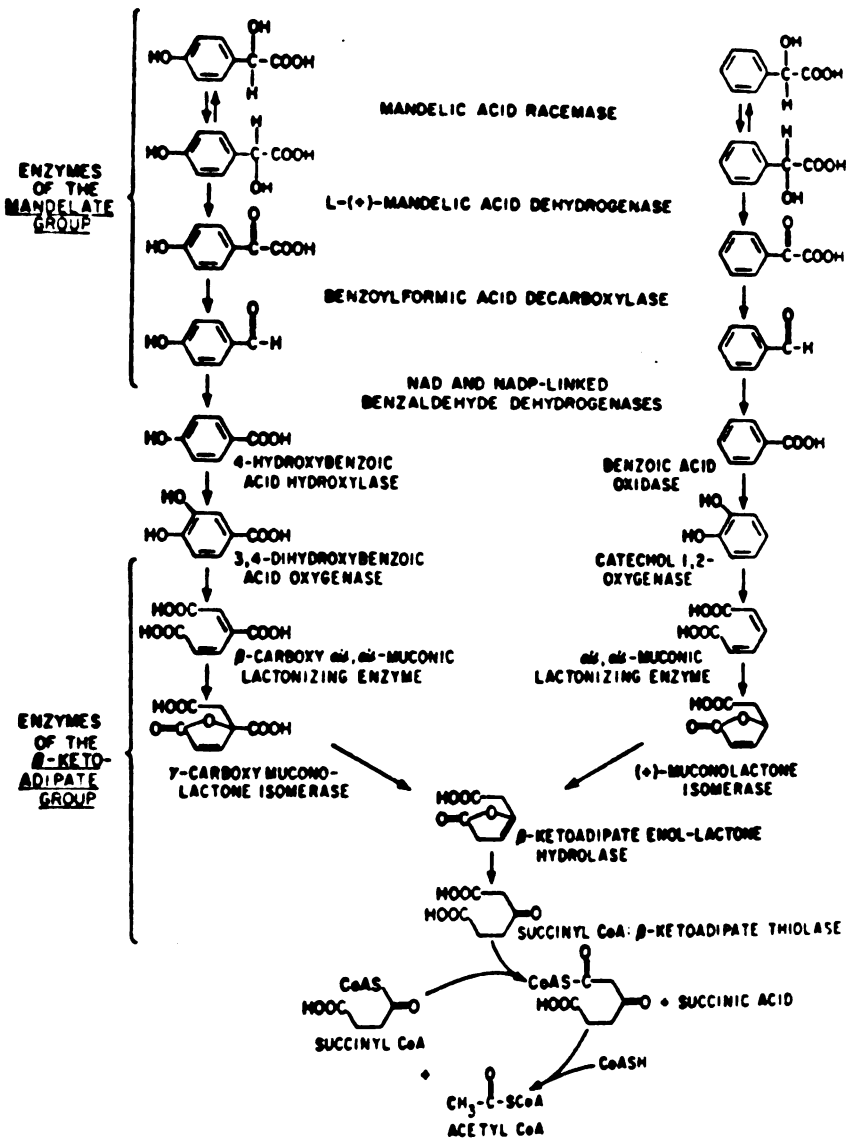
ENZYMES OF THE MANDELATE GROUP
MANDELIC ACID RACEMASE
L-(+)-MANDELIC ACID DEHYDROGENASE
BENZOYLFORMIC ACID DECARBOXYLASE
NAD AND NADP-LINKED BENZALDEHYDE DEHYDROGENASES
4-HYDROXYBENZOIC ACID HYDROXYLASE
BENZOIC ACID OXIDASE
3,4-DIHYDROXYBENZOIC ACID OXYGENASE
CATECHOL 1,2-OXYGENASE
β-CARBOXY cis,cis-MUCONIC LACTONIZING ENZYME
cis,cis-MUCONIC LACTONIZING ENZYME
ENZYMES OF THE β-KETO-ADIPATE GROUP
γ-CARBOXY MUCONO-LACTONE ISOMERASE
(+)-MUCONOLACTONE ISOMERASE
β-KETOADIPATE ENOL-LACTONE HYDROLASE
SUCCINYL CoA: β-KETOADIPATE THIOLASE
SUCCINYL CoA
+ SUCCINIC ACID
CoASH
ACETYL CoA

Figure II.1.

ion of enzyme synthesis, re-evaluation of sequential induction was necessary. If, indeed, the synthesis of each enzyme was induced by its substrate, then a very large number of operons for the metabolism of any compound of moderate complexity would be required. In the case of mandelate metabolism, for instance, eleven operons - each with its own regulatory and structural genes - would be necessary to regulate enzyme synthesis. A variety of later experiments (to be discussed below) showed that the enzymes of the mandelate group operated as a coordinate unit of expression and function, i.e. as an operon. Therefore, instead of five individual induction steps occurring as proposed by sequential induction, only one event was required for the synthesis of all five enzymes. Enzyme synthesis was therefore envisioned to occur by induction of functional groups of enzymes, separated in some cases by individual inductive steps, as was observed to be the case for the synthesis of enzymes capable of oxidizing aromatic compounds.

4. Regulation of Enzyme Synthesis.

Mandelic acid is metabolized in *P. putida* by the strictly inducible enzymes of two pathways: the mandelate group and the β-ketoadipate group. The mandelate group of enzymes converts D-mandelate to benzoic acid, which is metabolized by the benzoate oxidase system to catechol. The catechol branch of the β-ketoadipate group of enzymes

metabolizes catechol to β-ketoadipate which is converted by two more enzymes - a transferase and a thiolase - to succinate and acetyl CoA. These two final products of mandelate metabolism then enter general metabolism at the level of the tricarboxylic acid cycle. p-Hydroxymandelic acid is also a substrate of the mandelate group and is converted to p-hydroxybenzoate. The protocatechuate branch of the β-ketoadipate group converts this product to β-ketoadipate, converging with the catechol branch at the level of the enol-lactone.

Synthesis of the five enzymes of the mandelate group was found to be coordinately induced by the first three compounds of the pathway: D-mandelate, L-mandelate, and benzoylformate (Hegeman, 1966a). All three substrates were equally potent as substrate-inducers. Benzaldehyde was not tested due to its volatility and its toxicity to the cells. Phenoxyacetate, a nonmetabolizable substrate, also induced the synthesis of all five enzymes (but no others), although at a level of 10 - 20 % of the wild type. The specific activities of the enzymes (which reflects differential rates of synthesis) in induced cells increased approximately 2000-fold for mandelate racemase and L-mandelate dehydrogenase, and approximately 430-fold for benzoylformate decarboxylase over basal levels in noninduced cells.

Experiments using mutants of P. putida A.3.12 blocked for the synthesis of mandelate racemase (rac^-) and L-

mandelate dehydrogenase (md^-), respectively, produced results similar to those obtained with wild type cells (Hegeman, 1966b). Mutants in the presence of non-metabolizable substrates (D-mandelate + rac^- mutant; D,L-mandelate + md^- mutant) induced the synthesis of the respective four nondeleted enzymes. In the presence of metabolizable substrates (L-mandelate + rac^- mutant; benzoylformate + md^- mutant) the mutants synthesized the four nondeleted enzymes plus the enzymes of the catechol pathway (Hegeman, 1966b).

The results of the above experiments with both the blocked mutants and the wild type cells of P. putida show that the first five enzymes of mandelate metabolism are coordinately synthesized, and present evidence that the mandelate group comprises a regulatory unit sharing a common repressor.

Direct evidence that the structural genes coding for synthesis of the mandelate group are contiguous on the chromosome was not obtainable due to the lack of a genetic recombination system in P. putida at the time. However, existence of constitutive mutants would provide sufficient evidence for the existence of a regulatory (operator) gene. Mutants of P. putida, selected for constitutive synthesis of L-mandelate dehydrogenase after substrate-limited growth in a chemostat, were isolated. These mutants were capable of constitutive, coordinate synthesis of the five enzymes of

the mandelate group when grown in the absence of an inducer. In addition these mutants were superinducible by some inducers of the mandelate group of enzymes in the wild type; they also exhibited decreased specificity of induction in that they were inducible by a number of compounds which did not induce the wild type (Hegeman, 1966c). Two interpretations were possible based upon these results: (1) constitutivity could have been a result of broadened specificity of the repressor rather than of a mutation in the o-gene; (2) the mutants may have been operator constitutives (o^c), a result of a mutation in the o-gene (Hegeman, 1966c). A decision between these two interpretations would have to await development of a genetic recombination system in P. putida. However, these last studies provide further evidence that the synthesis of the enzymes which metabolize mandelic acid to benzoate is coordinately induced and is under genetic control by a complex operon.

Regulation of the sequences of the β-ketoadipate pathway has been elucidated by Ornston & Stanier (1966) and Ornston (1966a,b,c) in much the same manner as that described above for the mandelate enzymes. The control mechanisms present a highly complex picture, the details of which are summarized in Figure II.2 (Wheelis & Stanier, 1970). The syntheses of p-hydroxybenzoate hydroxylase (p-hydroxybenzoate to protocatechuate) and benzoate oxidase (benzoate to catechol) are each induced by their respective

Figure II.2.

The convergent protocatechuate and catechol branches of the β-ketoadipate pathway and their regulation in P. putida. Brackets denote coordinate synthesis of the enzymes.

(from Wheelis & Stanier, 1970).

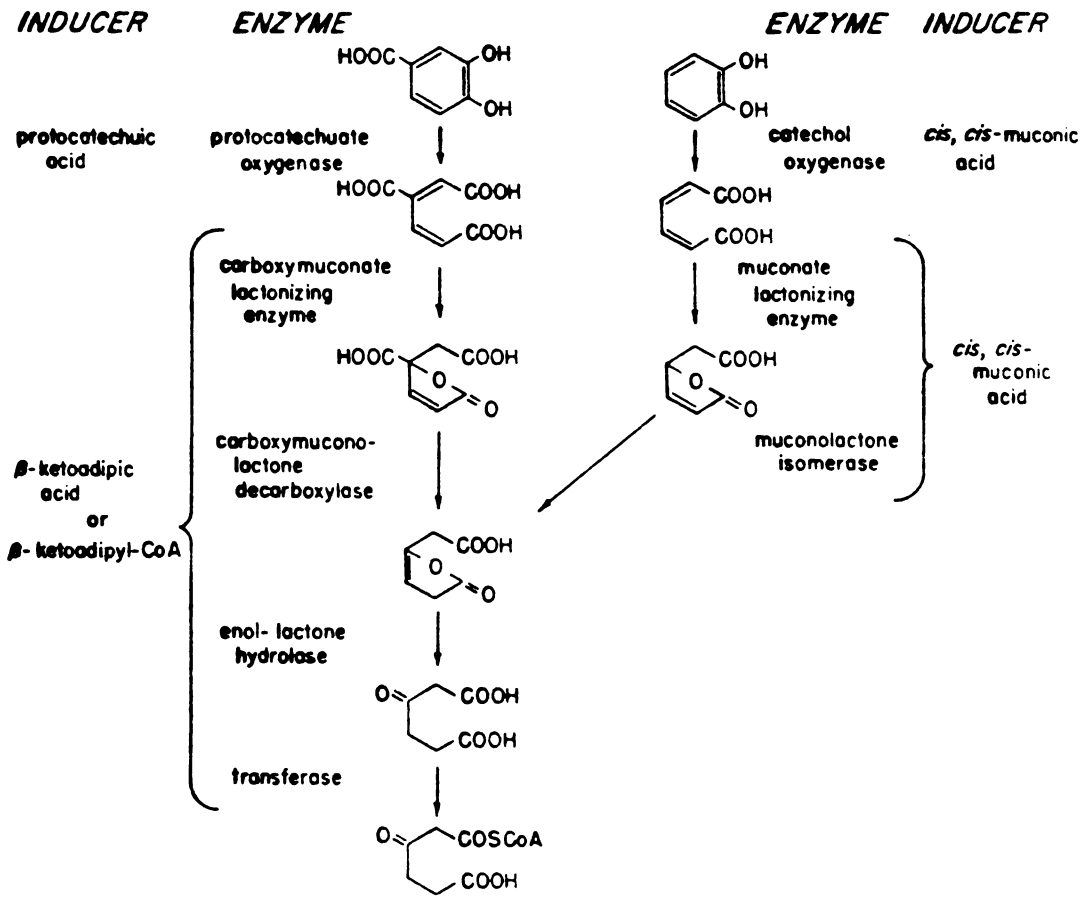
INDUCER
ENZYME
ENZYME
INDUCER
protocatechuic acid
protocatechuate oxygenase
catechol oxygenase
cis, cis-muconic acid
carboxymuconate lactonizing enzyme
muconate lactonizing enzyme
cis, cis-muconic acid
β-ketoadipic acid
or
β-ketoadipyl-CoA
carboxymucono-lactone decarboxylase
muconolactone isomerase
enol-lactone hydrolase
transferase
HOOC
OH
COOH
COSCoA
O

Figure II.2.

substrates. The enzymes which convert protocatechuate and catechol to β-ketoadipate form what has been described by Wheelis and Stanier (1970) as four distinct physiological regulatory units: the protocatechuate unit (protocatechuate oxygenase); the catechol unit (catechol oxygenase); the carboxymuconate unit (carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, enol-lactone hydrolase, and transferase); the muconate unit (muconate lactonizing enzyme and muconolactone isomerase). The protocatechuate unit is substrate-induced, whereas the carboxymuconate unit is coordinately induced by the final product - β-ketoadipate. Product induction is probably a result of the fact that the three immediate precursors of β-ketoadipate are unstable compounds, precluding their use as inducers. In the catechol branch cis,cis-muconate serves as the product inducer of catechol oxygenase and as the substrate inducer of the muconate unit whose enzymes are coordinately induced. These two units may share the same regulatory gene even though their synthesis is independently governed. A mutant of P. putida was isolated in Ornston's laboratory (Wu, et al., 1971) which constitutively synthesized the two enzymes of the muconate unit as well as catechol oxygenase. The two parallel branches of the β-ketoadipate pathway converge with the formation of β-ketoadipate enol-lactone. Low levels of enol hydrolase convert the lactone to β-ketoadipate; the product induces the coordinate synthesis of the

carboxymuconate unit. When the catechol branch is functioning, two enzymes of that unit (carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase) are gratuitously synthesized. The results of the experiments with blocked and permeability mutants (Ornston, 1966c) and with the constitutive mutant of the catechol branch (Wu, et al., 1971) provide evidence that the muconate unit and the carboxymuconate unit are each under the control of a complex operon.

5. Genetic Control of Enzyme Synthesis.

Evidence that the synthesis of the mandelate group and the muconate and carboxymuconate units are each controlled by an operon would be supplied if genetic analysis should reveal close linkage between any two structural genes within a complex physiological regulatory unit, but not necessarily between any two structural genes from different regulatory units. Genetic recombination by phage transduction results in the transfer of approximately 5% of the donor bacterial chromosome to the recipient cell (Gunsalus et al, 1968). Therefore, selection for a marker in the transductants, which was present only in the donor but not in the recipient prior to transduction, provides evidence for the transfer of a particular piece of bacterial chromosome.

These recombination experiments, performed in the laboratories of Gunsalus, Hegeman, and Stanier, provided

important information about the genetic linkages of the mandelate, benzoate, and p-hydroxybenzoate pathways. In the first of these experiments the host-range phage mutant pf16h2 was used to carry out interstrain gene transfer between PRS-1 (P. putida A.3.12) and PUG-2[2] (P. putida C1 tr), a camphor-degrading strain. This latter strain was unable to grow on D- or L-mandelate or benzoylformate, but it did grow on benzoate. A similar experiment was performed between the mutant PRS-3 (lacking L-mandelate dehydrogenase, MDH^-) and PUG-2. Activities of four enzymes in the mandelate cluster were analyzed in PRS-1, PUG-2, PRS-3 and the two transductants. PUG-2 contained none of the four activities and the cross PRS-1 x PUG-2 had cotransferred all four. PRS-3 (MDH^-) and the cross PRS-3 x PUG-2 contained three of the four enzymes, lacking only L-mandelate dehydrogenase activity. Thus a large segment of DNA containing at least four genes functional in the conversion of mandelate to benzoate was transferred in transduction. It was not clear if the regulatory genes had cotransduced with the structural genes (Chakrabarty et al., 1968). These results were extended by later studies of linkages in the mandelate gene cluster. Complete analysis has been limited by the lack of mutants containing deletions in genes coding for benzoylformate decarboxylase (mdlC) and benzaldehyde dehydrogenase

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[2]PUG: Pseudomonas, I. C. Gunsalus collection.

(mdlD). However, close linkage between $mdlA^+$ (mandelate racemase) and $mdlB^+$ (L-mandelate dehydrogenase) genes was observed in intra- and inter-strain transfers. It appears that a close linkage with all four genes does exist since transductants selected for $mdlC^+$ (decarboxylase) and $mdlD^+$ (dehydrogenase) invariably contained non-selected $mdlB^-$ or $mdlA^-$ characters. In another equally important experiment the operator constitutive (o^C) mutant PRS-11 (mdlR1010) isolated by Hegeman (1966c) was the donor in the phage-mediated transduction of the regulatory locus to strain PUG-2 and to the interstrain transductant PRS-628 [from PRS-3 ($mdlB^-$) x PUG-2 cross]. In the transfer to PUG-2 it appears that the entire mandelate operon was transduced as judged by the high cotransfer frequency value. There was also a high degree of linkage of the regulatory locus with the mdlB structural gene in the PRS-11 cross with the inter-strain transductant (Chakrabarty & Gunsalus, 1969). Even though the authors feel that these last results are tentative because of difficulties with the phenotypic scoring of the o^C mutant, there appears to be sufficient evidence that the genes which code for the synthesis of the mandelate enzymes in P. putida are closely linked - if not contiguous - on the genome and thus constitute an operon.

Further evidence of the close linkage of the racemase and mandelate dehydrogenase genes came from experiments with a non-metabolizable analog of mandelic acid, D,L-2,3,4,5,6-

pentafluoromandelic acid (PFM), which inhibits the growth of P. putida by competitive inhibition of mandelate dehydrogenase. Mutants which were resistant to this inhibition (PMF^r) were isolated. When transduced with $mdlA^-$ and $mdlB^-$ mutants using phage pf16h2, the PMF^r mutation mapped very near to the structural genes coding for the first two enzymes of the mandelate pathway (Hegeman & Root, 1976).

Just as regulation in the β-ketoadipate pathway is complex, so also is genetic control. Genetic analysis of this pathway was examined in Pseudomonas aeruginosa, a fluorescent pseudomonad lacking mandelate racemase, by Kemp & Hegeman (1968). The pattern of enzyme induction was shown to be the same as that in P. putida for the β-ketoadipate pathway but different in the pattern of induction and the number of enzymes in the mandelate pathway (Rosenberg & Hegeman, 1969). Mutants of P. aeruginosa were obtained for ten of the eleven structural genes of the β-ketoadipate enzymes, and the mutations in these genes were mapped by transduction using phage F116. Surprisingly, many of the genes coding for the metabolism of p-hydroxybenzoate and benzoate formed two clusters: one coding for the enzymes in the p-hydroxybenzoate pathway, and the other specifying several enzymes in the benzoate pathway. Close linkage was observed among the three genes of the carboxymuconate unit, as would be expected, but genes specifying p-hydroxybenzoate hydroxylase cotransduced with the carboxymuconate unit also. The

protocatechuate oxidase genes did not cotransduce with any of the structural genes in either cluster. Close linkage was observed between the two structural genes of the muconate unit. Genes coding for catechol oxygenase, anthranilate oxidase, and benzoate oxidase (all enzymes convergent with the benzoate pathway) were cotransducible with the muconate unit (Kemp & Hegeman, 1968). When mutations in the genes specifying the mandelate enzymes were mapped, it was found that the mandelate genes, mdlB and mdlC, cotransduced at a low frequency with the benzoate genes but not with each other. No linkage of the mandelate genes with p-hydroxybenzoate genes was observed (Rosenberg & Hegeman, 1969).

Wheelis and Stanier (1970) expanded the genetic analysis to a number of dissimilatory pathways in P. putida using the transducing phage pf16h2. A large number of mutants, derived from PRS-1, lacking enzymes in the mandelate and β-ketoadipate sequences as well as in a number of other sequences were prepared. Genes coding for mandelate racemase (mdlA) and L-mandelate dehydrogenase (mdlB) cotransduced with the two genes of the muconate unit and also with the benzoate oxidase gene. No linkage was detected between the mdlB gene and one of the genes of the protocatechuate sequence. As was reported for P. aeruginosa, two distinct gene clusters were observed in P. putida: a long cluster containing the genes coding for the

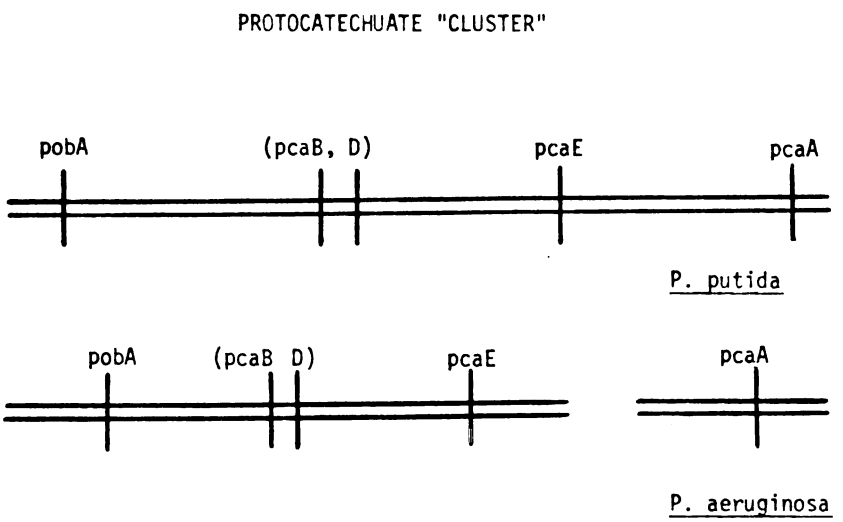
protocatechuate pathway and those sequences convergent on it; a shorter cluster containing the genes of the mandelate, benzoate, and catechol sequences. When genetic maps of these two clusters in P. putida and P. aeruginosa are compared, a high degree of homology is apparent with respect to the genes of both branches of the β-ketoadipate pathway (Figure II.3). Such genetic homologies support the hypothesis that the genes of the central sequences of the pathway have had a common evolutionary origin in the two species. With the mandelate genes, however, no homology is evident. In P. putida the mdlB and mdlC genes are close together and to one side of the benzoate and catechol genes, whereas in P. aeruginosa they are widely separated on each side of this cluster. This lack of genetic homology, along with differences in enzymology and regulation lead to the conclusion that the mandelate sequence may have evolved independently in the two species, subsequent to the already pre-existing homologous central sequences (Wheelis & Stanier, 1970). The existence of distinct regions of homology and nonhomology in the genomes of P. putida and P. aeruginosa has also been observed in genetic crosses by transduction between the two species (Chakrabarty & Gunsalus, 1970).

The existence of "supra-operonic" clustering of functionally related genes in the chromosome would appear to provide cells which contained such clusters with a distinct

Figure II.3.

Comparative genetic maps showing the genes of the β-ketoadipate pathway in P. putida and P. aeruginosa. The markers in parentheses are known to be cotransducible with genes of this cluster.

(from Wheelis & Stanier, 1970).



pobA	p-hydroxybenzoate hydroxylase	mdlB	L-mandelate decarboxylase
pcaB	protocatechuate oxygenase	mdlC	benzoylformate decarboxylase
pcaB-D-E	carboxy muconate unit	catB-C	catechol oxygenase
ben	benzoate oxidase		

Figure II.3.

evolutionary advantage. Bacterial transfer of genetic material, whether by transformation, conjugation, or transduction, usually occurs with the transfer of relatively short pieces of DNA from the donor to the recipient. Therefore, a loose clustering of functionally related operons increases the probability that many if not all of the determinants for a functional metabolic pathway would be provided to the recipient cell simultaneously (Wheelis & Stanier, 1970).

B. Physical Association of Enzymes

If the synthesis of functionally related enzymes is coordinately induced; if the genes which code for this synthesis are closely linked - or contiguous - on the chromosome; if these structural genes are closely linked to a regulatory (operator) gene forming a coordinate unit of genetic expression - an operon, then one would expect to observe a high degree of order among functionally related enzymes in the cell. An interesting relationship between the close linkage of functionally related genes and the closely aggregated enzyme products is demonstrated in the broad varieties of associations among the enzymes involved in the complete biosynthesis of tryptophan observed in different biological systems. For example, the tryptophan synthetase of Escherichia coli is a dimer of non-identical subunits which are noncovalently associated. The same multifunctional enzyme in Neurospora crassa, however, exists as a single polypeptide chain. In Euglena gracilis tryptophan synthetase appears to be part of a nondissociable aggregate of four other enzymes in the terminal pathway. This gene-enzyme inter-relationship as it applies to the tryptophan biosynthetic pathway in prokaryotes and eukaryotes has been extensively reviewed by Crawford (1975). Other examples of enzyme association include the observation of self-association among glycolytic enzymes (Mowbray & Moses,

1976), interactions of soluble enzymes with subcellular structures (Masters, 1978), and compartmentation and control of glycolytic enzymes (Ottaway & Moses, 1977). It is the increasing number of reports of the isolation of multifunctional proteins and multienzyme complexes, however, which is of major interest in this research. Occasionally there appears to be a tendency in the literature to use the terms "multifunctional protein" and "multienzyme complex" interchangeably. A multienzyme complex is "an aggregate of different functionally related enzymes bound together by noncovalent forces into a highly organized structure" (Ginsburg & Stadtman, 1970), whereas a multifunctional protein is a single covalent unit linked through α-peptide bonds, which has more than one enzyme activity (Stark, 1977). The term "multienzyme clusters" will be applied to physically associated enzymes in general, whether the association is covalent or noncovalent. Clustering of enzymes provides a distinct advantage to the cell in terms of metabolic economy and efficiency, the net result being localization or compartmentation of the enzymes. Thermodynamic and kinetic considerations aside, compartmentation of enzyme clusters may be a means first and foremost of reducing intracellular disarray due to freely dispersed enzymes and to freely diffusing substrates. Such a disorganized system would tax the solvent capacity of the cytoplasm (Atkinson,1969). The solvent capacity of water would be conserved by maintaining low

concentrations of all solutes. One mechanism by which the concentration of intermediate metabolites may be kept to a minimum in an enzyme complex is by "channeling", a process of "slow dissociation of a product from the complex to provide a high steady state concentration for the next enzyme (Stark, 1977). This mechanism works best to utilize intermediates of biosynthetic reactions, and also to isolate common intermediates of potentially competitive biosynthetic and degradative pathways (Ginsburg & Stadtman, 1970). The significance that multienzyme complexes hold for cellular regulation was summarized by Reed (1969) in a review of the pyruvate dehydrogenase complex. (1) Aggregation may itself produce, enhance, or modify a particular enzymatic activity. (2) Aggregation may stabilize the active conformation of one or more component enzymes of the complex. (3) Formation of a protein aggregate may confer new three-dimensional arrangements and thereby new modes of activity on the components of a complex. (4) If the activity of one of the component enzymes of a multienzyme complex is modulated by specific metabolites, then the physical association of the enzymes may allow coordinate control of all activities of the complex. In addition to the above advantages of enzyme complex-formation, enzyme clusters may also exhibit one or more of the following catalytic properties: (1) coordinate effects [cf. (4) above]; (2) reduction of the transient time - the time required for the system to undergo transitions

from one steady state to another; (3) reduction of diffusion or transit time; (4) rate enhancement (Gaertner, 1978). The role of multienzyme complexes in cellular metabolism has been extensively reviewed by Welch (1977). The areas discussed include enzyme organization in relation to the properties of the intracellular milieu; thermodynamic - kinetic aspects of enzyme aggregation and compartmentation; the evolution of aggregated multienzyme systems.

The isolation of multienzyme complexes and multifunctional proteins is complicated by a number of technical difficulties. Harsh methods of cell disruption, a decrease in intracellular viscosity by dilution, and a variety of isolation techniques all reduce the researcher's chances of preserving intact a noncovalently associated multienzyme complex. A number of enzyme aggregates, which had survived the isolation procedures, were discovered later to be multifunctional proteins. This phenomenon is the result of intracellular proteases which attack the exposed peptide bonds of the covalently linked enzymes. The arom cluster of Neurospora crassa was originally described as a multienzyme complex of five enzymes functioning early in the biosynthesis of tryptophan. Later reports demonstrated that N. crassa contained a protease which was responsible for peptide bond cleavage of a multifunctional protein. The arom aggregate, when isolated under protease-inhibiting conditions, proved to be a multifunctional protein with five

distinct sequential enzyme activities (Gaertner & Cole, 1976). Since proteases are present in nearly all cells, it is likely that a number of so-called multienzyme complexes are actually multifunctional proteins.

It appears that multifunctional proteins may have a selective advantage over the multienzyme complex (Stark, 1977). In the enzyme complex the noncovalent hydrophobic interaction must be stable enough to insure that the several different kinds of polypeptides stay together, and specific enough to form the correct complex forms. For prokaryotes with ca. 1000 proteins there are probably enough possibilities that stable and specific interactions would result. In eukaryotes which have many more kinds of proteins, covalent linkage of related enzymes would greatly aid the specificity of noncovalent interactions.

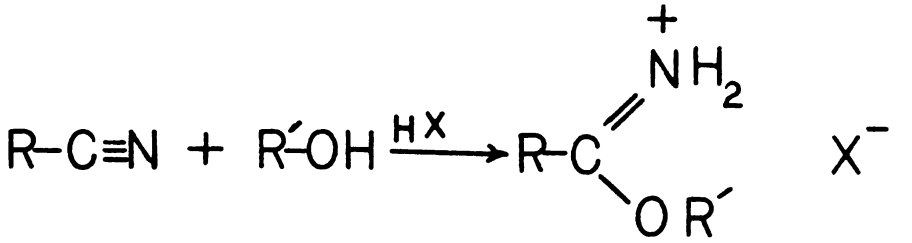
C. Imidoesters: Mechanism of Reaction with Proteins.

The rationale for this research project developed from the results of the regulation and genetics studies of the mandelate group of enzymes in P. putida, and from the considerations presented concerning multienzyme complexes. From the concept of "one gene, one enzyme" and the evidence that the genes coding for the mandelate enzymes almost certainly are members of an operon, it follows that the enzymes, as they are released from the ribosomes, may become closely associated within their environment. Because of the technical problems discussed above, the possibility of actually isolating a large complex of five enzymes appeared to be remote. If a technique could be developed whereby the presumed noncovalently associated enzymes could be covalently linked, then the chances of isolating a multienzyme complex would be somewhat improved.

Among the large number of compounds used to modify proteins chemically are the imidoesters. These compounds are reactive under relatively mild conditions, and more importantly, are available as bifunctional bisimidoesters which makes them desirable as cross-linking reagents.

The synthesis of imidoesters and their detailed study were carried out by A. Pinner in the late nineteenth century (1892). This synthesis consists of the hydrogen chloride or hydrogen bromide promoted addition of an alcohol to a

nitrile under anhydrous conditions to give the hydrohalide of the imidoester.



An extensive review of the chemistry of these imidoesters was reported by Roger and Neilson (1961). Unsubstituted imidoester salts when heated normally decompose into the corresponding amides and alkyl halides. The unsubstituted salts in water are hydrolyzed to the corresponding esters and ammonium salts (Pinner, 1892).

Formation of amidines by the imidoester-amine reaction was first investigated by Hunter and Ludwig (1962) as a means of specifically modifying proteins without altering the charge on the protein. The results showed that the rate of reaction of imidoesters with amino acids and proteins was pH-dependent, and that the pH-rate maximum was a function of the nature of both the amine and the imidoester being studied. The mechanism of the reaction was examined by Hand and Jencks (1962) by determining the rates and the products of the reaction of two benzimidate derivatives with several amines as a function of pH. The evidence strongly supported a mechanism involving a rate-determining decomposition of a

tetrahedral intermediate at low pH, and a change in the rate-determining step to a rate-determining formation of a tetrahedral intermediate from free amine and protonated imidoester at high pH (Hand & Jencks, 1962).

In addition to the use of imidoesters for the chemical modification of proteins, the bifunctional bisimidoesters (prepared from the dicyano-derivatives of dicarboxylic acids) have become extremely useful as cross-linking reagents in a variety of studies. The bisimidoesters form the cross-links by reacting with the ε-amino groups of lysine residues on the surface of enzymes resulting in covalent bisamidine products. Compounds of various chain lengths, e.g. dimethyl malonimidate, dimethyl adipimidate, and dimethyl suberimidate, have been used as probes of the nearest-neighbor relationship of proteins in ribosomes (reviewed in Kurland, 1974). Davies and Stark (1970) extended the use of these bifunctional reagents, particularly dimethyl suberimidate, to determine the subunit (or quaternary) structure of proteins. This technique has been widely used since its inception.

With the proliferating use of imidoesters in biochemical studies, it was becoming obvious from reports in the literature that some researchers were unaware of the earlier mechanistic studies of Hunter and Ludwig (1962) and Hand and Jencks (1962). The pH-optimum for imidoester-amine reactions ranges from 9 - 10. However, some reports showed

reaction conditions ranging from pH 7 to pH 10 and incubation periods of less than one hour to several days (Browne & Kent, 1975). In order to optimize conditions for the amidination of proteins with imidoesters, Browne and Kent (1975a,b) undertook studies using amines and the protein, liver alcohol dehydrogenase (LADH), to determine the stability of imidoesters in aqueous solutions at various pH's, and to determine the nature of the non-amidine products formed at lower pH. The results of the studies with the enzyme are summarized in Figure II.4. Treatment of the ε-amino groups of lysine residues in LADH with an imidoester at pH 10 resulted in rapid formation of a protein amidinated on 28 of its 30 lysine residues as determined by subsequent amino acid analysis. At pH 8.0 the initial reaction products were protein N-alkyl imidates rather than amidines. The protein N-alkyl imidate then appears to react equally with ammonia to form the protein N-alkyl amidine and with water to generate free protein plus unreactive ester. It is also possible for a free amino group on another enzyme (or another free amino group in the vicinity on the same enzyme) to react with the protein N-alkyl imidate to yield a disubstituted amidine. Optimum conditions for the amidination of LADH were either a single addition of imidoester to the enzyme at pH 10, or multiple additions at pH 8 until a stable preparation had been made.

In addition to the results of the Browne and Kent

Figure II.4.

Proposed mechanism for the reaction of an enzyme with imidoesters.

(redrawn from Browne & Kent, 1975b).

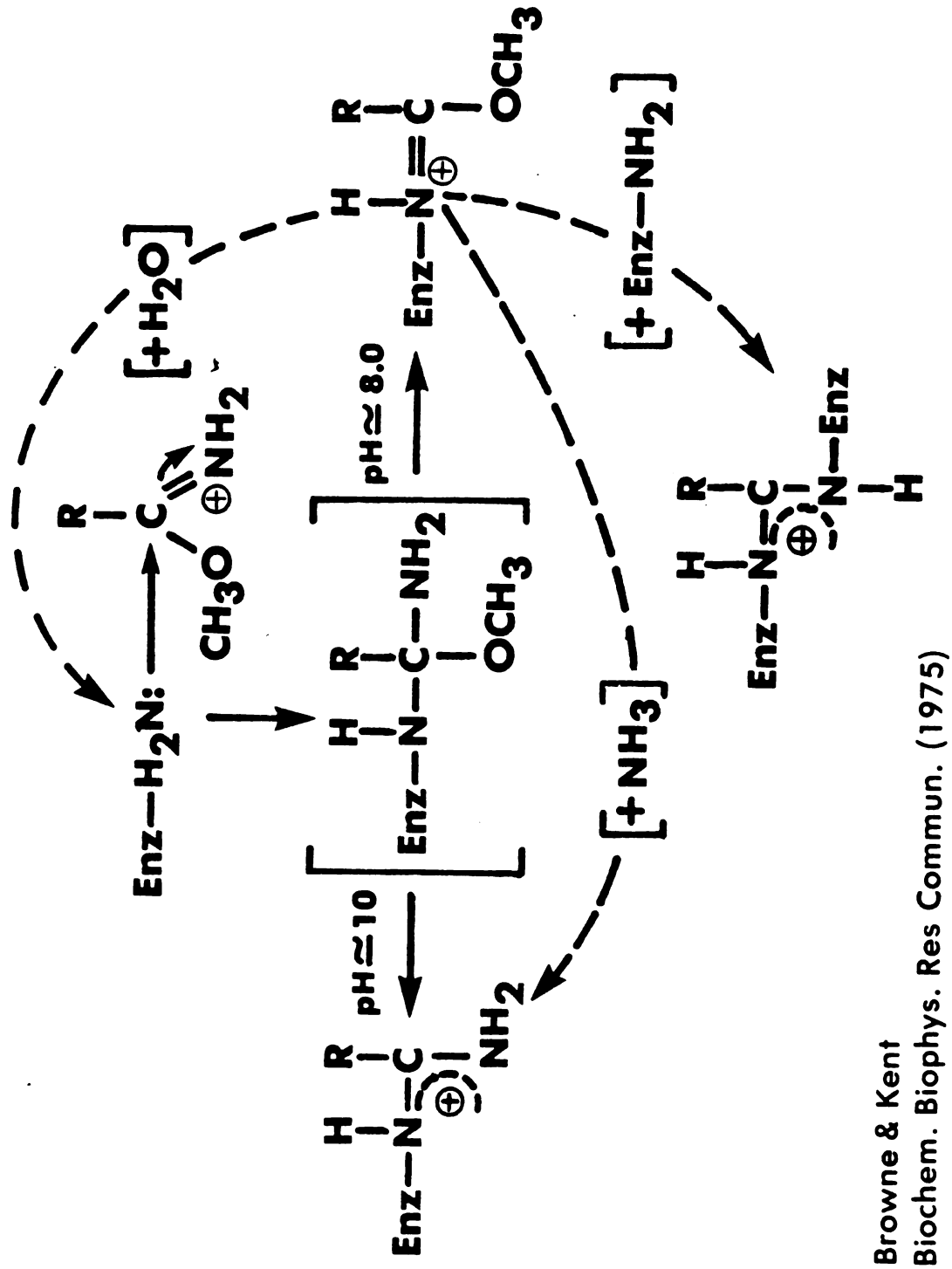

Enz—H2N:
CH3O ⊕NH2
R—C
[+H2O]
H—N—C—OCH3
Enz
R
pH≅8.0
Enz—N≡⊕
[+Enz—NH2]
[+NH3]
pH≅10
NH2
Enz—N=C
H
R
Browne & Kent
Biochem. Biophys. Res Commun. (1975)

Figure II.4.

study, it was necessary to be aware of several other points. (1) Many proteins are inactivated or denatured following incubation at pH 10. (2) The activity of benzoylformate decarboxylase, the third enzyme of the mandelate pathway, has a low pH-optimum of approximately 6.5. (3) The purpose of the cross-linking experiments in this project was not to amidinate completely all lysines of a specific protein, nor to cross-link completely all proteins in the proposed complex, but to provide sufficient linkages for stabilization of the complex to facilitate its isolation as such. Therefore, all cross-linking experiments described in this report were carried out at pH 8.0 with a single addition of the imidoester.

D. NMR Spectroscopy as a Probe of Metabolism in Living Systems..

Early uses of NMR spectroscopy as a probe of metabolism were limited, or almost impossible, due to the lack of sensitivity in the method. With the advent of Fourier transform NMR and superconducting magnets, it became possible to detect the weaker signals of such nuclei as phosphorus-31, carbon-13, and nitrogen-15. The report by Moon and Richards (1973) of the ^{31}P NMR spectrum of erythrocytes was rapidly followed by work from Radda's laboratory (Hoult *et al.*, 1974) identifying the major phosphate-containing compounds in intact muscle. The ^{31}P NMR spectra contained peaks identified as corresponding to inorganic phosphate, the sugar phosphates, phosphocreatine, and adenosine triphosphate. Wilkie and coworkers elegantly used the technique to measure the concentrations of the phosphorus-containing intermediates following contraction of skeletal muscle (Dawson, *et al.*, 1977). These early investigations of skeletal muscle and other intact systems using ^{31}P NMR have been recently reviewed by Burt *et al.*, (1979).

Shulman and coworkers have used both ^{31}P and ^{13}C NMR to study glycolysis and bioenergetics in a variety of whole cell systems, such as *E. coli*, yeast cells, rat liver cells, and purified rat liver mitochondria (reviewed in Shulman *et al.*, 1979). Their most recent work includes ^{13}C NMR studies

of gluconeogenesis in rat liver cells (Cohen et al., 1979) and ^{31}P NMR studies of adenine storage and secretion in platelets (Ugurbil et al., 1979). From ^{31}P NMR the Shulman group obtained a number of important results. They were able to measure the intracellular and extracellular pH in E. coli (Navon et al., 1977) and in yeast (Salhany et al., 1975); they were able to observe a pH difference between cytosol and mitochondria in rat liver cells, providing support for Mitchell's (1961) chemiosmotic model (Cohen et al., 1978); by using the saturation transfer technique they were able to measure the steady-state, unidirectional rates for ATPase-catalyzed synthesis of ATP from ADP and Pi in E. coli (Brown et al., 1977). This same group has also been able to follow metabolic pathways with ^{13}C NMR using ^{13}C-enriched substrates. By following glycolysis in E. coli (Ugurbil et al., 1978) and gluconeogenesis in rat liver cells (Cohen et al., 1979), it was possible to determine the distribution of the ^{13}C labels among the different carbons of intermediates and products.

Scott and coworkers were able to observe directly porphyrinogen biosynthesis in two bacterial strains from either ^{13}C-labeled δ-aminolevulinic acid or ^{13}C-labeled porphobilinogen using ^{13}C NMR (Scott et al., 1979). Further studies presented evidence that the transformation of porphobilinogen into uroporphyrinogens I and III occurred through a transient intermediate, pre-uroporphyrinogen (Burton et al.,

1979a). The structure of this intermediate was determined by monitoring its enzymatic synthesis from a doubly-labeled substrate ([1-^{15}N; 11-^{13}C]porphobilinogen) using both ^{13}C and ^{15}N NMR (Burton et al., 1979b).

These new applications of NMR spectroscopy provide researchers with unequivocal and noninvasive means of studying biochemical reactions in a large variety of cellular systems. It also appeared to be the obvious tool for characterizing the cross-linked, high molecular weight, multienzyme complex from P. putida. If the complex contained any or all of the enzymes capable of metabolizing mandelic acid, then it would be possible, in the presence of an appropiately labeled substrate, to follow the rise and fall of peaks corresponding to intermediates and substrate using ^{13}C NMR. There was already available in the laboratory from previous studies some 90%-enriched [^{13}C]mandelic acid labeled on the α-carbon (Maggio et al., 1975). With the label on the α-carbon it would be possible to follow changes in the oxidation state of the labeled carbon until it would be lost as [^{13}C]carbon dioxide in the conversion of benzoate to catechol (Figures II.5 & II.6). The monitoring of metabolism would be limited to the mandelate group of enzymes, which would greatly simplify the number of metabolites to be observed. The appearance in the NMR spectra of peaks corresponding to any intermediate would be conclusive evidence for the enzyme that produced it. The disappearance of

Figure II.5.

The metabolic pathway for the dissimilation of D,L-[2-^{13}C]mandelic acid to [α-^{13}C]benzoic acid.

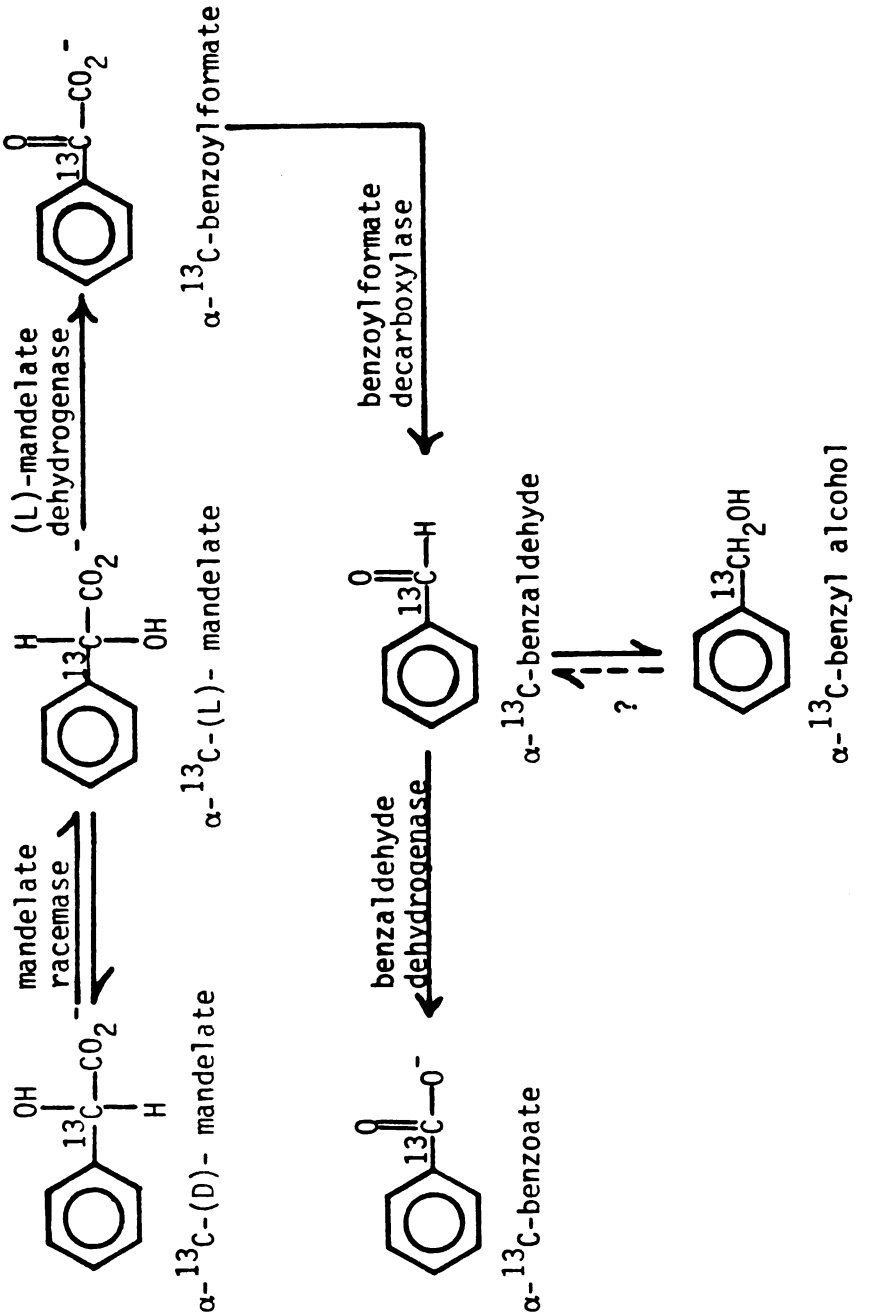

mandelate racemase
(L)-mandelate dehydrogenase
benzoylformate decarboxylase
benzaldehyde dehydrogenase
α-13C-(D)- mandelate
α-13C-(L)- mandelate
α-13C-benzoylformate
α-13C-benzoate
α-13C-benzaldehyde
α-13C-benzyl alcohol
?

Figure II.5.

Figure II.6.

The pathway for the conversion of [α-^{13}C]benzoic acid to [^{13}C]carbon dioxide and catechol.

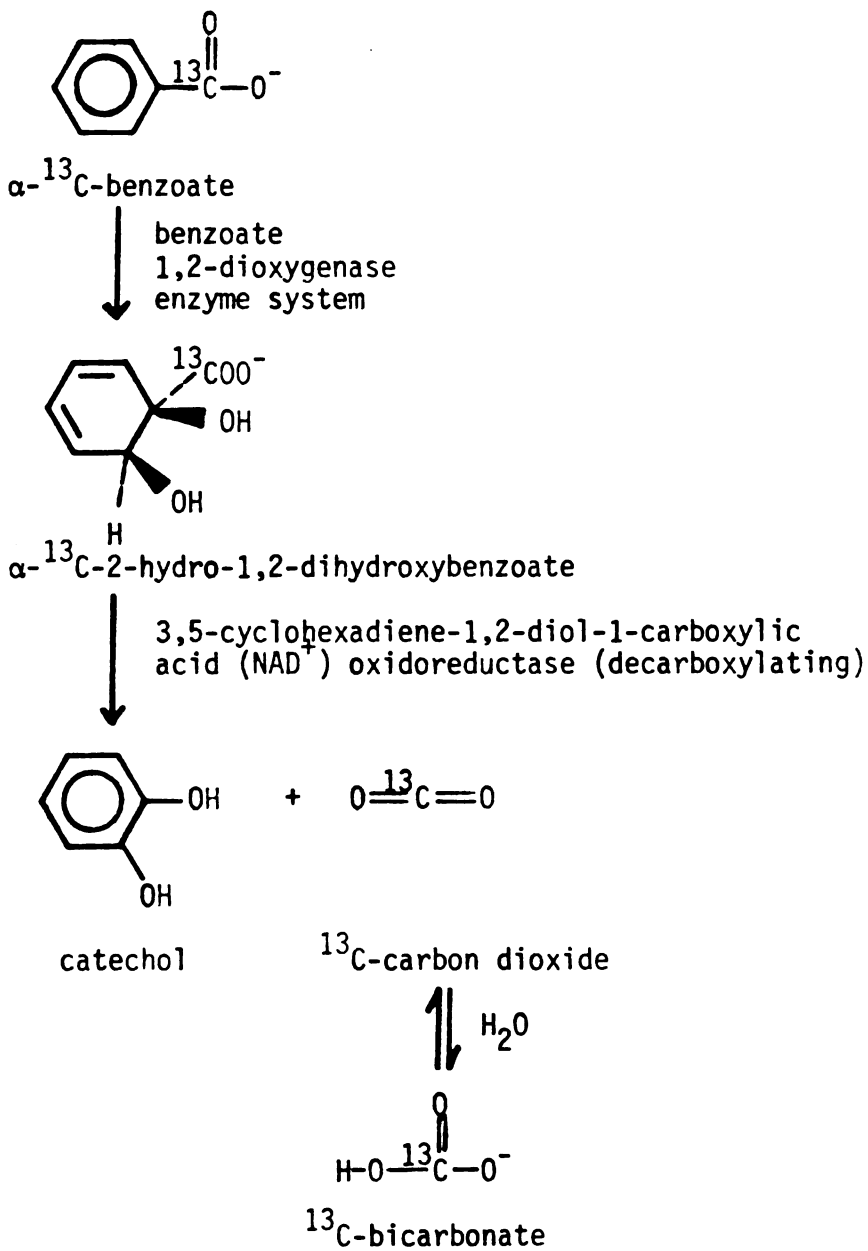

α-13C-benzoate
benzoate 1,2-dioxygenase enzyme system
α-13C-2-hydro-1,2-dihydroxybenzoate
3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (NAD+) oxidoreductase (decarboxylating)
catechol
13C-carbon dioxide
H2O
13C-bicarbonate

Figure II.6.

a peak from the spectra would provide evidence for the enzyme which metabolized the corresponding substrate. The ^{13}C NMR studies using whole cells of P. putida and the isolated cross-linked complex produced interesting as well as unexpected results, which are described in this thesis.

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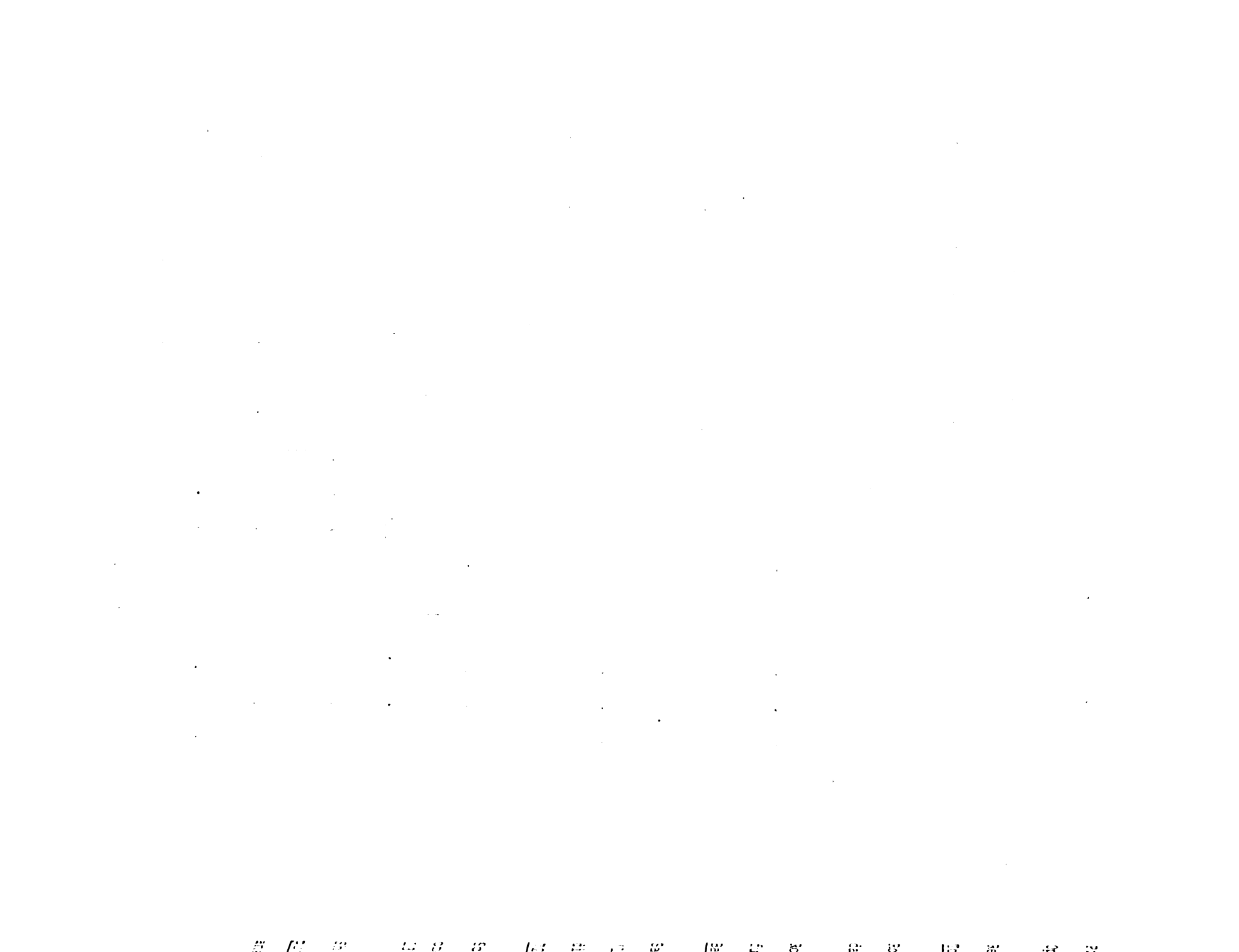

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III. MULTIENZYME COMPLEX: ISOLATION BY CHROMATOGRAPHY AND ULTRACENTRIFUGATION

A. Abstract

The physical association of intracellular enzymes has been investigated by treatment of whole bacterial cells with a cross-linking reagent followed by isolation of an enzymatically active, high molecular weight complex. Pseudomonas putida A.3.12 (ATCC 12633), a soil bacterium, is capable of utilizing mandelic acid as a sole source of carbon and energy. Five inducible enzymes (called the mandelate group) metabolize mandelate to benzoate. The second enzyme in the pathway, L-mandelate dehydrogenase, is membrane-bound; the other four enzymes are located in the cytosol. The benzoate oxidase system and the β-ketoadipate group of enzymes, also found in the cytosol, function in the further breakdown of benzoate to succinate and acetyl CoA, compounds readily able to enter general metabolism. The whole cells of P. putida were treated with the bifunctional bisimidoester, dimethyl suberimidate (DMSI), which forms cross-links between enzymes by reacting with free amino groups on the enzyme surface. The crude extract, obtained following sonic disruption of the cells and low-speed centrifugation to remove cellular debris, was subjected to gel permeation chromatography using LKB Ultrogel AcA22. Two peaks of enzyme activities were observed in the eluted fractions: a large peak of activity,

which also appeared in control samples, corresponding to uncross-linked enzymes, and a second, smaller peak which appeared in the fractions of the void volume. This second peak of enzymic activities was not observed in eluted extracts of untreated cells. A peak of L-mandelate dehydrogenase activity was also observed in the void volume fractions. A low level of this enzyme activity was present in the crude extracts of both untreated and DMSI-treated cells, even after low-speed centrifugation to remove cell membrane fragments. Larger quantities of the high molecular weight complex were isolated by ultracentrifugation of the crude extract at 100,000 x g for 1 h. Again appreciable levels of enzyme activities were observed in the 100,000 x g pellet from DMSI-treated cells, whereas, with the exception of L-mandelate dehydrogenase, little or no activities appeared in the control samples. DMSI-treatment of crude extract (instead of whole cells) followed by isolation of high molecular weight material by ultracentrifugation showed no increase of enzyme activities over untreated extracts. Ribonuclease treatment and lipase treatment of the *in vivo* cross-linked complex, isolated by ulracentrifugation, resulted in no loss of enzyme activity compared to the complex untreated by enzyme. The results are consistent with the idea that the enzymes of the mandelate group, and possibly one or more of the β-ketoadipate group, are all associated with the membrane-bound L-mandelate dehydrogenase.

B. Introduction

Biochemical reactions follow an orderly sequence of chemical events. It would appear that the enzymes involved in these sequential reactions would be organized or clustered in a particular manner to facilitiate the biosynthesis or the dissimilation of compounds essential for cellular vitality. A number of multienzyme complexes, usually containing from two to five enzymes in a cluster, have been isolated. But the question which still remains to be answered unequivocally is whether or not the enzymes of a given metabolic pathway associate using relatively weak hydrophobic interactions to form a catalytically efficient unit. We have undertaken the examination of this interesting problem in the relatively straightforward and dedicated pathway of mandelic acid metabolism in the bacterial strain, Pseudomonas putida.

Pseudomonas putida biotype A, strain 90 [ATCC 12633; also designated as P. putida (or fluorescense) A.3.12, and as PRS-1 (Chakrabarty et al., 1968, 1969)] is capable of utilizing D,L-mandelic acid as its sole source of carbon and energy by the strictly inducible pathway shown in Figure III.1. The metabolic sequence had been proposed by Stanier and coworkers (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 Gun-

Figure III.1.

The convergent pathways for the dissimilation of mandelic acid and p-hydroxymandelic acid in Pseudomonas putida.

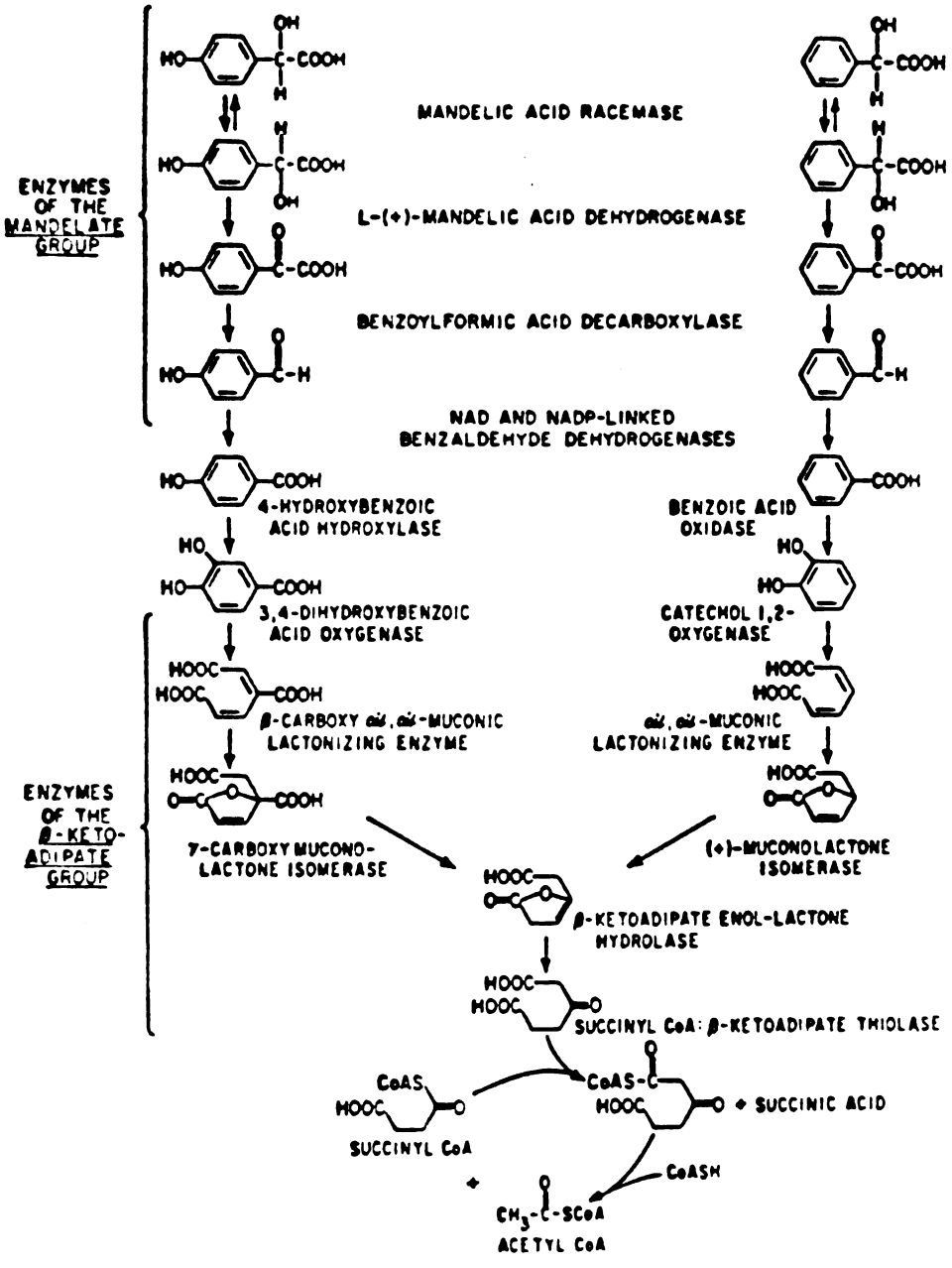
ENZYMES OF THE MANDELATE GROUP
MANDELIC ACID RACEMASE
L-(+)-MANDELIC ACID DEHYDROGENASE
BENZOYLFORMIC ACID DECARBOXYLASE
NAD AND NADP-LINKED BENZALDEHYDE DEHYDROGENASES
4-HYDROXYBENZOIC ACID HYDROXYLASE
BENZOIC ACID OXIDASE
3,4-DIHYDROXYBENZOIC ACID OXYGENASE
CATECHOL 1,2-OXYGENASE
β-CARBOXY cis,cis-MUCONIC LACTONIZING ENZYME
cis,cis-MUCONIC LACTONIZING ENZYME
ENZYMES OF THE β-KETO-ADIPATE GROUP
γ-CARBOXYMUCONO-LACTONE ISOMERASE
(+)-MUCONOLACTONE ISOMERASE
β-KETOADIPATE ENOL-LACTONE HYDROLASE
SUCCINYL CoA: β-KETOADIPATE THIOLASE
SUCCINYL CoA
SUCCINIC ACID
CoASH
ACETYL CoA

Figure III.1.

salus and Stanier (Gunsalus et al., 1953a,b; Stanier et al., 1953). 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 pathway: D-mandelate, L-mandelate, and benzoylformate (Hegeman, 1966a,b). Operator constitutive mutants, capable of constitutive, coordinate synthesis of the same five enzymes in the absence of an inducer, were isolated (Hegeman, 1966c). These studies provide evidence that the synthesis of the enzymes that metabolize mandelate to benzoate is coordinately induced and may be under genetic control by a complex operon.

The sequences of the β-ketoadipate pathway present a highly complex pattern of regulation. Synthesis of all the enzymes is strictly inducible, whereas synthesis of four enzymes of the protocatechuate branch (β-carboxymuconate lactonizing enzyme, γ-carboxy-muconolactone decarboxylase, β-ketoadipate enol-lactone hydrolase, and succinyl CoA: β-ketoadipate transferase) and two enzymes of the catechol branch (cis, cis-muconate lactonizing enzyme and muconolactone isomerase) are coordinately induced by β-ketoadipate (or β-ketoadipate CoA) and cis,cis-muconate, respectively. The genes specifying each of these two groups of enzymes may also be members of an operon (Ornston, 1966).

Genetic recombination studies using the host-range phage mutant of pf16h2 showed that a large segment of DNA

containing at least four genes functional in the conversion of mandelate to benzoate was transferred in intra- and inter-strain transduction (Chakrabarty et al., 1968). In the phase-mediated tranduction between the operator constitutive mutant isolated by Hegeman (1966c) and a camphor-degrading, mandelate-deleted C1 strain of P. putida, it appeared that the structural genes coding for the mandelate group plus a regulatory locus were transferred (Chakrabarty and Gunsalus, 1969). These genetic studies indicate that the genes specifying the synthesis of the mandelate enzymes are closely linked, if not contiguous, on the bacterial chromosome and therefore constitute an operon.

In an expansion of these genetic experiments to other dissimilatory pathways in P. putida Wheelis and Stanier (1970) observed a "supra-operonic clustering" of genes in two separate linkage groups. The smaller linkage group, comprising 3-4% of the bacterial chromosome, contained structural genes whose enzymes were associated with the catechol branch of the β-ketoadipate pathway, namely the mandelate sequence and benzoate oxidase genes and two genes of the catechol sequence. The larger linkage group, spanning nearly 5% of the chromosome, contained genes whose enzymes were associated with the protocatechuate branch of the pathway: an undetermined gene of the quinate pathway, a p-hydroxybenzoate hydroxylase gene, and four genes of the protocatechuate sequence. Observation of similar gene

clustering of the β-ketoadipate sequence in P. aeruginosa had been reported earlier by Hegeman and coworkers (Kemp and Hegeman, 1968; Rosenberg and Hegeman, 1979). During bacterial gene transfer only a small amount of the chromosome (about 3-5%) is transferred from the donor to the recipient. Therefore, a selective advantage exists if metabolically related genes are closely located in a region of the genome, favoring transfer of a complete metabolic pathway, or at least a physiologically functional segment of a pathway (Wheelis & Stanier, 1970).

The coordinate induction of enzyme synthesis, the close linkage of mandelate genes on the chromosome, and the clustering of the mandelate sequence and benzoate oxidase genes with two of the genes of the catechol pathway all pointed to the likelihood of organization among the resultant enzymes. Well-studied examples of the relationship of gene clustering and enzyme regulation with formation of enzyme complexes include the tryptophan biosynthetic enzymes in Escherichia coli and in Neurospora crassa and the prechorismate sequence of the polyaromatic biosynthetic pathway in fungi (reviewed in Ginsburg & Stadtman, 1970). A number of multienzyme complexes, such as the fatty acid synthetase and pyruvate dehydrogenase complexes, have been identified and studied because their inter-enzymic association is sufficiently strong to survive intact the harsh isolation and purification procedures. If the enzymes of the mandelate and β-

ketoadipate pathways are associated *in vivo* in a loose, non-covalent cluster, isolation of a functionally competent complex would be unlikely. Covalently linking these enzymes, however, would facilitate both the isolation and study of the complex.

Bifunctional bisimidoesters, 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 and the subunit structure of oligomeric proteins. Therefore, it was anticipated that one of these bisimidoesters would provide sufficient stabilization of the putative complex permitting its isolation and characterization. As a probe of *in vivo* macromolecular structure, we have employed imidoesters as gentle protein cross-linking reagents.

C. Materials and Methods

Materials were obtained from the following sources: all commonly used inorganic salts and organic solvents were analytical grade and obtained from Mallinckrodt or J.T. Baker; nitrilotriacetic acid, EDTA, triethanolamine, and Tris were purchased from J.T. Baker; yeast extract and agar were products of Difco Laboratories; D,L-mandelic acid, L(+)mandelic acid, D(-)mandelic acid, benzaldehyde, benzyl alcohol, and benzoic acid were from Aldrich; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) and 2,6-dichlorophenolindophenol (DCPIP) were from Calbiochem; Ultrogel AcA22 was from LKB; Blue Dextran 2000 was from Pharmacia; ribonuclease A and thiamine pyrophosphate hydrochloride were from Boehringer-Mannheim; dimethyl suberimidate dihydrochloride was from Pierce; 2,4-dinitrophenylhydrazine (DNP) was from Eastman; bovine serum albumin, bovine gamma globulin, brilliant Coomassie Blue G250, catalase, and lipase (free of α-amylase and protease) were from Sigma; cis,cis-muconic acid, L-mandelate dehydrogenase vesicles and a stock culture of P. putida A.3.12 were a generous gift of G.D. Hegeman. Water used for all solutions was distilled and deionized. Spectrophotometric assays were performed on a Gilford 2220A spectrophotometer.

1. 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 $NaKHPO_4$ 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 a mineral medium (Hegeman, 1966a) containing D,L-mandelate as the ammonium salt at a final concentration of 10 mM. (A detailed description of growth conditions is provided in the microbiological procedures of Chapter VI.) The cultures were incubated with vigorous shaking at 30° C. Growth was measured turbimetrically in a Klett-Summerson colorimeter, using a No. 54 filter. The cells were harvested in the late-log phase by centrifugation at 10,000 x g for 10 min. The sedimented cells were suspended in 0.05 M triethanolamine·HCl buffer (pH 8.0), containing 0.1 M NaCl and 10 mM $MgCl_2$ (TNM buffer), and centrifugation was repeated. Two pellets of packed cells, each of 0.7-0.9g wet weight, were obtained.

2. Preparation of Crude Extracts

For each experiment one pellet was suspended in 3.5-4.0 ml TNM buffer (pH 8.0) and disrupted by sonic oscillation for 1 min in a salt-ice bath at 100 watts using a probe-type oscillator (Branson Sonifier Cell Disruptor Model W350).

The cellular debris was removed by centrifugation at 10,000 x g for 10 min, and the supernatant (crude extract) was kept at 4° C, following dilution to 1.25 mg/ml with TNM buffer, until assayed for enzyme activities described below.

3. Dimethyl Suberimidate Treatment of Whole Cells

Freshly harvested whole cells (0.7-0.9g wet weight) were suspended in 3 ml 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 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 cold TNM buffer, followed by centrifugation at 10,000 x g for 10 min. The cells were resuspended in TNM buffer and centrifugation was repeated. Crude extracts from control and DMSI-treated cells were prepared as described above.

4. Gel Permeation Chromatography of Crude Extract

The crude extract (20 mg protein) was layered on Ultrogel AcA 22 [2% acrylamide, 2% agarose; 1.6 x (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 per 10-15 sec. Each fraction contained 40 drops (ca. 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.

5. 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 in a Beckman No.40 rotor at 100,000 x g for 1 h at 4° C. The supernatant was saved for enzyme analysis. The 100,000 x g pellet was suspended in TNM buffer using a Potter-Elvehjem homogenizer, diluted to 10 ml, and centrifuged again at 100,000 x g for 1 h. The resuspension-centrifugation step was repeated twice more. The supernatant washes were saved for qualitative assay of enzyme activity. After the third wash the 100,000 x g pellet was suspended by homogenization in TNM buffer to a final volume of 2 ml and assayed without dilution for enzyme activities. The suspension (10 mg protein) was also subjected to gel permeation chromatography as described above.

6. Enzyme Assays and Other Assays

cis,cis-Muconate lactonizing enzyme [EC 5.5.1.1, (+)-4-carboxymethyl-4-hydroxyisocrotonolactone lyase (decyclizing)], L-mandelate dehydrogenase [no EC number, L(+)-mandelate: (acceptor) oxidoreductase] and mandelate racemase (EC 5.1.2.2, mandelate racemase) activities were measured as described by Hegeman (1966a) (with modifications described in the biochemical procedures of Chapter VI). Benzoylformate decarboxylase (EC 4.1.1.7, benzoylformate carboxylase) activity was measured by a modification of the method of Jamaluddin et al., (1970) and details of the procedure are described in Chapters V and VI.

A qualitative assay of mandelate racemase activity was used to locate the enzyme in column eluants or to detect its presence in supernatant washes. The reaction mixture contained all the reagents of the quantitative assay. The reaction was started by addition of 0.1 ml sample, and the time required for bleaching of the DCPIP was measured relative to that of a blank tube. To detect the presence of small quantities of racemase in supernatant washes, 2.0 ml of the wash solution was substituted for the Hepes buffer, and reaction was initiated by addition of D-mandelate.

Protein determinations were measured by either the biuret method (Gornall et al., 1949) using bovine serum albumin as standard or the Coomassie Blue dye-binding pro-

cedures of Bradford (1976) using bovine γ-globulin as standard.

7. Dimethyl Suberimidate Treatment of Crude Extract

The crude extract of freshly harvested 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 100,000 x g for 1 h at 4° C. The pellet was suspended by homogenization in TNM buffer and centrifuged at 100,000 x g for 1 h. The suspension-centrifugation step was repeated twice more. The 100,000 x g pellet was suspended by homogenization to a final volume of 2.0 ml with TNM buffer, and assayed for enzyme activities without further dilution.

8. Ribonuclease Treatment of Isolated High Molecular Weight Complex

Freshly harvested cells were treated with DMSI, the crude extract prepared, and the 100,000 x g pellet isolated as described above except that Mg^{2+} was omitted from the buffer during the washing procedure of the 100,000 x g

pellet. The pellet was suspended by homogenization in 2.0 ml 0.05 M triethanolamine·HCl buffer (pH 8.0) containing 0.1 M NaCl, and a 0.1 ml aliquot was assayed for protein concentration. The suspension was divided into 2 - 1 ml aliquots and EDTA (pH 7.0) to a final concentration of 1 mM was added to each sample. To one sample (the control) was added 0.02 ml triethanolamine-NaCl buffer, and to the other sample was added 0.02 ml ribonuclease A, 10 mg/ml in triethanolamine-NaCl buffer. The mixture was incubated at 25° C with gentle swirling for 1 hr. The digestion was terminated by addition of $MgCl_2$ to a final concentration of 10 mM. The samples were diluted to 2 ml with cold TNM buffer and centrifuged in the Beckman No. 40 rotor fitted with adaptors at 100,000 x g for 1 h at 4° C. The supernatants and the pellets, suspended in 1 ml TNM buffer by homogenization, were assayed for protein concentration and enzyme activities.

9. Lipase Treatment of the Isolated High Molecular Weight Complex

The samples were prepared for lipase digestion as described for the ribonuclease treatment above, except that all buffers contained Mg^{2+}. Two 1 ml aliquots of DMSI-treated 100,000 x g pellet suspension were placed in small beakers. To one sample (the control) was added 0.025 ml TNM buffer and to the other sample 0.025 ml lipase, 10 mg/ml in TNM buffer, was added. The mixture was incubated at 25° C

with gentle swirling for 1 h. The reaction was terminated by dilution of the samples to 2.0 ml with cold TNM buffer followed by centrifugation in the Beckman No. 40 rotor fitted with adaptors at 100,000 x g for 1 h. The supernatants and the pellets, suspended in 1 ml TNM buffer by homogenization, were assayed for protein concentration and enzyme activities.

D. Results

1. Chromatography of Crude Extract from Untreated Whole Cells

The elution pattern of the crude extract from untreated bacterial cells is shown in Figure III.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 absorption peaks, three have 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 260/280 ratio of the first peak which appears at the void volume is very nearly 1:1, and the fractions in this region have a turbid, opalescent appearance. This material probably contains ribosomes along with other excluded high molecular weight material. In the region between the second and third absorbance peaks a small peak is observed with a 260/280 ratio of approximately 0.7 indicating the presence of proteins. Protein assays of each fraction (the results are not plotted) indicate 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

Figure III.2.

Gel permeation chromatography of crude extract from untreated cells was applied to the column using 0.05 M triethanolamine·HCl buffer (pH 8.0), 0.1 M NaCl, 0.01 M $MgCl_2$, 0.02% NaN_3 as eluant. Elution conditions are described in "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 (▲---▲).

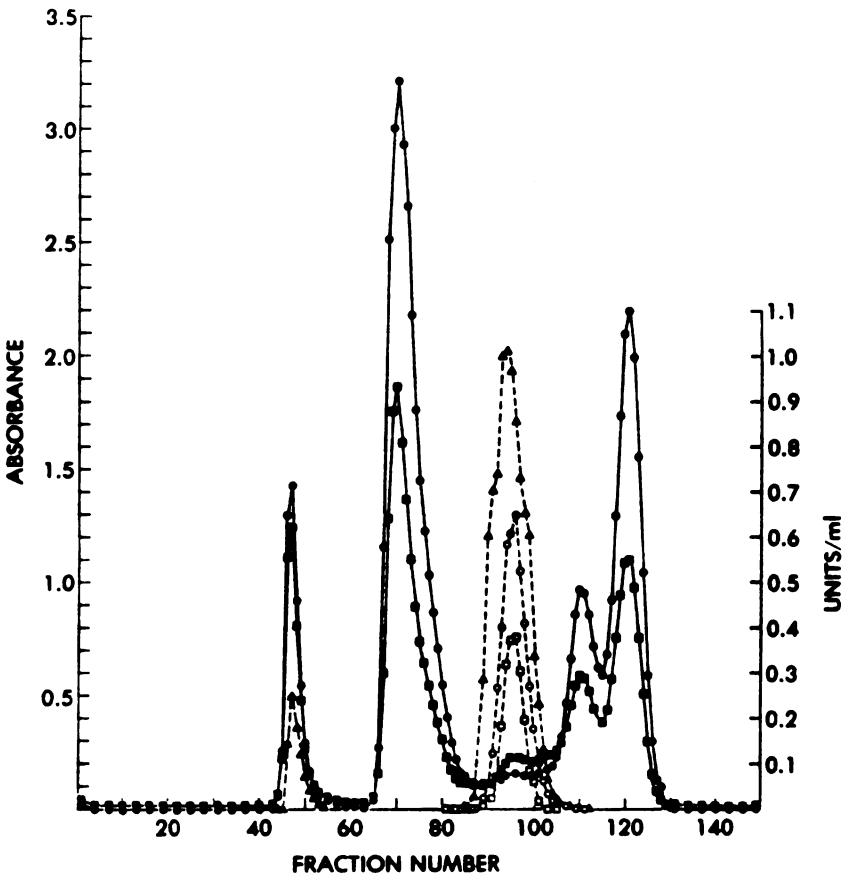
ABSORBANCE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
UNITS/ml
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
FRACTION NUMBER

Figure III.2.

rapidly falls to zero in the beginning region of the third peak. It is in this region of high protein concentration that the activities of benzoylformate decarboxylase, cis,cis-muconate lactonizing enzyme, and mandelate racemase - all enzymes found in the cytosol - are observed. The activity of L-mandelate dehydrogenase, a membrane-bound enzyme, appears solely in the fractions of the exclusion volume.

2. Chromatograhy 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 III.3. The overall absorption pattern and the location of the uncross-linked enzymes are nearly identical to those observed in Figure III.2. The activity of cis,cis-muconate lactonizing enzyme, however, appears to be inhibited to some degree by the DMSI treatment. The major difference between the DMSI-treated sample and the control is the appearance in the exclusion volume of low levels of activities corresponding to benzoyl-formate decarboxylase, cis,cis-muconate lactonizing enzyme, and mandelate racemase. There is also a low plateau of mandelate racemase activity through the fractions of the second peak, which is not observed in the control fractions. The activities of the other two enzyme were not observed in this region, either because they are not present or because

Figure III.3.

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

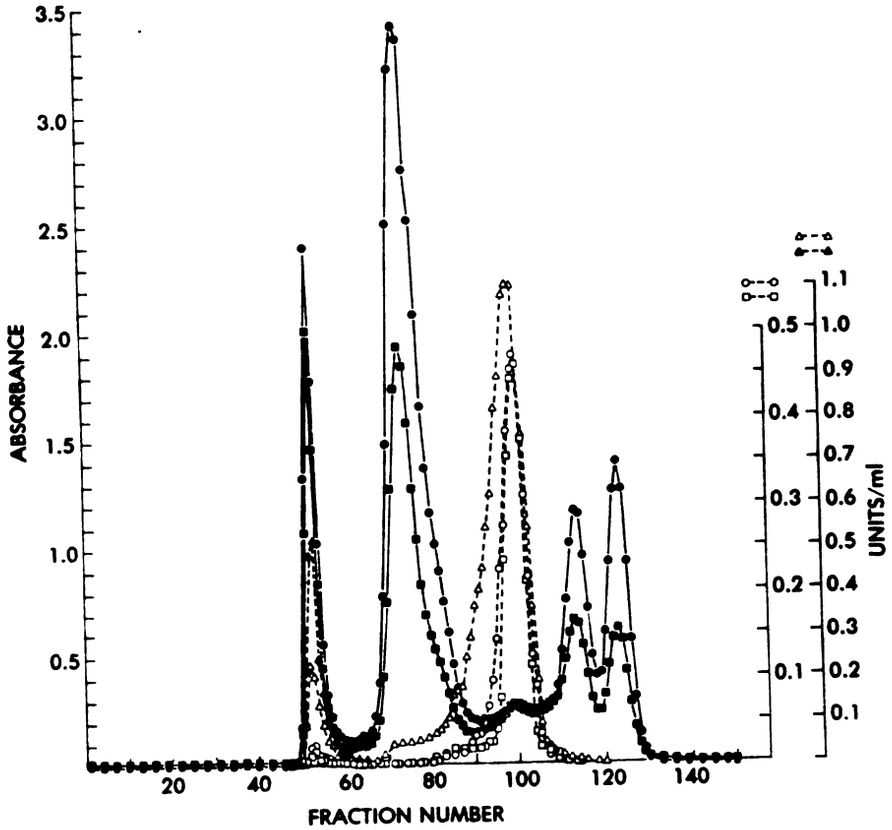
ABSORBANCE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.5
0.4
0.3
0.2
0.1
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
UNITS/ml
20
40
60
80
100
120
140
FRACTION NUMBER

Figure III.3.

ity levels are so low as not to be detectible.

3. Isolation of the High Molecular Weight Multienzyme Complex by Ultracentrifugation

he high molecular weight material isolated by centri-
on at 100,000 x g for 1 h was washed three times with
to remove extraneous, loosely bound enzymes. The
atant washes were qualitatively analyzed for mandelate
se activity. The time required for the second and
washes of the untreated sample to bleach the DCPIP was
imately 45-60 min. The second and third washes of the
reated sample, however, bleached the DCPIP within 5
indicating that significant amounts of mandelate
se were still being removed from the high molecular
material. No further washes of the isolated DMSI-
material were carried out in order to avoid loss of
activities present in small amounts.

e suspension of the washed 100,000 x g pellet was
for enzyme activities and the results from untreated
I-treated samples are summarized in Table I. Barely
ble levels of the soluble enzymes are observed in the
, whereas, a 9- to 25-fold increase in enzyme activi-
observed in the DMSI-treated sample. The difference
ndelate dehydrogenase activities in the untreated and
eated materials shows only a small decrease following
eatment.

TABLE I

Specific activities of enzymes in untreated and DMSI-treated suspensions of 100,000 x g pellets

Enzymes	Specific activities (μmole·min^{-1}·mg protein^{-1}) Control	DMSI-treated
Benzoylformate decarboxylase	0.002	0.018
cis,cis-Muconate lactonizing enzyme	0.002	0.023
Mandelate racemase	0.007	0.173
L-Mandelate dehydrogenase	0.746	0.629

. Dimethyl Suberimidate Treatment of Crude Extract

The crude extract, rather than intact bacterial cells, ubjected to DMSI-treatment followed by isolation of the 00 x g material. The specific activities of the four es in this material were not increased above the con- levels shown in Table I.

5. Chromatography of 100,000 x g Pellet Suspension Isolated from Dimethyl Suberimidate-treated Whole Cells

The elution pattern of the 100,000 x g pellet suspen- isolated from DMSI-treated cells (Figure III.4) is ar to that of Figure III.3 except that the peaks

ıre III.4.

Gel permeation chromatography using Ultrogel AcA22 of the 100,000 x g pellet suspension isolated from dimethyl suberimidate-treated cells. The suspension (10 mg protein) was applied to the column using 0.05 M triethanolamine·HCl buffer (pH 8.0), 0.1 M NaCl, 0.01 M $MgCl_2$, 0.02% NaN_3 as eluant. Each fraction was assayed for absorbances at 260 and 280 nm (not shown), for protein concentration (●——●), and for the enzyme activities of cis,cis-muconate lactonizing enzyme (O---O), mandelate racemase (△---△), and L-mandelate dehydrogenase (▲---▲).

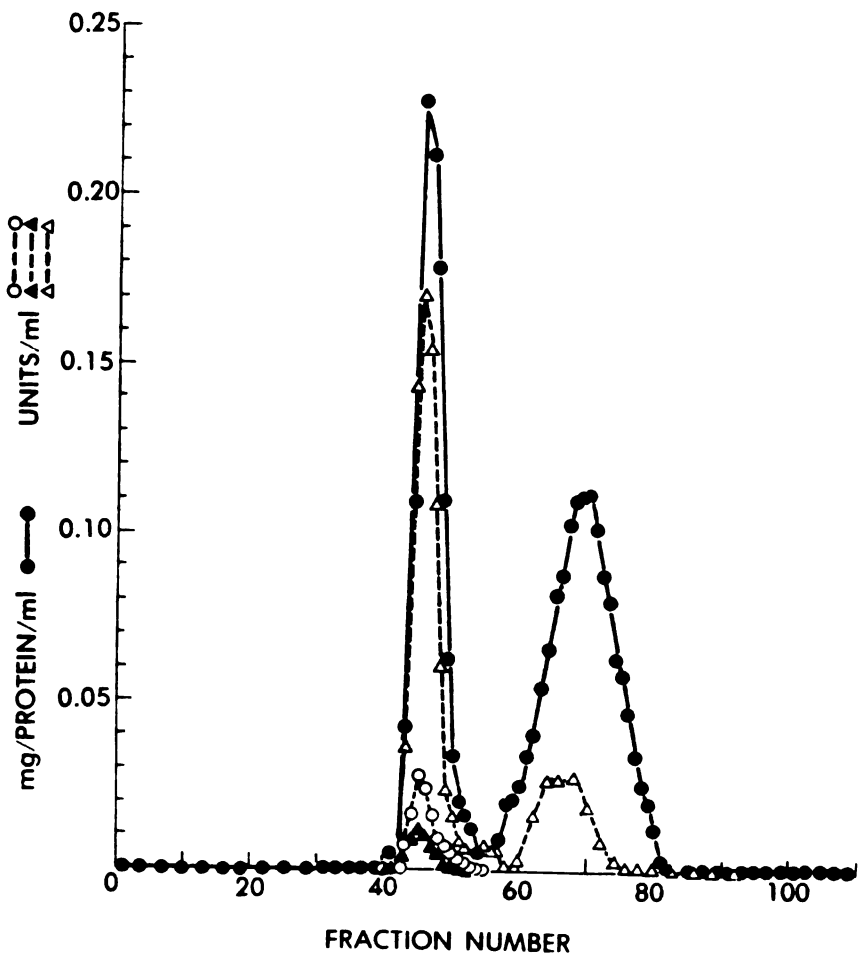
0.25
0.20
0.15
0.10
0.05
0
20
40
60
80
100
UNITS/ml
mg/PROTEIN/ml
FRACTION NUMBER

Figure III.4.

esponding to the uncross-linked enzymes and low molecu- weight nucleic acids are not observed. The absorbance es are not plotted here; however, the 260/280 ratio es are essentially the same as those observed for the st two peaks of eluted crude extracts. The protein con- tration of the fractions, as was observed for the crude racts, is much higher in the void volume peak than in the cond peak. Benzoylformate decarboxylase activity was not tected in the exclusion volume fraction. Only cis,cis- conate lactonizing enzyme and mandelate racemase along ith L-mandelate dehydrogenase, were observed in the high olecular weight protein peak. As in Figure III.3 a low peak of only mandelate racemase activity appeared in the second peak, but the activity tapered off to zero.

6. Ribonuclease and Lipase Treatment of the 100,000 x g Pellet Suspension Isolated from Dimethyl Suberimidate-treated Whole Cells

Ribonuclease and lipase treatments of the high molecular weight enzyme complex appeared to have no appreciable effect on the enzyme activities of the resuspended 100,000 x g pellets when the results of the control and enzyme-treated samples are compared (Table II). Similar results are observed in the supernatants obtained from these same samples (Table III).

TABLE II.

Specific activites of high molecular weight complex following enzyme treatment

(μmole·min^{-1}·mg protein^{-1})

;	Control	RNase	Control	Lipase
lformate oxylase	0.013	0.015	0.020	0.023
s-Muconate izing enzyme	0.020	0.027	0.031	0.035
ate racemase	0.199	0.186	0.164	0.192
elate ogenase	0.524	0.627	0.505	0.621

TABLE III.

Specific activities of supernatants from enzyme-treated complexes

(μmole·min^{-1}·mg protein^{-1})

s	Control	RNase	Control	Lipase
lformate oxylase	0.067	0.076	0.099	0.087
s-Muconate izing enzyme	0.135	0.164	0.137	0.113
ate racemase	1.425	1.704	1.096	0.987
elate ogenase	0.524	0.755	0.117	0.097

E. Discussion

he use of imidoesters as chemical modifiers of lysine
es in proteins was first reported by Hunter and Ludwig
. The mechanism of the reaction was found to be pH-
ent involving a change in the rate-determining step,
at low pH was the decomposition of the tetrahedral
on intermediate and at high pH involved the rate-
ining formation of the tetrahedral intermediate from
ree amine and protonated imidoester (Hand & Jencks,
. The bifunctional bisimidoesters have become
nely useful as probes of macromolecular structure.
reagents form cross-links by reacting with the ε-amino
s of lysine residues on the surface of enzymes result-
n covalent bis-amidine products. Compounds of various
lengths have been used as probes of the nearest-
bor relationship of ribosomal proteins (Kurland, 1974).
s and Stark (1970) extended the use of the bisimidoes-
particularly dimethyl suberimidate, to determine the
rnary structure of oligomeric proteins.

In studies undertaken to optimize amidination condi-
s Browne and Kent (1975) found that the half-life of
oesters in aqueous solutions was both temperature- and
pendent and that complete amidination of the protein,
r alcohol dehydrogenase, occurred either with a single
tion of imidoester at pH 10 or with multiple additions

at pH 8 until a stable preparation had been made.

Our own cross-linking experiments were carried out with a single addition of dimethyl suberimidate to whole bacterial cells at pH 8.0 for two reasons. First, one of the enzymes of the mandelate pathway, benzoylformate decarboxylase, has a pH-optimum of only 6.0-6.5 (Gunsalus, et al., 1953a). Incubation of the cells at pH 10 would probably inactivate this enzyme and possibly others. Second, the purpose of the cross-linking experiments was not to amidinate a protein completely or to cross-link all the possible proteins, but simply to provide sufficient covalent linkages for stabilization of the putative complex of enzymes. Comparison of the elution profiles of crude extracts from control (Figure III.2) and DMSI-treated (Figure III.3) cells shows that treatment of P. putida whole cells with dimethyl suberimidate permitted isolation of an enzymatically active, high molecular weight complex of proteins. Treatment of the crude extract from cells disrupted by sonic oscillation does not yield the high molecular weight complex since the disruption process and the concomitant dilution by the buffer solution in which the cells were suspended apparently serve to perturb the internal organization of the enzymes.

Larger quantities of this complex, which has a molecular weight estimated to be 2.5×10^6 (Kenyon et al., 1976), were isolable by ultracentrifugation of the crude extract at 100,000 x g for 1 h. The amount of enzymatically active

complex obtained by chromatography contained approximately 2 mg protein, whereas ca. 20 mg protein was present in the material isolated by ultracentrifugation.

Gel permeation chromatography of the 100,000 x g pellet isolated from DMSI-treated cells (Figure III.4) demonstrates the effectiveness of ultracentrifugation in the separation of cross-linked and uncross-linked enzymes. Benzoylformate decarboxylase activity was not observed in the fractions of the void volume peak probably due to loss of activity during the course of the experiment.

The appearance of low levels of mandelate racemase activity in the second peak of the eluted crude extract (Figure III.3) and the 100,000 x g pellet suspension (Figure III.4) from DMSI-treated cells may be due to random cross-linking of mandelate racemase or 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 their concentrations were diluted during elution from the column. That the latter may be true is substantiated by the appearance of all four enzyme activities in the supernatants (Table III) of the control and enzyme-treated high molecular weight complexes. The supernatants obtained from the three buffer washes of the 100,000 x g pellets prior to enzyme treatment had been assayed qualitatively for mandelate racemase activity only. The 10 ml-volume would probably have

precluded detection of the low levels of benzoylformate decarboxylase and possibly the cis,cis-muconate lactonizing enzyme activities which were observable in the 2 ml-supernatant obtained following ribonuclease and lipase treatments of the isolated complex. Obviously small amounts of the enzymes are being removed continuously from the high molecular weight complex with repeated supension and centrifugation of the 100,000 x g pellets. Therefore, it appears likely that dimethyl suberimidate acts to trap a "cluster" of enzymes within a net, but chromatography or repeated centrifugation allows the more loosely held enzymes to escape.

A question may be raised as to the nature of the cross-linked 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? To test the possibility that the enzymes may be attached to either ribosomes or a segment of the menbrane, aliquots of the in vivo cross-linked complex, isolated by ultracentrifugation, were treated with ribonuclease and with lipase. The results of these experiments, however, were inconclusive. There appeared to be no difference in the specific activities of the enzymes assayed in treated (RNase and lipase) and untreated (control) complex suspensions. It will be necessary to design other experiments which will better determine the composition of the cross-linked complex.

From the results of these experiments we conclude that

dimethyl suberimidate is sufficiently lipophilic to penetrate the cell membrane, and that it covalently links enzymes of the mandelate pathway that may be "clustered" about, or loosely associated with the membrane-bound L-mandelate dehydrogenase. This enclosed group of enzymes is sufficiently stable to survive the disruption and isolation procedures.

Experiments are in progress to purify the isolated, in vivo cross-linked multienzyme complex and to identify the components contained within it. It has been possible, however, to identify conclusively some of the enzymes present in the crude complex using ^{13}C NMR spectroscopy. These results are described in the following chapter.

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IV. MULTIENZYME COMPLEX: CHARACTERIZATION BY ^{13}C NMR SPECTROSCOPY

A. Abstract

The composition of the *in vivo* cross-linked complex of enzymes, the isolation of which was described in the previous chapter, was characterized by ^{13}C NMR spectroscopy. Initial experiments to determine which metabolites would be observable by NMR were carried out using whole cells of *Pseudomonas putida* in the presence of 90%-enriched [2-^{13}C] D,L-mandelic acid. Following periods of oxygenation, the signal for the labeled α-carbon of mandelate gradually decreased while signals corresponding to the α-carbons of benzaldehyde, benzoic acid, and benzyl alcohol, a previously unreported metabolite of mandelate metabolism, appeared and increased. [^{13}C]Bicarbonate, a product of the decarboxylation of benzoate, was also observed. When the starting concentration of [α-^{13}C]mandelate was reduced from 50 mM to 12.5 mM the amount of benzyl alcohol produced was significantly reduced. Oxygenation of the isolated *in vivo* cross-linked enzyme complex in the presence of either 50 mM or 12.5 mM [α-^{13}C]mandelate also resulted in the metabolism of mandelate to benzoate as followed by ^{13}C NMR spectroscopy. The metabolites observed in addition to the substrate, [α-^{13}C]mandelate, were [α-^{13}C]benzoylformate, [α-^{13}C]benzaldehyde, and [α-^{13}C]benzoate. ^{13}C NMR spectra of

isolated, untreated high molecular weight material showed no greater than 50% reduction of peak intensity of the mandelate signal, and [α-^{13}C]benzoylformate was the only product observed. This result was due to the presence of the membrane-bound, stereospecific L-mandelate dehydrogenase which had been observed in the 100,000 x g pellets of both untreated and DMSI-treated cells. From the ^{13}C NMR data it is clearly evident that all five enzymes of the mandelate group are present in the isolated, in vivo cross-linked multienzyme complex. These results support the conclusions of the previous chapter that the enzymes of the mandelate group are all associated with the membrane-bound L-mandelate dehydrogenase. In vivo treatment of this group of associated enzymes with dimethyl suberimidate may entirely cross-link these enzymes, but results appear to indicate that they are only partially cross-linked entrapping other enzymes in a loosely entwined net.

B. Introduction

In less than a decade NMR spectroscopy has proved to be an excellent tool for the study of a variety of processes in biological systems. The technique is non-destructive and non-invasive; and with the advent of Fourier Transform NMR, superconducting magnets, and enrichment techniques it is possible to detect the weaker signals of such biologically significant nuclei as phosphorus-31, carbon-13, and nitrogen-15. The initial use of ^{31}P NMR by Moon and Richards (1975) to study the intracellular pH and phosphorylated metabolites of erythrocytes was rapidly followed by work from Radda's laboratory (Hoult *et al.*, 1974) identifying the major phosphate-containing compounds in skeletal muscle. Wilkie and coworkers designed an elegant experimental chamber in which to study levels of phosphorus-containing metabolites in skeletal muscles during contraction (Dawson *et al.*, 1977). These early investigations of metabolism in skeletal muscle and other intact systems with ^{31}P NMR have recently been reviewed by Burt *et al.* (1979).

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 coworkers have 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). Their most recent work includes ^{13}C NMR studies of gluconeogenesis in rat liver cells (Cohen et al., 1979) and ^{31}P NMR studies of adenine nucleotide storage and secretion in platelets (Uburgil, et al., 1979).

Scott and coworkers were able to observe directly the biosynthesis of porphyrinogen in two bacterial strains from ^{13}C-enriched substrates using ^{13}C NMR (Scott et al., 1979). Further studies presented evidence that the transformation of porphobilinogen into uroporphyrinogens I and III occurred through a transient intermediate, pre-uroporphyrinogen (Burton et al., 1979a). The structure of this intermediate was determined by monitoring its synthesis from a doubly-labeled substrate ([1-^{15}N, 11-^{13}C]porphobilinogen) using both ^{13}C and ^{15}N NMR (Burton et al., 1979b).

Further study and characterization of the cross-linked, high molecular weight, multienzyme complex (isolated from Pseudomonas putida A.3.12 as described in the previous chapter) was hampered by difficulties in attempts to purify the complex. ^{13}C NMR spectroscopy of the crude complex, however, in the presence of an appropriately enriched mandelic acid, would provide conclusive evidence of the presence of any or all of the enzymes functional in the metabolism of the substrate. Biochemical analysis showed that three enzymes of the mandelate group (mandelate racemase, L-mandelate dehydrogenase, and benzoylformate decarboxylase)

and one enzyme of the β-ketoadipate group (cis,cis-muconate lactonizing enzyme) were present in the complex, but the question remained as to how many other functioning enzymes had been trapped in the cluster by cross-linking with dimethyl suberimidate.

The metabolism of D,L-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. The sequence of reactions would be limited to the mandelate group enzymes (plus the benzoate oxidase system if benzoate was metabolized), which would greatly simplify the number of intermediates to be observed in the NMR spectrum. In order to determine which metabolites would be observable by NMR, however, and also to optimize NMR parameters, initial experiments were carried out using whole cells from P. putida.

C. Materials and Methods

Materials were obtained from the following sources: deuterium oxide, 99.8 atom % was purchased from Aldrich; [2-^{13}C]D,L-mandelic acid was synthesized by Dr. E. T. Maggio (Maggio et al., 1975); all other materials were obtained as described in the previous chapter.

1. Conditions of Cultivation

Stock cultures of P. putida A.3.12 were maintained, and cells were grown as described in the previous chapter (and in chapter VI) with the exception that cells were harvested in the mid-log phase when turbidity measured 100 Klett units. The harvested cells were washed three times with 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$ with centrifugation at 10,000 x g for 10 min. From a 500 ml culture one pellet of packed cells, 0.5-0.7g wet weight, were obtained.

2. Preparation of Whole Cells for ^{13}C NMR

The washed cells, untreated and DMSI-treated, were suspended with 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, 20% D_2O to a final volume of 2.5 ml and placed in a 12 mm NMR tube. [2-^{13}C]D,L-mandelic acid, sodium salt (pH 7) was added to a final concentration of 50 mM as indicated in the text. The tube was equipped with a vortex plug.

3. Preparation of Suspensions of 100,000 x g Pellets from Untreated and DMSI-treated Cells for ^{13}C NMR

Stock solutions of NAD^+, 0.15 M, and thiamine pyrophosphate, 0.1 M, were prepared immediately prior to use in 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, 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 which 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 0.1M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$. The cells were disrupted by sonic oscillation for 1 min, and the cellular debris was removed by centrifugation at 10,000 x g for 10 min. The crude extract (supernatant) was dialyzed against 1 liter distilled-deionized water for 24 h in the cold. 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 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, 20% D_2O.

The 100,000 x g pellet was isolated and washed as described in the previous chapter with the exception that 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$ was used. The pellet was suspended by homogenization in 1 ml 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 dialyzate solution. The sample was diluted to 2.5 ml with 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, 20% D_2O solution. The final concentration of cofactors were 60 mM NAD^+, 10 mM FMN and 10 mM thiamine pyrophosphate. [2-^{13}C]D,L-mandelic acid was added to the suspension to a final concentration of 50 mM. The sample was placed in a 12 mm NMR tube equipped with a vortex

plug.

4. Oxygenation of Samples

After each NMR spectrum was obtained, the whole cells or the 100,000 x g pellet suspensions were removed from the NMR tube, placed in a 10 ml round bottom flask containing a small stirring bar, and allowed to come to 25° C. 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 sec and the air vent was closed. The balloon was allowed to fill with oxygen, and the system remained under oxygen atmosphere for the times indicated in the text. The timing period began with the initial flow of oxygen. The sample was place again in the NMR tube, cooled to 5° C, and sealed with the vortex plug.

5. ^{13}C NMR Spectra

Carbon-13 NMR spectra were obtained at a frequency of 25.158 MHz using 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 usec rf pulse) and 20 sec delay between

pulses (giving equilibrium intensities) were accumulated as 8K data points. The acquisition time was 0.68 sec and a total of 200 scans were accumulated per spectrum in a one-hour period. Broad band proton-decoupling was employed, but the decoupling power was gated for obtaining spectra without NOE. All chemical shifts were measured with respect to internal dioxane, but the reported chemical shifts are relative to TMS. The dioxane carbon chemical shift in H_2O is 67.4 ppm downfield from TMS (Johnson and Jankowski, 1972). The sample temperature was maintained at 5^{o} C during data acquisition.

D. Results

1. Metabolism of [2-^{13}C]D,L-Mandelate by Cells of Pseudomonas putida

^{13}C NMR spectra of whole cells of P. putida initially containing 50 mM [2-^{13}C]D,L-mandelate are shown in Figure IV.1. The upper spectrum, obtained prior to oxygenation, contains a single proton-decoupled ^{13}C-signal for the α-carbon of D,L-mandelate at 75.8 ppm. In the lower spectrum following 30 min of oxygenation a number of signals are observed corresponding to the α-carbons of several metabolites. The signal for the α-carbon of mandelate is reduced by more than 50%. Spectra obtained after longer oxygenation periods show complete disappearance of this peak. The other signals are 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 IV.2, the benzaldehyde is a doublet; the benzoate peak remains a sharp singlet; the mandelate peak becomes a doublet; the benzyl alcohol is split into a triplet. In obtaining the coupled spectrum the decoupling power was gated so as to enhance the

Figure IV.1.

Proton-decoupled, ^{13}C NMR spectra (25.2 MHz) of P. putida cells prior to (upper) and following (lower) aeration with oxygen. The cells were suspended in 0.1 M $NaKHPO_4$ buffer (pH 6.8), 10 mM $MgCl_2$, 20% D_2O. [2-^{13}C]D,L-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 in "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.

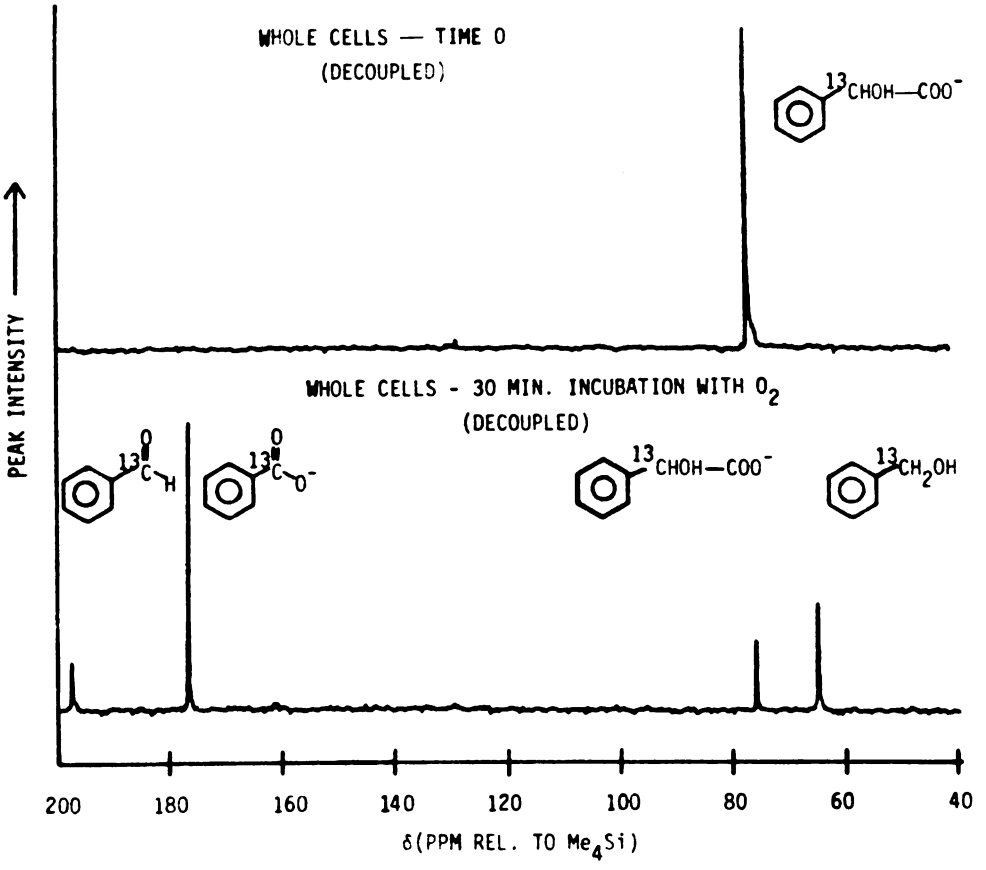
WHOLE CELLS — TIME 0
(DECOUPLED)
^{13}CHOH—COO$^-$
PEAK INTENSITY
WHOLE CELLS - 30 MIN. INCUBATION WITH O_2
(DECOUPLED)
^{13}CHOH—COO$^-$
^{13}CH$_2$OH
200
180
160
140
120
100
80
60
40
δ(PPM REL. TO Me_4Si)

Figure IV.1.

Figure IV.2.

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 IV.1. The decoupling power was gated to provide NOE enhancement of CH- and CH_2-resonances.

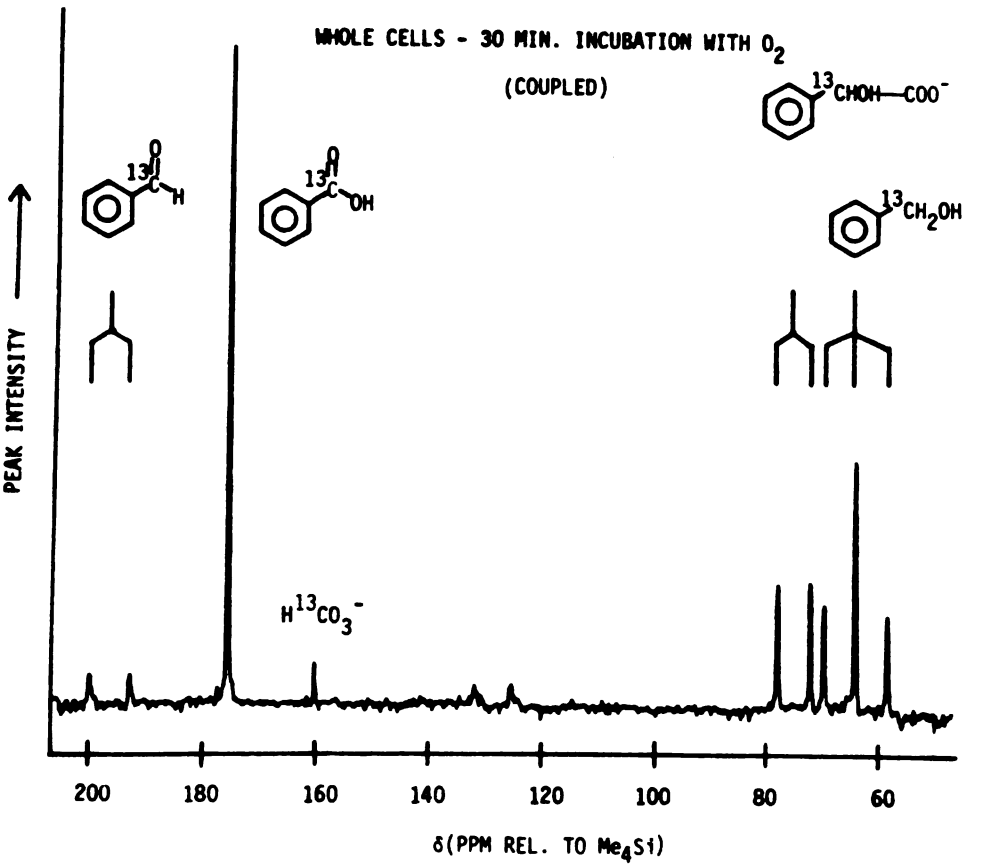
WHOLE CELLS - 30 MIN. INCUBATION WITH O_2
(COUPLED)
$^{13}CHOH—COO^-$
$^{13}CH_2OH$
PEAK INTENSITY
$H^{13}CO_3^-$
200
180
160
140
120
100
80
60
δ(PPM REL. TO Me_4Si)

Figure IV.2.

nuclear Overhauser 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 [2-^{13}C]D,L-mandelate as a function of oxygenation time is shown in Figure IV.3. At t=0 only the single peak for mandelate is observed. Already at t=2 min small peaks for benzyl alcohol and benzoate appear. With increasing periods of oxygenation the mandelate peak decreases in intensity by more than 50% and the intensities of the benzyl alcohol and benzoate peaks increase. A small peak corresponding to benzaldehyde is observed at t=30 min.

By using appropriate pulse sequences and delays to allow for equilibrium intensities it is possible to obtain quantitative information on the time course of D,L-mandelate metabolism. The plot of relative peak intensities vs. time of oxygenation of whole cells in the presence of 50 mM mandelate (Figure IV.4) shows a fairly rapid decrease in the concentration of mandelate, while the levels of benzyl alcohol and benzoate increase and reach a plateau.

Figure IV.3.

Time-lapse plot of proton-decoupled ^{13}C NMR (25.2 MHz) spectra of P. putida cells as a function of the time of oxygenation. The sample is the same as in Figure IV.1. 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.

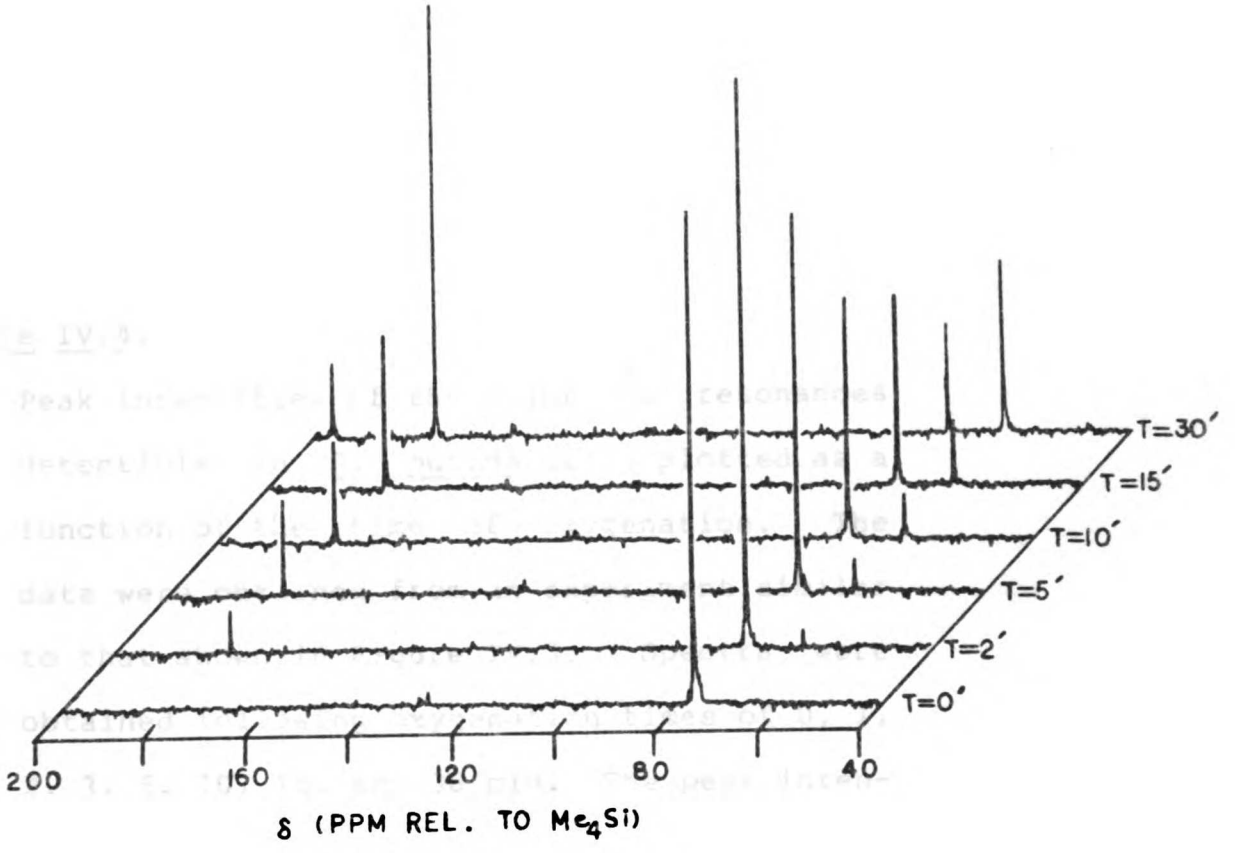

Figure IV.3.

Figure IV.4.

Peak intensities of the major ^{13}C resonances detectible in P. putida cells plotted as a function of the time of oxygenation. The data were obtained from an experiment similar to that shown in Figure IV.3. Spectra were obtained following oxygenation times of 0, 1, 2, 3, 5, 10, 15, and 30 min. The peak intensities are given in arbitrary units.

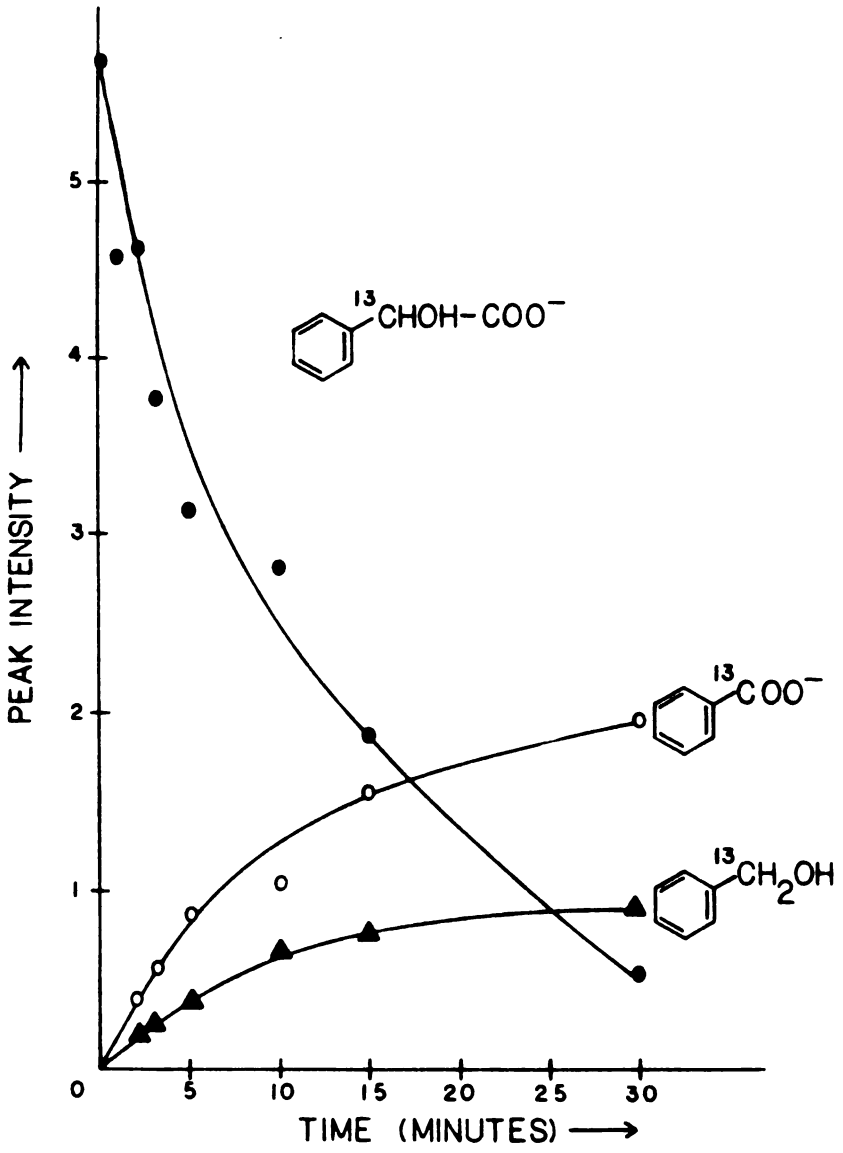
PEAK INTENSITY
TIME (MINUTES)
0
1
2
3
4
5
10
15
20
25
30
^{13}CHOH-COO$^-$
^{13}COO$^-$
^{13}CH$_2$OH

Figure IV.4.

2. Metabolism of [2-^{13}C]D,L-Mandelate by Suspensions of 100,000 x g Pellets Isolated from Untreated and DMSI-Treated Cells.

Addition of ^{13}C-mandelate (to either 50 mM or 12.5 mM final concentration) to the isolated cross-linked complex followed by oxygenation results in fairly rapid formation of 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 have the same chemical shift value at 198 ppm. It is necessary, therefore, to obtain a coupled spectrum to demonstrate the presence of both compounds. After 1 h oxygenation mandelate and benzoylformate concentrations are reduced, benzaldehyde concentration is increased, and a sharp peak for benzoate appears. A proton-coupled ^{13}C spectrum of the cross-linked complex after 1 h oxygenation is shown in Figure IV.5. Mandelate is a doublet; benzoate is a sharp singlet; the singlet of benzoylformate is surrounded by the doublet of benzaldehyde. [^{13}C]Bicarbonate has also appeared in another spectrum but is not observed here.

In a 100,000 x g pellet isolated 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 IV.6), but no other metabolites were observed. Proton-

Figure IV.5.

Coupled ^{13}C NMR (25.2 MHz) spectrum of the suspension of the 100,000 x g pellet isolated 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 $MgCl_2$, 20% D_2O containing 60 mM NAD^+, 10 mM FMN, 10 mM thiamine pyrophosphate, and 0.5 ml dialyzate solution. [2-^{13}C]D,L-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 in "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 CH- and CH_2-resonances.

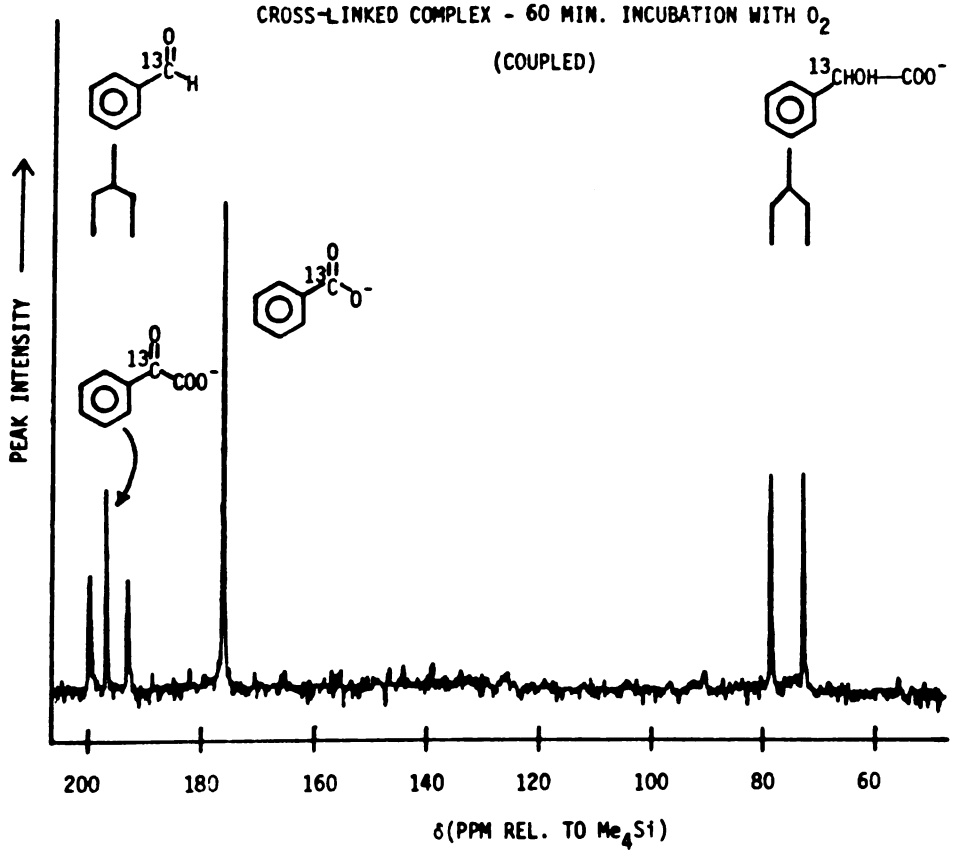
CROSS-LINKED COMPLEX - 60 MIN. INCUBATION WITH O_2
(COUPLED)
PEAK INTENSITY
200
180
160
140
120
100
80
60
δ(PPM REL. TO Me_4Si)

Figure IV.5.

Figure IV.6.

Proton-decoupled ^{13}C NMR (25.2 MHz) spectrum of the suspension of the 100,000 x g pellet isolated from untreated cells of P. putida following 30 min of oxygenation. The sample was suspended in the buffer solution described in Figure IV.5. Other details are the same as described in Figure IV.5 except that the decoupling power was gated to obtain the spectrum without NOE enhancement.

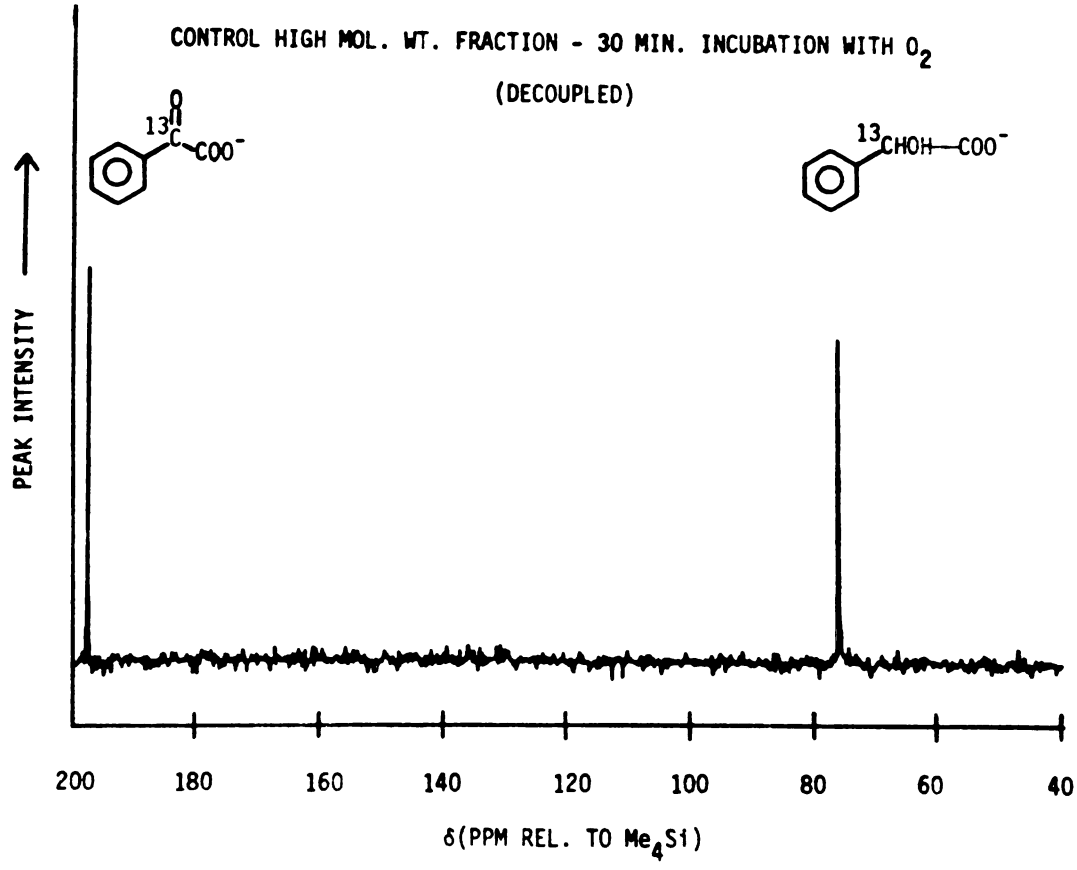
CONTROL HIGH MOL. WT. FRACTION - 30 MIN. INCUBATION WITH O_2
(DECOUPLED)
^{13}C
COO$^-$
^{13}CHOH—COO$^-$
PEAK INTENSITY
200
180
160
140
120
100
80
60
40
δ(PPM REL. TO Me_4Si)

Figure IV.6.

coupled spectra were obtained but no benzaldehyde doublet emerged from the singlet at 198 ppm.

E. Discussion

^{13}C NMR spectroscopy has demonstrated its efficiency as a probe of the *in vivo* metabolism of D,L-mandelate in *P. putida*. In the experiments with whole cells all the metabolites, with the exception of benzoylformate, were observed in the conversion of [α-^{13}C]D,L-mandelate to [α-^{13}C]benzoate. Benzoylformate was not detected, either because its conversion to benzaldehyde was too fast to be observed on the NMR time scale, or because it remained bound as an enzyme-product or an enzyme-substrate complex. The complete disappearance of the 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. It is likely that small amounts of [^{13}C]carbon dioxide dissolved in the medium, and ^{13}C-bicarbonate was detected.

The observance of benzyl alcohol as a major metabolite in the dissimilation of D,L-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 m*M* mandelate, but the concentration of the substrate in the cell suspension in the NMR studies was 50 m*M*. This higher concentration was chosen in order that possible low

levels of intermediates would be detected by NMR. Thus experiments in which the cell suspension contained only 12.5 mM [2-^{13}C]D,L-mandelate showed that the amount of benzyl alcohol which appeared was 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 which saturates the benzaldehyde dehydrogenases. The "spill-over" benzaldehyde is then reduced to benzyl alcohol by constitutive, non-specific dehydrogenases present in the cell. At lower mandelate concentrations little or no such saturation of benzaldehyde dehydrogenase occurs. Further experiments are in progress to test the possibility that benzyl alcohol results from the reduction of benzaldehyde, and that this reaction is reversible.

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 reduction of the mandelate concentration to 12.5 mM metabolism of all intermediates was complete in approximately 15-20 min. It is known that benzaldehyde and benzyl alcohol are cytotoxic. Therefore, it appears that addition of 50 mM mandelate overloads the capacity of the enzymes to maintain low concentrations of toxic intermediates.

^{13}C NMR spectroscopy was particularly valuable for the characterization of the crude, in vivo cross-linked multienzyme complex. Preparation of the 100,000 x g samples from control and DMSI-treated cells, however, involved a complex addition of cofactors and other small intracellular molecules which 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 is a flavo-protein; 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 in "Materials and Methods") was added to provide the preparation with other necessary small molecules.

Spectra of the high molecular weight complex showed, after periods of oxygenation, all the intermediates produced in the metabolism of [α-^{13}C]D,L-mandelate to [α-^{13}C]benzoate. [^{13}C]Bicarbonate was observed in one experiment providing tentative evidence for the presence of benzoate oxidase in the complex. It is perhaps significant, though, that no benzyl alcohol was formed in the cross-linked complex. This suggests that enzymes not involved in mandelate metabolism may be absent. This possibility may be

further examined by testing the complex for the presence or absence of enzymes involved in glycolysis by the Entner-Doudoroff pathway; e.g. 6-phosgluconate dehydrogenase. No differences were observed in the spectra of the complex in the presence of either 50 m$\underline{M}$ or 12.5 m$\underline{M}$ D,L-mandelate.

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, the soluble enzymes of the mandelate group were not present. The sample, however, did contain some of the membrane-bound, stereospecific L-mandelate dehydrogenase which converted the L-isomer of mandelate to benzoylformate. Low-speed centrifugation of the crude extract following sonic disruption of the cells removes a large amount of the particulate fraction which contains this enzyme. Assays of the remaining extract, however, demonstrate that the enzyme has not been completely eliminated, and it is this portion of the dehydrogenase remaining in the extract which appears in the high molecular weight samples following centrifugation at 100,000 x g (cf. to Table I of the previous chapter).

These NMR experiments provide conclusive evidence that this high molecular weight material, isolated from cells treated with dimethyl suberimidate, contains all the enzymes

necessary to convert mandelate to benzoate. The results support the conclusions presented in the previous chapter, that the enzymes of the mandelate group are all in close association with the membrane-bound L-mandelate dehydrogenase, and that treatment of P. putida whole cells with dimethyl suberimidate provides sufficient covalent linkages to entrap these enzymes.

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 containing the carboxylate function.

Multienzyme complexes do not survive the usual isolation and purification procedures - unless their protein-protein interactions are reasonably stable - and therefore, are infrequently observed. Treatment of whole cells with a cross-linking reagent prior to sonic disruption has provided a means of stabilizing the putative mandelate complex, thus permitting its isolation. Organization of enzymes involved in a sequence of reactions may provide the cell with a number of distinct advantages. First, aggregation of the separate proteins may produce, enhance, or modify a particular enzymatic activity. Second, enzymes functioning in close proximity to each other may improve the overall efficiency of a reaction sequence, even if the catalytic activities of the individual enzymes are not changed by the

aggregation (Reed & Cox, 1966). Multienzyme complexes may also provide some or all of the following features: a channeling mechanism whereby a high steady state concentration of a substrate is provided for the next enzyme, or separation between competitive biosynthetic and catabolic pathways is maintained; conservation of the solvent capacity of the cell (Atkinson, 1969); coordination of metabolic processes (Lynen, 1964); increased catalytic properties (Gaertner, 1978).

It would be interesting to speculate about the nature and function of a tentative "mandelate complex" in P. putida. One may propose that a cluster of the mandelate enzymes would rapidly turnover substrate (mandelate) to product (benzoate) utilizing a channeling mechanism not so much as 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 completely characterized, and a purified multienzyme "mandelate complex" isolated, with further analysis as to structure, kinetics, etc., it is not possible to make any conclusive statements at this time.

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V. BENZOYLFORMATE DECARBOXYLASE ASSAY

A. Abstract

Benzoylformate decarboxylase catalyzes the conversion of benzoylformate to benzaldehyde and CO_2. An improved spectrophotometric assay for benzoylformate decarboxylase has been developed, and it has been used to monitor the activities of this enzyme in extracts of Pseudomonas putida. By converting the unreacted benzoylformate to the sodium salt of its 2,4-dinitrophenylhydrazone, a strong, water-soluble chromophore with an absorbance shoulder at 540 nm (ϵ=13,000) is generated. The 2,4-dinitrophenylhydrazone of benzaldehyde, on the other hand, is insoluble in aqueous base, and may be removed either by filtration or centrifugation.

B. Introduction

Benzoylformate decarboxylase (benzoylformate carboxy-lyase, EC 4.1.1.7) catalyzes the thiamine pyrophosphate-dependent decarboxylation of benzoylformate (phenylglyoxy-late) to benzaldehyde and CO_2. The enzyme is one of a group of five coordinately inducible enzymes, called the mandelate group, that dissimilates D,L-mandelate to benzoate. It was first isolated by Gunsalus et al. (1953) from the soluble component of crude extract of Pseudomonas putida A.3.12. The enzymatic activity was originally assayed manometrically by measuring the rate of CO_2-evolution at pH 6.0 and 30° C. This rather cumbersome method was replaced by a spectropho-tometric procedure developed by Hegeman (1966;1970), in which the disappearance of benzoylformate is monitored at 334 nm. This latter assay is not very sensitive, however, due to the small molar extinction coefficient of benzoylfor-mate at this wavelength (ϵ=81).

Recently, we have become involved in cross-linking the enzymes of the mandelate group together in intact cells of P. putida (Kenyon et al., 1976). These experiments led us to seek an assay for benzoylformate decarboxylase activity capable of detecting even small amounts of the enzymatic activity in crude extracts. The assay procedure of Jamalud-din et al. (1970), which involves formation of the 2,4-dinitrophenylhydrazone of benzoylformate, appeared promis-

ing. In this paper, we describe modifications of their procedure which make the assay more suitable and convenient for accurate and rapid multiple determinations of benzoylformate decarboxylase activities, even in crude extracts.

C. Materials and Methods

1. Reagents

Benzoylformic acid (97%) was purchased from Aldrich Chemical Co. and was carefully dried *in vacuo* at room temperature before use. Benzaldehyde was also purchased from Aldrich and was freshly distilled. 2,4-Dinitrophenylhydrazine was the product of Eastman-Kodak Co. Thiamine pyrophosphate hydrochloride was obtained from Boehringer-Mannheim Biochemicals. All other reagents were of analytical grade.

2. Preparation of Crude Extract.

Stock cultures of *Pseudomonas putida* A.3.12 (ATCC 12633) were maintained on solid medium containing 1% yeast extract and 2% agar (both from Difco Laboratories) in 0.01 M $NaKHPO_4$ buffer (pH 7.0). Cells of *P. putida* were grown in a mineral medium (Hegeman, 1966) containing D,L-mandelate as the ammonium salt at a final concentration of 10 mM. The cultures were incubated with vigorous shaking at 30° C, and cells were harvested in late-log phase by centrifugation at

10,000 x g for 10 min. The sedimented cells were suspended in 0.05 M triethanolamine·HCl buffer (pH 8.0) containing 0.1 M NaCl and 10 mM $MgCl_2$ and disrupted by sonic oscillation. The cellular debris was removed by centrifugation at 10,000 x g, and the supernatant (crude extract) was kept at 4° C until assayed.

3. Assay for Benzoylformate Decarboxylase Activity.

The reaction mixture (0.5 mL total volume) contained 1.0 µmole of sodium benzoylformate, 40 µmoles phosphate buffer, (pH 6.0), 50 µg thiamine pyrophosphate chloride, and crude extract in the range of 0.01 to 0.15 mg protein. The mixture was incubated at 25° C for 30 min, and then 0.5 mL of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was added. After incubation at 25° C for 15 min, 9 mL of 2 N NaOH was added, and the mixture was allowed to stand for an additional 15 minutes at 25° C. The insoluble 2,4-dinitrophenylhydrazone of benzaldehyde was removed either by filtration or by centrifugation. The absorbance of the sodium salt of the 2,4-dinitrophenylhydrazone of benzoylformate was measured in a Gilford spectrophotometer (Model 2220A) at 540 nm. The reference cell contained all of the reagents except benzoylformate and crude extract; the standard contained all of the reagents except the crude extract.

Protein determinations were performed using either the biuret method (Gornall et al., 1949) with bovine serum

albumin as standard or the Coomassie Blue dye-binding procedure of Bradford (1976) using bovine γ-globulin as standard.

D. Results

1. Linearity of Assay.

In Figure V.1-A is shown a plot of the absorption of the sodium salt of the 2,4-dinitrophenylhydrazone of benzoylformate at 540 nm *vs.* concentration, demonstrating linearity in the range 0.1 to 1.0 µmole added per assay. In Figure V.1-B is shown a plot of µmole benzoylformate decarboxylated per 30 min *vs.* protein concentration. Up to *ca.* 0.06 to 0.08 mg of crude extract protein per assay tube, the latter plot shows excellent linearity.

2. Interference by Products.

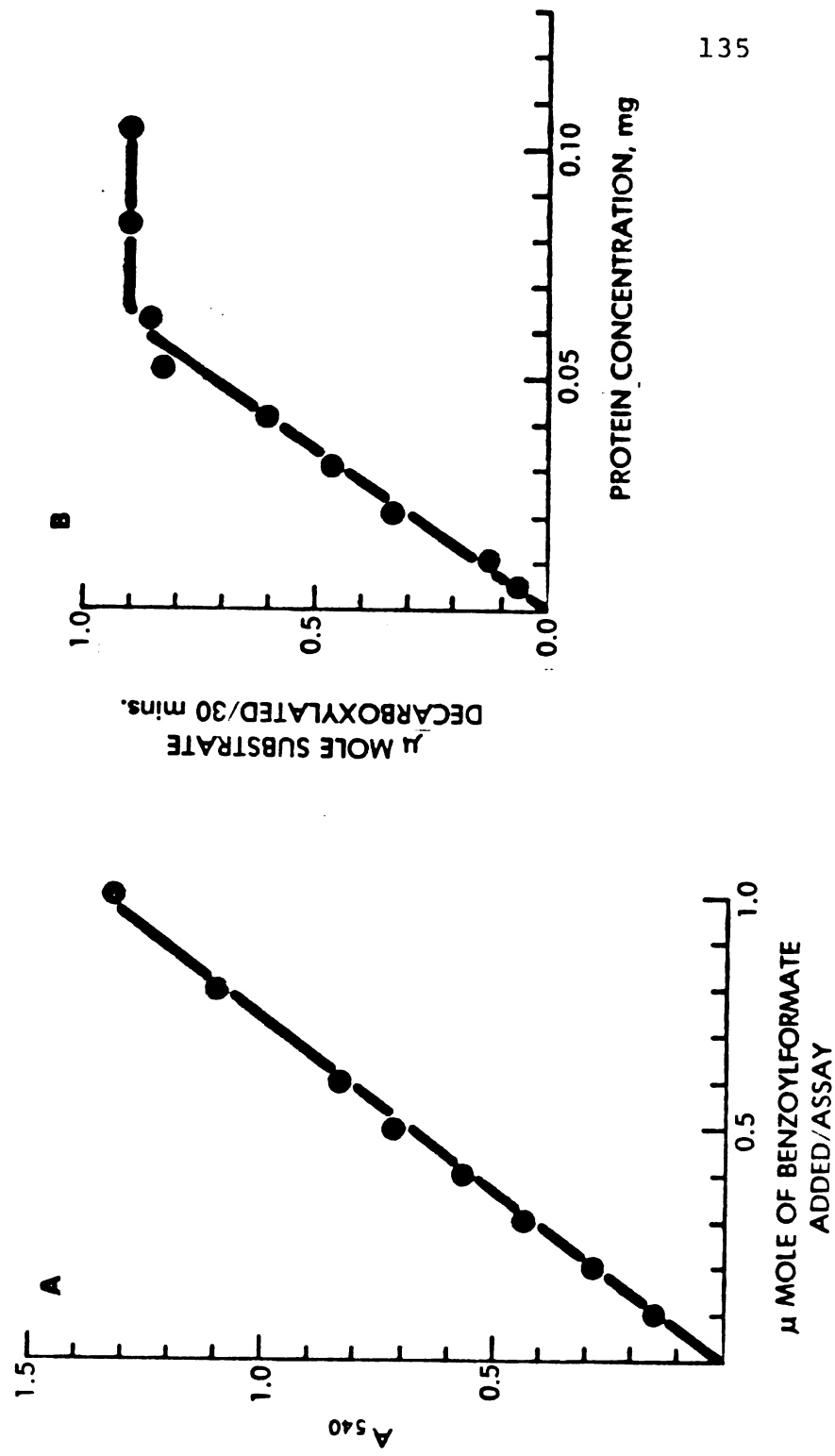
In Figure V.2 are shown visible spectra of the 2,4-dinitrophenylhydrazones of both benzoylformate and pyruvate as well as those of the 2,4-dinitrophenylhydrazones of their decarboxylation products, benzaldehyde and acetaldehyde, respectively. At 540 nm the 2,4-dinitrophenylhydrazones of benzaldehyde and acetaldehyde have relatively low absorbance values compared to those for the corresponding derivatives of benzoylformate and pyruvate. Nevertheless, in order to optimize the sensitivity of the assay it is necessary to correct for the presence of the small amount of the 2,4-dinitrophenylhydrazone of benzaldehyde that remains in the assay mixture after filtration or centrifugation.

Figure V.1-A.

The absorbance at 540 nm of the sodium salt of the 2,4-dinitrophenylhydrazone of benzoylformate as a function of the number of μ moles of benzoylformate added per assay [r^2 (correlation of determination) = 0.994; slope = 1.36; intercept = 0.01].

Figure V.1-B.

Plot of μmoles of benzoylformate decarboxylated per 30 minutes as a function of protein concentration. Components of the assay are described in "Materials and Methods". (r^2 = 0.9994; slope = 10.69; intercept = 0.0003).



Figures V.1 A-B.

Figure V.2.

Spectra of the 2,4-dinitrophenylhydrazones of benzoylformate, pyruvate, acetaldehyde and benzaldehyde. Samples were prepared and dissolved in 2 N NaOH as described in "Materials and Methods". The 2,4-dinitrophenylhydrazone of benzaldehyde was relatively insoluble, and this sample was filtered prior to measurement of its spectrum.

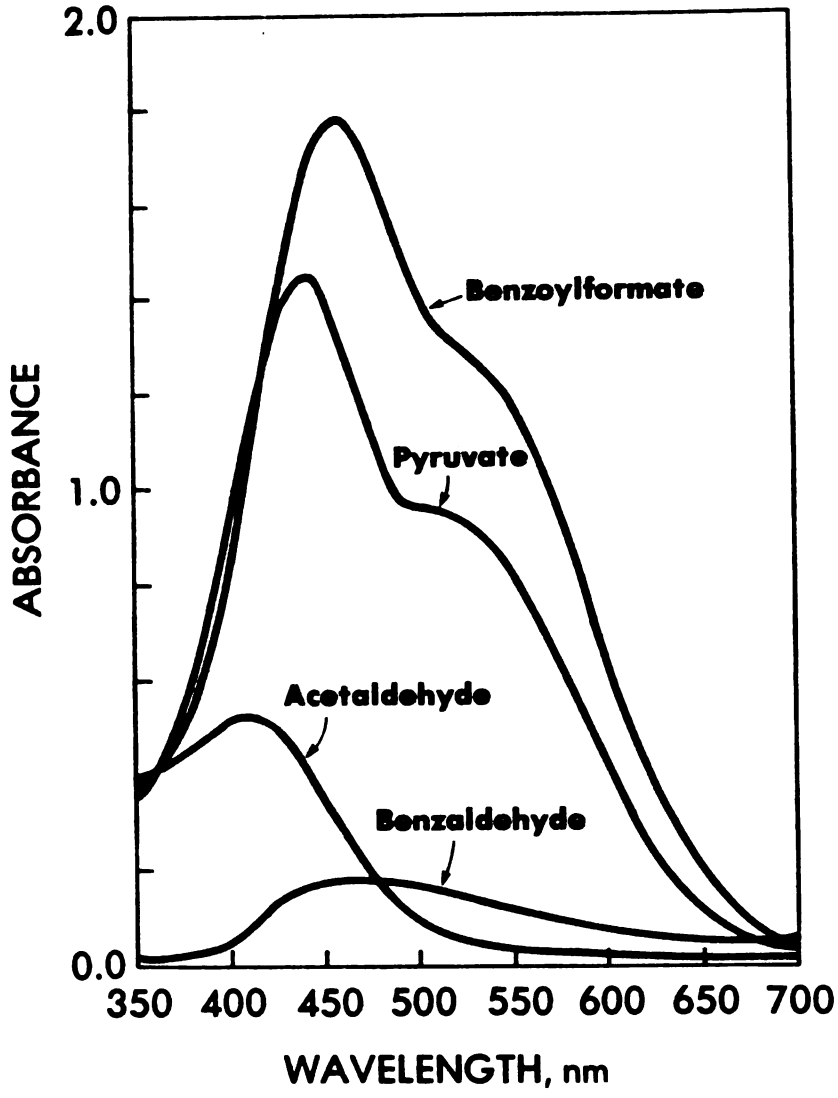

2.0
1.0
0.0
ABSORBANCE
Benzoylformate
Pyruvate
Acetaldehyde
Benzaldehyde
350 400 450 500 550 600 650 700
WAVELENGTH, nm

Figure V.2.

To determine the amount of dissolved 2,4-dinitrophenylhydrazone of benzaldehyde present in the assay mixtures, a standard curve was constructed by carrying out the usual assay on a series of solutions containing 0.1 to 1.0 μ mole of freshly distilled benzaldehyde. At the end of these assays, each sample was either filtered or centrifuged, and the absorbance of the resulting solution was measured at 540 nm. Absorbance values in the range 0.05 to 0.06 (relative to blanks) were found for each of the samples. Therefore, the average absorbance of 0.055 was subtracted from absorbance readings of enzymatic reaction samples measured at 540 nm, and these corrected values were used to obtain the amount of substrate remaining from the standard curve for benzoylformate. Failure to make this correction for interference by the product results in false values for the disappearance of substrate, especially at low protein concentrations, and plots of μmoles of benzoylformate decarboxylated per 30 min. *vs.* protein concentration have an apparent "sigmoidal" shape instead of the characteristic hyperbolic shape seen in Figure V.1-B.

3. *Effects of pH, Incubation Time and Added Cofactors.*

The pH-range used in examining the activity of purified benzoylformate decarboxylase from *P. putida* is reported to be 6.0-6.5 (Gunsalus *et al.*, 1953) with an optimum at pH 6.2

(Hegeman, 1966). In crude extracts the range appears to extend to pH 6.8 in phosphate buffer, but activity declines at pH 7.0 in Na^+ N-hydroxyethylpiperizine-N'-ethanesulfonate (Hepes) buffer and more rapidly at pH 8.0 in triethanolamine buffer.

A time-course was measured over a range of 60 minutes at several different protein concentrations, and for each the optimal enzymatic activity was obtained at an incubation time of ca. 30 minutes.

Gunsalus et al. (1953) previously showed that benzoylformate decarboxylase requires thiamine pyrophosphate and that the K_m of the enzyme for this co-factor is approximately $4x10^{-6}$ M. Addition of Mg^{2+} to the manometric assay system reportedly did not stimulate activity (Gunsalus et al., 1953).

We find with crude extracts that if thiamine pyrophosphate is not added, only approximately one-half of the optimal activity can be detected. Also, enzymatic activity is completely lost if the crude extract is either dialyzed against cold H_2O for 24 h or subjected to gel-permeation chromatography on Ultrogel AcA22 using 0.05 M triethanolamine buffer, (pH 8.0), containing 0.1 M NaCl. Approximately 60% of this lost activity may be restored to the dialyzed crude extract if Mg^{2+} is added to the extract in 10 mM final concentration and if 50 µg thiamine pyrophosphate is added to the assay system. Either Mg^{2+} or thiamine pyrophosphate

added alone has no effect on restoring activity. Similarly, decarboxylase activity of crude extract fractions eluted from the Ultrogel AcA22 column can be retained if 10 m$\underline{M}$ $MgCl_2$ is present in the eluting buffer. Once again, thiamine pyrophosphate must be added to the assay medium for this activity to be expressed.

E. Discussion.

The assay procedure described here had its origins in the work of Friedman and Haugen (1943) and Jamaluddin *et al.* (1970). Friedman and Haugen (1943) utilized the solubility properties of nitrophenylhydrazones in both sodium carbonate solutions and in organic solvents to measure pyruvate in blood and urine. Jamaluddin *et al.* (1970) used 2,4-dinitrophenylhydrazone formation to follow both the enzymatic oxidation of D-mandelate to benzoylformate by D-mandelate dehydrogenase, a particulate enzyme in *Aspergillus niger*, and also the decarboxylation of benzoylformate by benzoylformate decarboxylase, a soluble enzyme in the supernatant fluid of extracts of *A. niger*. In their D-mandelate dehydrogenase assay the enzymatic reaction was stopped by the addition of 2,4-dinitrophenylhydrazine, the particulate protein was removed by centrifugation following heat-denaturation, and the absorbance of the hydrazone dissolved in 2.5 *N* NaOH was determined at 470 nm. A similar procedure was used to follow the decarboxylase activity, except that the enzymatic reaction was stopped by addition of 2 *N* HCl, and the benzaldehyde formed was removed by extraction with CCl_4 prior to addition of the 2,4-dinitrophenylhydrazine. The benzoylformate decarboxylase assay of Jamaluddin *et al.* (1970) was only rather briefly described in their article, and no information was given on either the linearity or sen-

sitivity range of their procedure.

When large numbers of samples are to be assayed, extraction steps in the assay procedure are very time-consuming. In the procedure described here, the extraction step is omitted and the 2,4-dinitrophenylhydrazone of benzaldehyde, which is practically insoluble in NaOH solution, is removed by either centrifugation or by filtration. Removal of protein appears to be unnecessary except in cases of low activity in turbid, crude extracts or where the enzyme may be located in a particulate fraction, thereby contributing turbidity to the samples.

With the A. niger enzyme, Jamaluddin et al. (1970) measured the absorbance of the sodium salt of the 2,4-dinitrophenylhydrazone of benzoylformate at 470 nm, which, as is apparent from the visible spectrum shown in Figure V.2, is the λ_{max} for that derivative. In our procedure absorbance measurements are made using the absorbance shoulder (also a strongly absorbing peak) at 540 nm to reduce somewhat interference due to the residual 2,4-dinitrophenylhydrazone of benzaldehyde.

From the absorption spectra shown in Figure V.2, it appears that these procedures may be adapted to the measurement of pyruvate decarboxylase as well. Pyruvate decarboxylase is commonly assayed in a coupled procedure with lactic dehydrogenase in which NAD^+ reduction is monitored spectrophotometrically at 340 nm (Holzer et al., 1965; Bergmeyer et

al., 1974). Such coupled assay procedures, while convenient, are often not suitable for assays in crude extracts owing to the presence of interfering NAD-dependent dehydrogenases.

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VI. EXPERIMENTAL

A. General Methods and Procedures.

Spectrophotometric assays were performed on a Gilford 2220A spectrophotometer equipped with a Beckman DU Model 2400 monochrometer and a Gilford 6040 recorder. The cell compartment was equipped with thermo-spacers and thermostatted at 25°C.

Bacterial cell cultures were incubated in a New Brunswick Environmental Incubator Shaker, Model G25 (floor model) or Model G24 (bench model). Bacterial growth was followed by turbidity readings taken on a Klett-Summerson colorimeter, using a No. 54 (520-580 nm) filter.

Low-speed centrifugation was carried out in a Sorvall R-2 refrigerated centrifuge using a SS34 (8 x 40 mL capacity) or a GSA (6 x 250 mL capacity) rotor. High-speed centrifugation was carried out in either a Beckman-Spinco Model L or a Beckman Model L2-65B refrigerated ultracentrifuge using a Beckman No. 40 rotor (12 x 10 mL capacity). For the high-speed centrifugation of quantities smaller than 10 mL per tube appropriate tubes with adapters for the No. 40 rotor were used.

^{13}C-T_1 (spin-lattice) relaxation time experiments were perfomed on a Varian XL-100 spectrometer at a frequency of 25.2 MHz, interfaced with a Nicolet Instrument Corporation Model NIC-80 data processor and modified with a Nicolet

'Multi-Observe Nuclei Accessory' (MONA).

All reagents were prepared using distilled-deionized water.

B. Microbiological Procedures.

1. Maintenance of Stock Culture.

Stock cultures of Psedomonas putida A.3.12 (ATCC 12633) were maintained on solid medium containing 1% yeast extract and 2% agar in 0.01 M $NaKHPO_4$ buffer (pH 7.0). The solid medium was prepared as described below.

a. Preparation of Solid Medium for Slants and Plates.

Yeast extract (1%) and agar (2%) were dissolved in 0.01 M $NaKHPO_4$ (pH 7.0) by boiling. The mixture was prepared in a large beaker to reduce the likelihood of the solution's boiling over. Slants were prepared by adding approximately 5 mL yeast-agar-phosphate (YAP) solution to screw-cap culture tubes. The caps were replaced loosely and the tubes were autoclaved at 125°C (250°F) for 30 min and cooled by slow exhaust. When the tubes were removed from the autoclave, the caps were tightened and the tubes were placed on their sides with the cap-end raised 5-10° from the bench top (so that the top of the medium is a slant) until cooled to room temperature. The slants were stored on their sides in

a cool, dark cabinet.

In order to prepare the YAP culture plates, the medium was prepared as described above. While the solution was still hot, the beaker was covered with aluminum foil, and the medium was autoclaved at 125°C (250°F) for 30 min and cooled by slow exhaust. When removed from the autoclave, the covered medium was allowed to cool to approximately 40-50°C (several hours) for ease of handling and reduction of condensation on the covers of the plates. (If the medium becomes too cool, it solidifies and must be reheated.) Disposable Petri dishes, supplied in sterile packages of 20 dishes per package, required approximately 30 mL medium. To minimize contamination while transferring the medium to the plates, the aluminum foil cover was removed only sufficiently to expose the lip of the beaker, and the cover of the Petri dish was opened sufficiently wide to permit pouring of the medium until the dish was half-full. The plates were stacked until the medium had solidified, repackaged with the original plastic sleeve, and stored in a cool, dark cabinet.

b. Transfer of Stock Culture.

To maintain the stock culture an inoculum of P. putida was transferred from the most recently inoculated slant to a new YAP slant and culture plate. The transferred culture was allowed to grow 6-10 h at 30°C or 12-18 h at 25°C, after

which time the slant was stored at 4°C. The culture plate was examined for possible contamination or other changes in the culture, and also for the characteristic yellow-green fluorescent substance exuded by P. putida when grown on solid medium. To maintain the culture, the transfer was carried out once a month. (NOTE: Slants with fresh cells should be refrigerated before the yellow-green fluorescent material is observed in the slant - before 10 h at 30°C or 20 h at 25°C.)

2. Growth of P. putida.

Cells of P. putida were grown in a mineral base containing D,L-mandelate as the ammonium salt at a final concentration of 10 mM.

a. Formulation of Mineral Base.

Preparation of the modified Hutner's mineral base and the culture medium is described below. Formulation of the mineral base involved the preparation of a series of stock solutions.

(1.) Hutner's "Metals 44".

The following were dissolved in the order given; additional components were not added until the previous one had dissolved. In 800 mL water were dissolved:

EDTA (free acid, not sodium salt)	2.50g
The solution was heated slightly; the acid dissolved when the remaining metals were added.	
$ZnSO_4 \cdot 7H_2O$	10.95g
$FeSO_4 \cdot 7H_2O$	5.00g
$MnSO_4 \cdot H_2O$	1.54g
$CuSO_4 \cdot 5H_2O$	0.392g
$Co(NO_3)_2 \cdot 6H_2O$	0.250g
$Na_2B_4O_7 \cdot 10H_2O$	0.177g

A few drops of conc. H_2SO_4 were added to retard precipitation. The solution, which should be a clear, lime-green color, was diluted to a final volume of 1 liter with water. The solution should store indefinitely at room temperature.

(2.) Concentrated Base (Hutner's Base).

The following components were dissolved in the order given.

In 600 mL of water were dissolved:

nitrilotriacetic acid (NTA - free acid)	20.0g
The acid was dissolved and neutralized by the addition of KOH (14.6g).	
$MgSO_4$ anhydrous (or equivalent heptahydrate)	28.9g
The pH of the solution decreased to ca. pH 5 but was not re-adjusted.	
$CaCl_2 \cdot 2H_2O$	6.67g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0185g
$FeSO_4 \cdot 7H_2O$	0.198g
"Metals 44"	100 mL

The solution was adjusted to pH 6.8 by slowly adding 50% KOH and waiting for the white flocculent precipitate which formed to dissolve before further addition of base. The final volume was made to 1 liter with water and the solution was stored at 4^oC.

(3.) 1 M $NaKHPO_4$ Buffer, pH 6.8.

To 700 mL water 68.045g (0.5 M) KH_2PO_4 (monobasic) and 71.00g (0.5 M) Na_2HPO_4 (dibasic) were added. When both salts were dissolved, the pH was adjusted to 6.8 if necessary and the solution was diluted to 1 liter with water. The solution was 1 M with respect to phosphate concentration.

(4.) 10% (w/v) $(NH_4)_2SO_4$, Aqueous.

(5.) 1 M D,L-Mandelate, Ammonium Salt, pH 7.0.

This solution was prepared in 50 mL quantities and stored in an aluminum foil-wrapped or in a dark bottle. Larger quantities stored for longer than a few months had to be discarded due to bacterial contamination.

b. Preparation of Culture Medium.

The culture medium was prepared (per liter) as described below.

To approximately 800 mL water were added

1 M $NaKHPO_4$ buffer, pH 6.8	40 mL
10% $(NH_4)_2SO_4$	10 mL
Concentrated Base	20 mL
1 M D,L-Mandelate, NH_4 salt, pH 7.0	10 mL

The solution was adjusted to pH 6.8 if necessary and diluted to 1 liter with water.

The medium was distributed either to Fernbach or to nephelometer flasks (see below) which were then stoppered with cheesecloth-wrapped cotton plugs, covered with aluminum foil, and autoclaved at 125°C (250°F) for 30 min with cooling by slow exhaust. During autoclaving a white precipitate formed in the solution which slowly dissolved as the medium cooled to room temperature. The dissolution was facilitated by occasionally swirling the flasks. The pipettes necessary for transferring cells to the culture medium were autoclaved for 20 min at 125°C (250°F) and oven-dried.

The 2.8 liter Fernbach flask is a broad-based Erlenmeyer-type flask with baffles incorporated into the base which improves aeration of the medium during cell

growth. This flask is used for growing large volumes of bacterial cells (500 mL medium per flask) and fits only the Model G25 New Brunswick incubator. The nephelometer flask is an Erlenmeyer flask with a test tube fused into the side of the flask, a design which permits turbidity readings of samples to be taken without opening the flask. The flask is available in a variety of sizes and may be used, with the proper clamps, in either the Model G24 or G25 incubator. The 500 mL nephelometer flask was the most commonly used size to which was added 90 mL culture medium. The 125 mL flasks were used for the blank (10-20 mL medium) and the preculture (usually 20 mL medium) samples.

c. Procedure for Growth of P. putida.

The total amount of time required to prepare fresh cells is two days. The entire procedure may be divided into four parts:

a.) preparation of actively dividing cells;

b.) preparation of fully induced cells (Preculture);

c.) growth of the cells;

d.) harvesting of the cells.

The schedule is described below.

(1) At a minimum of 18 h prior to the growing of cells, a fresh YAP slant and plate are streaked with P. putida from the most recently prepared slant and incubated either at 30°C for 6-10 h (or at 25°C for 16-20 h, beginning proportionately earlier).

(2) The culture medium is prepared and autoclaved as described above. At least 6 h is necessary for the medium to cool and for the precipitate to dissolve. The usual volume required when fresh cells are prepared for each experiment is 500 mL culture medium, which is divided as follows: a.) 90 mL to each of 5 - 500 mL nephelometer flasks (for growth of cells); b.) 20 mL to 1 - 125 mL nephelometer flask (for the preculture); c.) the remainder to another 125 mL nephelometer flask

which is used as the blank for turbidity readings. When larger quantities of cells are required, the necessary amount of medium is prepared and distributed as follows: a.) 500 mL to each Fernbach flask necessary; b.) 90 mL to 1 - 500 mL nephelometer (used for following cell growth by turbidity readings; c.) sufficient volume to 1 - 125 mL preculture flask to provide 5 mL preculture to each 500 mL medium and 1 mL for the 500 mL nephelometer flask; d.) the remaining amount of medium to 1 - 125 mL flask for the blank.

(3) For the usual cell preparations 5 - 1 mL pipettes are sterilized as described above. For the larger quantities a sufficient number of 5 mL pipettes and a 1 mL pipette are autoclaved.

(4) When the slant (prepared in step 1.) is covered with cells, 1 - 2 loopsful of cells (using a heat-sterilized nickle loop) are transferred to the preculture flask under sterile conditions. (For standard microbiological techniques refer to any microbiology text or laboratory manual.) Both the blank and preculture flasks are placed in the shaker-incubator at 30^{o}C with the shaker speed set at 350 rpm for approximately 12 h.

(5) After the induction period the turbidity of the cells in the preculture is measured against the medium blank in the Klett-Summerson colorimeter using a No. 54 green filter. (A No. 66 red filter (640 - 670 nm) may be used if available.) Under usual conditions the value of the reading is 260 - 280 Klett units.

(6) Aliquots of the preculture are transferred to the culture medium in the proportion of 1 mL preculture to 100 mL culture medium using sterile conditions. (One mL is usually added to the 90 mL in the 500 mL flasks.)

(7) Flasks are placed in a balanced manner in the shaker-incubator at 30°C and the shaking speed is set to 450-500 rpm when using the nephelometer flasks in the Model G24 incubator. In the Model G25 incubator using the Fernbach flasks with the baffles, a shaking speed of 300-350 rpm is sufficient to aerate the medium.

(8) Turbidity measurements are taken every hour and plotted on semi-log paper as Klett units vs. time, or on linear paper as ln Klett units (2.303 log Klett units) vs. time. If there is sufficient oxygenation of the medium by vigorous mixing, the curve during exponential growth will be linear. When the curve is no longer linear, (usually after 5.5-6 h) growth has reached what

is called the late-log phase signifying that the carbon source - in this case D,L-mandelate - has been exhausted. Also, the culture medium in the later linear stage takes on a blue-gray color typical of a catechol-iron complex. This phenomenon is characteristic of cells which metabolize compounds via a catechol-intermediate, and the disappearance of the color is an indicator of the exhaustion of the carbon source.

(9) For all the biochemical studies carried out the cells are harvested at this late-log phase. For the ^{13}C NMR experiments the cells are harvested at mid-log phase (after approximately 4 h). To standardize conditions a turbidity measurement of 100 Klett units is used as the mid-log point.

(10) The cells are harvested by centrifugation of the cell suspension at 10,000 x g for 10 min in a refrigerated centrifuge.

(11) The supernatant is discarded. The pellets of cells are suspended in an appropriate cold buffer to a final volume of approximately 70 mL for cells grown to late-log phase, and approximately 35 mL for cells grown to mid-log phase. Centrifugation is repeated as above.

The total yield from cells grown to late-log phase is 1.4 to 1.8g wet weight (2 pellets weighing 0.7 to 0.9g each). The yield from cells grown to mid-log plase is one pellet of 0.6 to 0.8g wet weight. The average wet weight of cells obtained is 2-4g cells per liter of culture medium.

(12) The number of cells obtained can be calculated from this equation:

$$30\ Klett\ units = 1 \times 10^8\ cells/mL \qquad (1)$$

(note from G. D. Hegeman.)

EXAMPLE: Reading of sample is 180 Klett units.

$$\frac{1 \times 10^8\ cells/mL}{30\ Klett\ units} = \frac{x}{180\ Klett\ units} \qquad (2a)$$

$$x = 6 \times 10^8\ cells/mL$$

$$6 \times 10^8\ cells/mL \times 450\ mL = 2.7 \times 10^{11}\ cells \qquad (2b)$$

(13) Equations for the calculation of the specific growth rate constant (μ) and for the doubling time (g) are as follows:

a.) specific growth rate constant (μ)

$$\log Z - \log Z_0 = \frac{\mu}{2.303}(t - t_0) \qquad (3)$$

where Z = some quantity measuring growth (i.e. Klett units) and t = time.

b.) doubling time (g)

$$g = \ln \frac{2}{\mu} = \frac{0.693}{\mu} \qquad (4)$$

The average doubling time of *P. putida* is 45 min. (Stanier *et al.*, 1976). The values obtained ranged from 35 to 55 min.

C. Biochemical Procedures.

1. Preparation of Ultrogel AcA 22 Column.

An excellent source of general information concerning the use of gel permeation chromatography using dextrans (Sephadex) and agarose (Sepharose) is available on request from Pharmacia Fine Chemicals Inc. The titles of a few of the booklets are "Sephadex: Gel Filtration in Theory and Practice", "Blue Dextran 2000", "Beaded Sepharose 2B - 4B - 6B", and "Sepharose CL" (Pharmacia). Also, explicit directions for their assembly and care are supplied with columns and adaptors purchased from this company. LKB provides an instruction manual containing general and practical information about Ultrogel with each bottle purchased. Since such detailed information is available, only a brief description about the procedures used will be given here.

The columns used in this project were the Pharmacia K16/100 (1.6 x 100 cm) and K26/45 (2.6 x 45 cm) columns. The best resolution of samples was obtained using the long, narrow K16/100 column. All the column experiments were carried out at room temperature. Several days were allotted for preparation of the column which involved removal of dissolved gases from the gel, packing the column, and equilibration of the gel with buffer.

a. Preparation of Ultrogel AcA 22.

The amount of gel required for the column was calculated from the equation for the volume of a rod, $V = \pi r^2 h$, where r is the radius of the column and h is the desired height of the gel. For a column of the approximate dimensions of 1.6 x 90 cm, 200 mL Ultrogel AcA 22 (stored at $4^{\circ}C$) was required. A slurry was prepared by suspending the gel with gentle swirling in no more than 100-150 mL eluting buffer. The slurry was placed into a vacuum flask fitted with a one-hole, rubber stopper containing a glass tube. A short piece of polyethylene tubing with an adjustable clamp was attached to the glass tube. The flask was evacuated using line vacuum and, to prevent sudden bubbling in the gel, the clamp was closed gradually. Occasional swirling of the gel suspension facilitated the degassing procedure. The slurry was allowed to degas (and also to equilibrate with room temperature) for 4-6 h. When degassing was complete, the suspension was allowed to stand 6-8 h at room temperature prior to pouring into the column. Temperature equilibrium was important in order to prevent formation of air bubbles in the gel after it was packed into the column. (If the column was prepared during one of San Francisco's rare heat waves, air bubbles formed all through the gel after the temperature returned to normal, and the column had to be repacked.)

b. Preparation of the Column.

The column was mounted vertically and assembled according to instructions provided by the supplier. The attachment of an extension tube or a gel reservoir to the column was recommended in order that the gel slurry could be poured all at one time. Neither piece of equipment was available. Attempts to use a large funnel as a modified reservoir resulted in breaking the top of the column. Therefore, the column was packed as described below. Air was removed from the nylon net screen of the gel bed support in the bottom column piece by injecting 5-10 mL buffer into the column through the outlet tubing.

c. Packing the Column.

The column was packed using hydrostatic pressure rather than a pump. The slurry of gel was mixed gently by swirling and then poured slowly into the column down a glass rod held in contact with the column wall. When the column was filled, the gel was allowed to settle for 5-10 min. The flow rate of the buffer, regulated by the flow-control valve was adjusted to no more than 1 drop per 15 sec. Buffer was added periodically to the column to keep the gel from drying. As the gel settled in the column, more slurry was added after first resuspending the upper layer of gel in the buffer to prevent uneven packing. The mixing of the gel in

the column was best accomplished by gently swirling a length of tygon tubing (the type supplied with the column) in the upper layer of gel until an even suspension was obtained. This method was also most appropriate for the removal of entrapped air bubbles. The gel slurry was added until the packed gel bed was approximately 5 cm above the desired height. Some compression of the gel occurred when the buffer reservoir was attached to the column.

d. Connection of Column with Buffer Reservoir.

The column assembly was completed by one of two procedures: (1.) use of the column top piece; (2.) introduction of the adaptor (necessary after the top of the column was broken).

(1.) Use of Column Top Piece.

The tubing from the buffer reservoir (fitted with a clamp) was connected to the tubing from the column top piece. The entire length of tubing to the end of the column top piece was filled with buffer. The top of the column was carefully filled with buffer so as not to disturb the gel bed. The column outlet was closed. The air vent in the column top piece was opened and the top piece was screwed onto the column end piece. A slow flow of buffer from the reservoir was begun to remove through the air vent any remaining air from the top of the column or from the tubing.

Then the air vent was closed. The buffer flow was adjusted to the desired rate by the flow-control valve.

(2.) Introduction of Adaptor.

When the top of the column was broken, a complete seal was not obtained when the column top piece was connected to the column. Use of the adaptor (A16) solved the problem since the seal with the O-ring was made just above the gel bed and not at the top of the column as with the column top piece.

Explicit instructions for the assembly and insertion of the adaptor are provided by the supplier. The tubing from the buffer reservoir was connected to the tubing from the adaptor by means of a three-way tubing connector. The outlet tubing of the connector was sealed with a metal stopper (provided with the adaptor). The instructions for insertion of the adaptor specified that there should be no liquid space between the gel bed surface and the adaptor net. This precaution was not observed here. A buffer space of 3-4 mm was maintained between the gel bed and the adaptor net. Since the density of the sample applied to the gel was much greater than that of the buffer, it was assumed that little dilution or mixing of sample with the buffer occurred. It was necessary to maintain this space between gel and adaptor in order to monitor the gel bed for any gross bacterial contamination (see discussion below).

e. Equilibration of the Gel Bed.

When the column assembly was completed, the gel was equilibrated with the buffer by passing 2-3 gel bed volumes of buffer through the column (400-600 mL for a 200 mL-bed volume). The flow rate was maintained at approximately 1 drop per 15 sec. The hydrostatic pressures recommended (LKB) for packing and equilibration of the gel were not observed due to space and safety limitations. The distance between the buffer level and the outlet, however, was held relatively constant. The level of the buffer in the reservoir averaged 25-30 cm above the gel bed; the outlet (in the fraction collector) was 90 cm below the top of the gel bed. The total hydrostatic pressure, as measured from the buffer to the outlet, was approximately 120 cm H_2O. Such a high pressure probably contributed to the gradual compression of the gel, but it appeared to have no adverse effect on the resolution of the sample.

2. Application of the Sample to the Column.

The volume of sample applied to the column ranged from 0.7-1.0 mL (0.5% or less of the total gel bed volume). The amount of protein contained in this volume was 20 mg. The average protein concentration of the sample was 25 mg/mL. The sample was applied either under the eluant or through the adaptor.

a. Application under the Buffer.

This method was used when the column top piece was used to seal the column. The column outlet was closed, and the outlet tubing was positioned in the fraction collector. The flow from the buffer reservoir was stopped, and the column top piece was removed. A syringe was fitted with a needle to which was attached a tight-fitting piece of tygon tubing. The premeasured sample was drawn into the syringe through the tubing and a small air space was left at the tubing outlet. The tubing was lowered into the buffer and held a few mm above the gel bed. (The sample applicator cup, provided by Pharmacia, was not used because it tended to settle a few mm into the gel bed.) The sample was released from the syringe at an even rate using care not to disturb the gel. When the application was complete, the tubing was carefully removed from the column. Buffer was added to the top of the column if needed, and the column top piece was replaced as described above. The clamp controlling the flow of buffer from the reservoir was opened, the flow rate was adjusted by the flow-control valve, and the fractions were collected using the drop-counter mode.

b. Application through Adaptor.

Application of the sample through the adaptor required more time but provided better control of the process. The

flow-control valve was closed, and the outlet tubing was positioned in the fraction collector. The flow of buffer was reduced. The metal stopper in the outlet tubing of the three-way connector was removed, and a sample reservoir (a syringe barrel fitted with a needle) was quickly inserted. The buffer was allowed to enter the syringe to the bottom of the barrel and the clamp was closed. The sample was measured into the syringe, and the application was initiated by opening the flow-control valve and adjusting the flow to the desired elution rate. Fraction collection was begun at this time using the drop-counter mode. When the sample level reached the bottom of the syringe barrel (but had not entered the needle), 0.5-1.0 mL eluting buffer was added to the syringe to wash the remaining sample into the gel. This washing step was repeated twice more. When the application process was completed, the sample reservoir was removed from the tubing, and the metal stopper was quickly inserted to prevent air bubbles from entering the system. Buffer flow from the reservoir was resumed immediately.

3. Elution Conditions.

The following conditions were maintained for all the experiments. The column dimensions were 1.6 x 87 cm (the column height ranged between 85-90 cm) with a total bed volume averging between 170-180 mL. The eluting buffer was 0.05 M triethanolamine·HCl (TEA), 0.1 M NaCl, 0.01 M $MgCl_2$,

0.02% NaN_3. The flow rate was maintiained at 1 drop per 10-15 sec. Each fraction contained 40 drops (approximately 1 mL), and 160-200 fractions were collected over a priod of 20-24 h.

4. Elution of Molecular Weight Markers.

A high molecular weight polysaccharide, Blue Dextran 2000, was eluted from a freshly prepared Ultrogel AcA 22 column to determine the exclusion (void) volume of the column. Proteins of various molecular weights were individually eluted from the column to determine the approximate location of each peak in the elution pattern. Since the fractionation range of Ultrogel AcA 22 is 1×10^5 to 1.2×10^6 daltons, commercially available proteins of high molecular weights were chosen. The markers and their molecular weights were as follows: Blue Dextran 2000, MW 2×10^6 (Pharmacia); catalase, MW 250,000; bovine gamma globulin, MW 150,000; and bovine serum albumin, MW 66,000 (Sober, 1968).

Each sample (10 mg Blue Dextran and 20-25 mg each of the proteins) was dissolved in 1 mL eluting buffer and filtered through glass wool if any insoluble material remained. The samples were applied and eluted individually from the column as described above. The absorbance value of each fraction containing Blue Dextran was read at 260 nm. The fractions containing the proteins were read at 280 nm. The absorbance values were plotted against fraction number,

which was equivalent to the volume in mL. A column with an average gel height of 87 cm had an exclusion volume, as indicated by Blue Dextran, of approximately 45 mL. The proteins, even though the molecular weights ranged from 66,000 to 250,000, eluted from the column in the narrow range of 90-100 mL. The catalase elution pattern showed two peaks: one peak plus a shoulder in the 90-100 mL volume; the second in the low molecular weight region of 115 mL. This peak may have been due to an impurity in the sample.

5. Maintenance of the Column.

Between experiments the Ultrogel column was preserved by maintaining a slow flow of buffer through it. The eluting buffer contained sodium azide (0.02% final concentration) to retard bacterial growth. A number of precautions were observed to prevent further contamination of the Ultrogel. The buffer reservoir and tubing were thoroughly washed each time that a fresh column was prepared, or whenever the buffer appeared to be contaminated. The buffer supply was replenished in only 500 mL volumes in order to provide rapid turnover of the buffer. The buffer was frequently checked visually for bacterial growth, especially during periods of warm weather.

The major source of gross contamination of the Ultrogel, however, was the sample itself - the crude extract from disrupted bacterial cells. The problem was particularly

severe when the sample was applied under the buffer directly upon the gel bed.

The only remedy was to remove the contaminated gel after each experiment and replace it with fresh gel. The top of the column was disassembled and most of the buffer above the gel was removed. The top 2-3 cm of gel was siphoned by syringe without stirring the gel. Fresh buffer (2-5 mL) was added to the column and 2-3 cm of the upper gel bed was evenly suspended in the buffer. Freshly degassed and temperature-equilibrated gel was added to the column to the desired height. The column was reassembled as described above.

Bacterial degradation of Ultrogel appeared to be minimized when the sample was applied through the adaptor. The only problem resulting from this procedure was the eventual clogging of the nylon net screen of the adaptor. Therefore, the nylon net was changed when each experiment was completed. Removal of the adaptor caused minor disturbances of the gel bed. The upper layer of gel (1-2 cm) was evenly suspended in the buffer and allowed to settle before the adaptor was re-inserted.

6. Enzyme Assays.

a. Benzoylformate Decarboxylase.

A general description of this assay is provided in the previous chapter. The conditions under which the various samples obtained during the course of this research were assayed are described below.

The usual conditions involved preparation of a bulk substrate solution, and the addition of water and varying amounts of protein to the sample tubes (0.1 mL total volume).

The substrate solution contained the following reagents:

Reagents	Volume per tube	Conc. per tube
Benzoylformate, 0.025 M pH 6.0	0.04 mL	1 µmole
Phosphate buffer 40 mM, pH6.0	0.10 mL	40 µmole
Thiamine PPi, 1 mg/mL	0.05 mL	50 µg
H_2O	0.21 mL	
Total volume	0.40 mL	

Each volume was multiplied by the number of samples to be assayed plus the standard to make up the bulk solution. The crude extract (control or DMSI-treated) was diluted with TNM buffer (pH 8.0) to a concentration of 1.25 mg/mL. The usual protein range assayed was 0.01 to 0.15 mg and usually nine

points were determined.

The tubes were prepared in the following manner:

	Blank	Standard	Samples
	(1)	(2)	(3)
H_2O	0.50	0.10	0.095-0.0
Crude Extract 1.25 mg/mL	----	----	0.005-0.1
Substrate Solution	----	0.40	0.40

Incubation time: 30 min at 25°C

The remaining procedure was carried out as described in the previous chapter.

When the fractions eluted from the Ultrogel column were assayed, the substrate solution was prepared as above and the volume of each fraction assayed was 0.1 mL. Incubation time was initiated by addition of the substrate solution to the sample. If the absorbance value of the benzoylformate-2,4-dinitrophenylhydrazone was less than 0.200, the assay was repeated using 0.05 mL fraction plus 0.05 mL H_2O. Incubation was initiated by addition of 0.40 mL substrate solution.

When the *in vivo* cross-linked crude extract was eluted from the Ultrogel column, assay conditions were slightly altered in order to detect low levels of decarboxylase activity in the void volume fractions. The substrate solu-

tion was prepared as described except that 0.11 mL H_2O per tube was added to the solution instead of 0.21 mL. The reaction was initiated by addition of 0.30 mL substrate solution (instead of 0.40 mL) to 0.20 mL eluted fraction. The incubation time was increased to 60 min.

In the case of the control and DMSI-treated 100,000 x g pellets, barely detectible (control) and low (DMSI-treated) levels of enzyme activity were observed. Although the protein concentrations of these samples were 8-10 mg/mL, the samples were not diluted. The usual volumes assayed ranged from 0.05-0.10 mL and 0.40 mL substrate solution was added to start the reaction. The DMSI-treated samples were incubated for 30 min, but the incubation time for the control samples was 60 min.

The calculation of enzyme activity was carried out as follows. The absorbance value of the samples, but not the standard, at 540 nm was corrected for the presence of a small amount of dissolved benzaldehyde-2,4-dinitrophenylhydrazone (see previous chapter) by the subtraction of 0.055 (the average absorbance value of the dissolved material) from each reading. The amount of benzoylformate present in the standard and in each sample was obtained from the standard curve using the corrected absorbance values. The amount of benzoylformate present in each sample was subtracted from the total in the standard to give the μmoles benzoylformate catalyzed per 30 min (or 60 min).

This last value was divided by the incubation time (30 or 60 min) to obtain μmoles benzoylformate catalyzed per min (or units). For the crude extracts and the 100,000 x g pellets the μmole/min values were divided by the amount of protein in each sample to give the enzyme activity as μmole/min/mg protein. The μmole/min values of the eluted fractions were divided by the volume assayed yielding the enzyme activity as μmole/min/mL fraction.

b. cis,cis-Muconate Lactonizing Enzyme

The activity of this enzyme was measured by monitoring the disappearance of the substrate in the spectrophotometer at 260 nm. The spectrophotometer was set up to record the rate of the decrease in substrate concentration as described inside the panel behind the switch labeled "Off, UV or Vis, UV and Vis". The ratio switch was set to 0.1, the 100 chart divisions were set to equal a change of 2.000 absorbance units, and the wavelength was set at 260 nm with the deuterium lamp on for the ultraviolet region.

The following reagents were added to the cuvettes:

Reagents	Volume/ cuvette	Conc./ cuvette	Final conc.
cis,cis-Muconate 1 mM, pH 8.0	0.30 mL	0.3 µmole	100 µM
$MnCl_2$, 10 mM	0.30 mL	3.0 µmole	1 mM
Tris, 33.3 mM, pH 8.0	to total of 3.0 mL when sample is added	100 µmole	33.3 mM

Crude extracts (1.25 mg/mL protein concentration) were assayed in the range of 0.01 to 0.15 mg protein per sample, and usually eight points were determined. The protein was added to begin the reactions. No changes were required during the assay of the eluted fractions from which 0.1 mL was used to initiate the reaction.

It was difficult to assay the 100,000 x g pellets for lactonizing enzyme activity due to the turbidity of the sample. Volumes in the range of 0.02 to 0.07 mL could be assayed, but a series of manipulations on the spectrophotometer were necessary in order to blank out the turbidity. The reaction was initiated by addition of substrate rather than addition of protein. To the cuvettes were added the following reagents:

$MnCl_2$, 10 mM	0.30 mL
Tris, 33,.3 mM	to total 3.0 mL
100,000 x g Pellet	0.02 to 0.07 mL

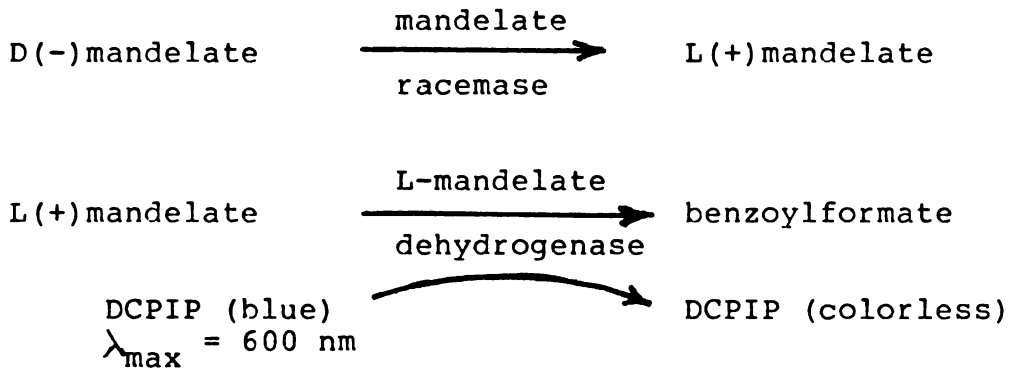
The cuvettes were placed in spaces 2, 3, or 4 of the cuvette holder. The cuvette to be monitored was moved into the light path. The slit width was opened to 1.8 mm, and the proper recorder offset dial was adjusted until both the spectrophotometer and the recorder pen were zeroed. The reaction was started by addition of 0.3 mL cis,cis-muconate.

The enzyme activity was calculated for each of the samples in the following manner. The change in absorbance was determined by drawing a straight line through the linear portion of the recorded curve. The number of chart divisions (estimated to the 0.1 decimal) along this line in a one-inch span was multiplied by 0.02 (100 chart divisions = 2.000 absorbance units), and divided by the chart speed in min/inch to give the change in absorbance/min. The disappearance of 1 µmole of cis,cis-muconate was equivalent to a decrease of 5.75 absorbance units under the conditions of the assay. The absorbance/min value was divided by 5.75 absorbance units/µmole yielding the enzyme activity in µmole/min, or units. The enzyme activities in the crude extracts and in the 100,000 x g pellets were reported as µmole/min/mg protein. The activity in the eluted fractions was reported as µmole/min/mL fraction.

c. Mandelate Racemase

The activity of this enzyme was measured in a coupled assay in which the bleaching of the blue dye 2,6-dichlorophenolindophenol (DCPIP) by L-mandelate dehydrogenase was monitored at 600 nm when D-mandelate was added to the reaction mixture (Hegeman, 1966a).

The reaction scheme is shown below.



The difficulties and disadvantages of this assay are discussed by Sharp *et al.*, (1979). However, the problems which must be corrected in each assay are detailed here:

(1) the presence of a small amount of L-mandelate in the substrate D-mandelate;

(2) the presence of low levels of racemase activity in the L-mandelate dehydrogenase (L-MDH) "particles";

(3) the turbidity of the assay mixtures.

Two other usual sources of difficulty - the reoxidation of the DCPIP by the electron transport system present in the

particulate fraction, and the variable quality of DCPIP - were readily solved. First of all, the current supply of DCPIP was of good quality. It contained no water-insoluble material and gave reproducible results at the concentrations used. Second, the reoxidation of DCPIP was eliminated by poisoning the electron transport system by addition of KCN to the assay system.

The commercially available D-mandelate contained small amounts of the L-isomer. This contaminant was removed from each assay mixture by the L-MDH "particles" prior to beginning the racemase reaction.

L-mandelate dehydrogenase is a membrane vesicle-bound protein which is isolated as the particulate fraction from the disrupted cells of P. putida A.3.12. The enzyme may also be isolated from cells of the rac^- (PRS-2) mutant derived from the above strain of P. putida (Hegeman, 1966b). The enzyme used in the racemase assays of this research, however, was isolated from the racemase-containing P. putida A.3.12.

The high turbidity of the assay mixtures is due to the light scattering of the L-mandelate dehydrogenase-containing vesicles present in the mixture. The effects of the light scattering were reduced by using the turbid assay mixture (minus the DCPIP and the racemase sample) to zero the spectrophotometer.

Addition of DCPIP to the assay mixture provided an

electron acceptor for the L-mandelate dehydrogenase conversion of the L-isomer contaminant to benzoylformate, and of the low level of L-mandelate produced by the racemase activity present in the L-MDH "particles". This racemase activity was measured and subtracted from that of the samples assayed.

The spectrophotometer was prepared for recording the decolorization of DCPIP with the ratio switch set at 0.1, the change of absorbance set equal to 2.000 units per 100 chart divisions, and the wavelength set to 600 nm with the tungsten lamp on for the visible region. To reduce the amount of time required for each assay, four cuvettes were set up containing the following reagents:

Reagents	Volume/ cuvette	Conc./ cuvette	Final conc.
Hepes, 0.1 M pH 7.0	2.00 mL	200 µmole	66.7 mM
$MgCl_2$, 0.1 M	0.1 mL	10 µmole	3.33 mM
KCN, 0.5 M	0.10 mL	50 µmole	16.7 mM
D-Mandelate, 0.1 M pH 7.0	0.25 mL	25 µmole	8.33 mM
H_2O	0.19-0.10 mL (for sample volume range of 0.01-0.10 mL)		
L-MDH "Particles"	0.05 mL		

The reagents were well mixed and placed in the cuvette holder. The spectrophotometer was zeroed using cuvette #1.

DCPIP, 1 mM	0.30 mL	0.3 µmole	0.10 mM

The dye was well-mixed in each cuvette. The L-MDH activity followed by the racemase activity of the "particles" were measured for at least 15 min in all four cuvettes using the automatic sample changer.

Crude Extract (1.25 mg/mL)	0.01-0.10 mL	0.013-0.125 mg added
Total volume	3.0 mL	

The reaction was started by addition of the protein and each sample was assayed individually. There was an initial lag time in the reaction course while sufficient levels of L-mandelate were accumulating before conversion by the L-MDH, resulting in sigmoid-shaped curves.

No changes in the assay procedure were required for the determination of enzyme activity in the eluted fractions or in the 100,000 x g pellets. The volume of the eluted fractions added to the assay mixture was 0.1 mL, while a volume range of 0.05-0.10 mL 100,000 x g pellet was used for the racemase assay.

Calculation of mandelate racemase activity is described below. For each sample assayed a straight line was drawn through the linear portion of the sigmoid curve. The change in absorbance/inch was determined by counting the number of chart divisions along the line in one inch and multiplying this number by 0.02 (100 chart divisions = 2.000 absorbance units). The change of absorbance/inch was divided by the chart speed in min/inch to obtain the change of absorbance/min. The change of absorbance/min was calculated

in the same manner for the small amount of racemase activity present in the L-MDH "particles". This value was subtracted from the absorbance/min obtained for each sample. A decrease of 6.72 absorbancy units at 600 nm under the conditions of the assay corresponds to the conversion of 1 µmole of mandelate from the D(-) to the L(+)isomer. The absorbance units/min value was divided by 6.72 absorbance units/µmole to give the enzyme activity in µmole/min, or units. The enzyme activities of the crude extracts and the 100,000 x g pellets were reported as µmol/min/mg protein. The activity in the eluted fractions was reported as µmole/min/mL fraction.

Mandelate racemase activity was also determined in a qualitative manner. The procedure was to locate the enzyme in column eluants or to detect its presence in supernatant washes. The following reagents (the same as used in the quantitative assay) were added to test tubes:

Hepes	2.00 mL
$MgCl_2$	0.10 mL
KCN	0.10 mL
D-Mandelate	0.25 mL
H_2O	0.10 mL
L-MDH	0.05 mL
DCPIP	0.30 mL
Sample	0.10 mL

(2.0 mL supernatant wash was substituted for the buffer and the reaction was initiated with substrate addition.)

After the sample (or substrate) addition, the time period for decolorization of the sample was measured relative to a blank tube, which contained all the reagents except the sample.

d. L-Mandelate Dehydrogenase

The activity of this enzyme was determined by monitoring the decolorization of DCPIP at 600 nm. The spectrophotometer was prepared in the same manner as described for mandelate racemase. The following reagents were added to each cuvette:

Reagents	Volume/ cuvette	Conc./ cuvette	Final conc.
Hepes, 0.1 M pH 7.0	2.00 mL	200 µmole	66.7 mM
L-Mandelate 0.1 M, pH 7.0	0.25 mL	25 µmole	8.33 mM
H_2O	0.40-0.05 mL		
DCPIP, 1 mM	0.30 mL	0.3 µmole	0.10 mM
Crude Extract 1.25 mg/mL	0.05-0.40 mL	0.06-0.50 mg	

The reaction was started with the addition of protein and the bleaching of the dye was monitored at 600 nm. No changes in the assay procedure were required. The volume of the eluted fractions assayed was 0.1 mL and the volume range

was 0.01-0.05 mL for the 100,000 x g pellets.

The calculations were carried out in the same manner as described for mandelate racemase. The decrease of 6.72 absorbance units corresponds to the oxidation of 1 μmole of L(+)-mandelate to benzoylformate.

The activity for each enzyme in μmole/min was plotted as a function of the amount of protein added per sample (Figure VI.1).

cis,cis-Muconate lactonizing enzyme and L-mandelate dehydrogenase activities were linear through 0.125 mg and 0.50 mg protein per sample, respectively. Linearity for benzoylformate decarboxylase extended only to ca. 0.06-0.08 mg protein per assay tube. The lack of linearity in the mandelate racemase is probably due to the light-scattering effects of the L-MDH vesicles in the assay mixture and to the competing racemase activity present in the L-MDH particulate fraction.

Statistical Evaluation

Enzymes	r^2	slope	intercept
L-MDH	0.9934	0.203	-0.002
Benzoylformate decarboxylase	0.9938	0.388	0.000
cis,cis-Muconate lactonizing enzyme	0.9932	0.621	0.000
Mandelate racemase	average specific activity = 1.423 μmole/min/mg protein		

Figure VI.1.

A plot of μmole activity for each enzyme *vs.* the amount of protein added per sample. The enzymes assayed are benzoylformate decarboxylase (□——□), *cis,cis*-muconate lactonizing enzyme (O——O), mandelate racemase (△——△), L-mandelate dehydrogenase (▲——▲).

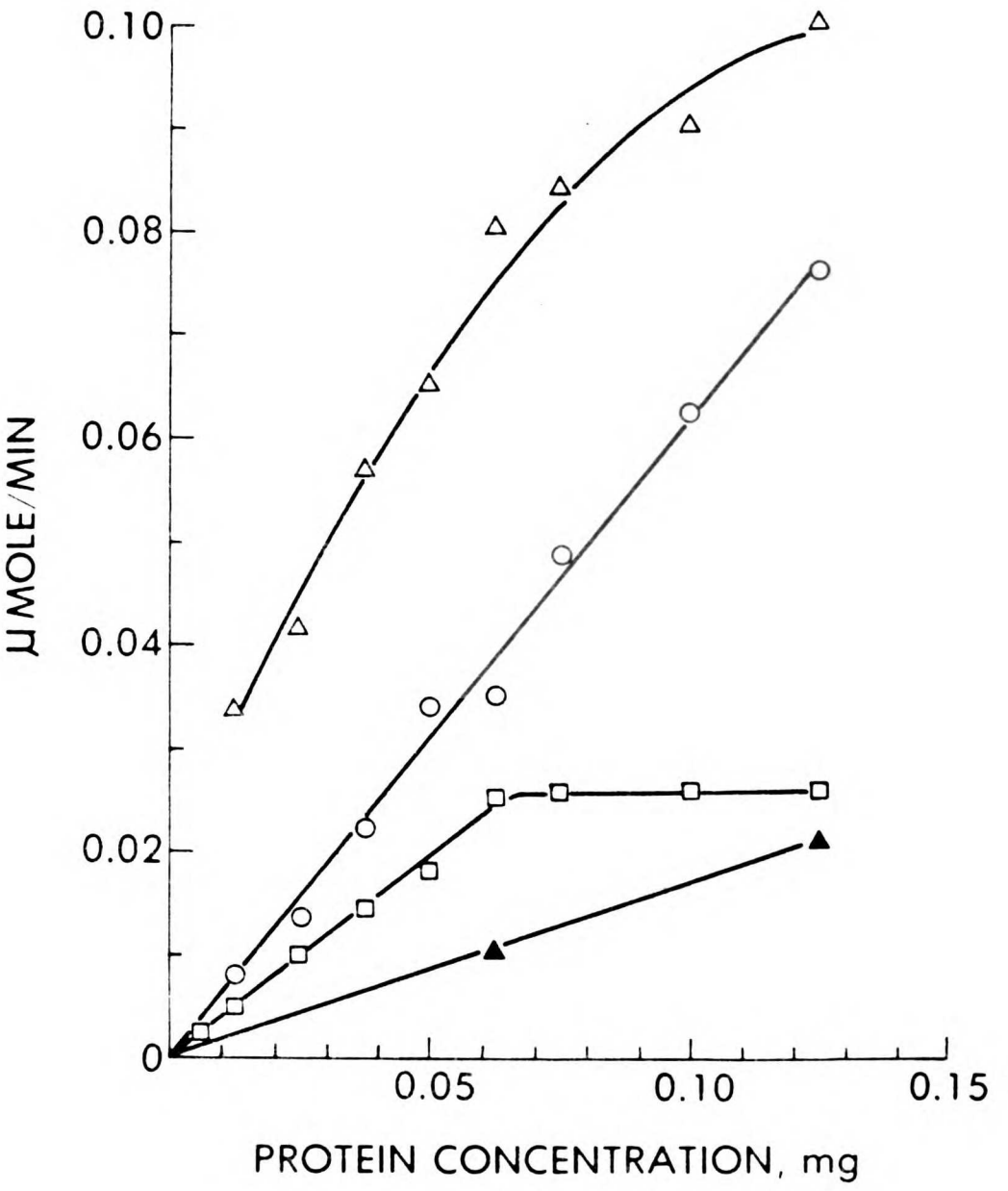

0.10
0.08
0.06
0.04
0.02
0
μ MOLE/MIN
0.05
0.10
0.15
PROTEIN CONCENTRATION, mg

Figure VI.1.

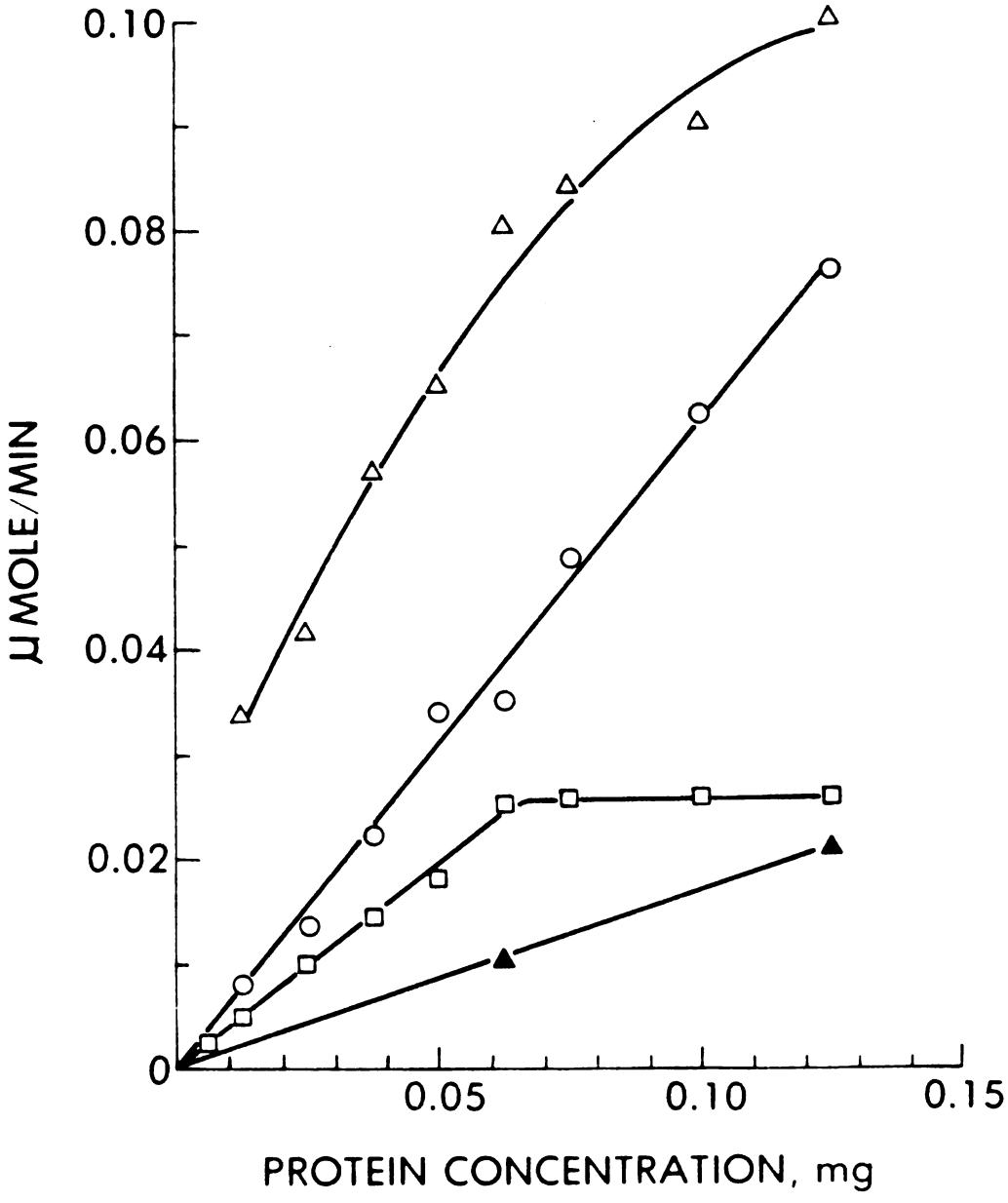

0.10
0.08
0.06
0.04
0.02
0
μMOLE/MIN
0.05
0.10
0.15
PROTEIN CONCENTRATION, mg

Figure VI.1.

D. ^{13}C T_1 (Spin-Lattice) Relaxation Time Determination

The initial ^{13}C NMR studies of mandelate metabolism in whole cells and in isolated, in vivo cross-linked enzymes were performed using a rapid pulse sequence with a pulse angle of approximately 80^{o}. These parameters did not provide optimum conditions for spectral observation of the labeled carbons of all the products of mandelic metabolism by the mandelate group of enzymes. The expected labeled carbons included the α-keto carbon of benzoylformate, the aldehydic carbon of benzaldehyde, and the carboxylate carbon of benzoic acid. From a table of representative ^{13}C T_1 values (Mullen & Pregosin, 1976) the relaxation times of these carbons would be expected to range from 30-50 sec or longer. Therefore, since the carboxylate carbon was readily observed in the NMR spectra and the aldehydic carbon contained a hydrogen (facilitating its relaxation), it appeared that the α-keto carbon of benzoylformate would have the longest T_1 relaxation time.

The relaxation time determination was performed on the Varian XL-100 spectrometer at a frequency of 25.2 MHz. Since the T_1 value was expected to be reasonably long, the experiment performed was the "T_1 by Progressive Saturation with Homospoil" (T_1HS) as described in the NTCFT instruction manual (Nicolet, 1976). The theory and method are explained

with references by James (1975) and in the instruction manual for the FT-80 NMR spectrometer (Varian, 1977).

The pulse sequence for the T_1HS experiment is

$$(P_1, P_3, D_3, D_1, P_2, \text{sample and add}, D_3)_{NA}.$$

P_1 = 10 μsec is a 90° pulse which flips the magnetization into the x'-y' plane. The leading edge of P_1 triggers the homospoil sequence, consisting of a delay P_3 = 0.0001 sec followed by the spoiling pulse D_3 = 0.01 sec. After a series of delay times (τ) D_1 = 20, 60, 90, 200, 300, 500 sec, the spin system gradually relaxes, resulting in a new magnetization along the z-axis. P_2 = 10 μsec is the 90° pulse which flips the magnetization into the x'-y' plane for sampling. After this measure sweep another spoiling pulse D_3 = 0.01 sec occurs to dephase the spin. The advantage to the homospoil sequence lies in the fact that long delays of $5T_1$ (used in T_1 experiments by inversion recovery) are unnecessary.

Benzoylformate, 1 M pH 5.0 (5 mL) was prepared in D_2O and poured through a Pasteur pipette containing chelex-100 in D_2O to remove a yellow contaminant and any possible paramagnetic metal ions. The solution was oxygenated for ca. 15 min, and placed in a 12-mm NMR tube fitted with a vortex plug. The experiment was carried out as described above with the temperature control set for 5°C at the probe. The peak heights for the α-keto carbon (197.5 ppm) were measured using the peak printout (PP) command. These

numbers plus the τ values (D_1 delay times in sec) were used to calculate the T_1 value by the following equation:

$$M_\tau = M_o\,(1 - e^{-\frac{\tau}{T_1}}) \qquad (5)$$

which is integrated to

$$\ln (M_\tau - M_o) = \ln M_o - \frac{\tau}{T_1}. \qquad (6)$$

M_τ is the magnetization along the z-axis at time τ ;

M_o is the magnetization along the z-axis at τ = 0 (or $5T_1$).

A plot of ln (M_τ - M_o) vs. τ gives a slope of $1/T_1$ with the intercept equal to ln M_o. The T_1 value obtained for the α-keto carbon of benzoylformate (in the presence of oxygen) was calculated to be 56.3 sec.

By setting the tilt angle (α_E) to 45° and the T_1 value to 60 sec, it was possible to calculate the delay time between pulses required to obtain the maximum signal from the following equation:

$$\cos\alpha_E = e^{-\frac{(t_{acq} + t_{post})}{T_1}} \qquad (7)$$

where α_E is the Ernst (tilt) angle, t_{acq}, is the acquisition time, and t_{post} is the delay time between pulses. The value obtained for the delay time was 20 sec.

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VII. SYNTHESIS OF A LIGAND FOR AFFINITY CHROMATOGRAPHY.

A. Introduction

All experiments to date on the isolated, in vivo cross-linked multienzyme complex have been performed on "crude" material, which contained not only the complex of enzymes but also ribosomes, membrane fragments, nucleic acids, and other high molecular weight material. Preliminary attempts to purify the cross-linked complex (gel filtration on Sepharose 4B, polyacrylamide gel electrophoresis) were unsuccessful.

A recently-developed technique, affinity chromatography, appears to have great potential for two reasons: (1.) isolation and purification of the cross-linked complex; (2.) further purification of mandelate racemase, which by current methods is obtained containing about 5% impurities.

A brief description of affinity chromatography will be given here, but a number of excellent books and earlier review articles are available to the reader (Cuatracasas & Anfinsen, 1971; Lowe & Dean, 1974; Jakoby, 1971; Jakoby & Wilchek, 1974).

B. Affinity Chromatography.

Affinity chromatography has been defined as "...a separation process whereby the material to be separated is

selectively retarded by complex formation to an insolubilized binding ligand. High selectivity is achieved by using binding ligand materials with specific or highly selective affinity for the desired substance" (Baum & Wrobel, 1975). The isolation is by biochemical function rather than by separation based on the physical and chemical differences between substances.

There are five components of affinity chromatography which must be considered, whether the procedure involves isolation and purification of an enzyme by an immobilized ligand, or separation of a substrate from a mixture by an immobilized enzyme. These components are the following:

a.) the support and its activation;

b.) a spacer arm with the necessary functional groups at each end;

c.) synthesis of a ligand with the required functional groups by which it can be covalently bound to the spacer arm, and which has the necessary affinity or specificity for the substance to be isolated;

d.) adsorption conditions;

e.) elution conditions.

1. The Support

The characteristics of a favorable support include

a.) minimal interactions with proteins in general,

b.) formation of a loose, porous network allowing easy entry and exit of macromolecules,

c.) retention of good flow properties during use,

d.) a chemical structure that permits convenient and extensive attachment of specific ligands under mild conditions,

e.) absence of charged or hydrophobic groups.

The matrix of choice, particularly for isolation and purification of enzymes, is generally Sepharose, a "beaded" form of the cross-linked dextran of highly porous structure, agarose. Agarose is a linear polysaccharide consisting of alternating residues of D-galactose and 3,6-anhydro-L-galactose (Araki, 1937). Its gelling properties are attributed to extensive hydrogen bonding. Its hydrophilic nature and lack of charged groups cause very little denaturation and non-specific adsorption of sensitive biochemical substances.

2. The Spacer Arm.

Initial procedures in affinity chromatography called for the immediate addition of the ligand to the activiated Sepharose. Such conditions required that the ligand have a high affinity for the substance to be isolated; $K_i \approx 10^{-9}$ M for unbound ligand (Cuatracasas, 1968). Within a short period of time experimenters became aware that enzymes which

showed weak affinity for the ligand on the matrix were in reality poorly and incompletely (if at all) resolved. Extension of the distance between the matrix and the ligand improved resolution capabilities showing that strong inhibitors need not be required as a ligand. Indeed, a ligand with $K_i \approx$ 1-5 m$\underline{M}$ could effect good resolution.

The first compounds to be used as spacer arms were diaminoalkyl compounds, such as ethylenediamine and hexamethylenediamine, the monoaminoalkyl compound ε-amino caproic acid, as well as 3,3'-diaminodipropyl amine. A major disadvantage of these simple spacer arms was the resultant non-specific hydrophobic interaction with enzymes. (This problem later turned to an advantage and was developed as a new technique - hydrophobic chromatography.)

A large number of spacer arms have been developed, many for specific ligand or protein interactions, e.g. $\underline{p}$-chloromercuribenzoic acid which reacts with sulfhydryl groups of proteins; $\underline{p}$-nitrobenzoyl azide which, following reduction and diazotization of nitro groups, forms an azo derivative with the ligand; a tripeptide with tyrosine at the end to which a diazotozed ligand can be attached. Bis-oxiranes, such as 1,4-butanediol diglycidyl ether, provided a more hydrophilic milieu to reduce or eliminate hydrophobic interactions.

3. Adsorption and Elution

For adsorption of the material to be isolated batch or column conditions may be used following procedures for gel filtration chromatography. A variety of elution methods are available to remove the isolated material from the ligand. If the affinity of the enzyme for the ligand is not too great, the substance eventually elutes out. A change in the pH, temperature, or ionic strength of the eluant may remove the substance from the ligand, as well as addition of substrate or a stronger competitive inhibitor. If the affinity is very great, it may be necessary to use denaturants such as urea or guanidine hydrochloride to perturb the enzyme-ligand interaction. Following such treatment the enzyme must be neutralized, diluted, or extensively dialyzed to promote renaturation.

4. The Ligand

The heart in the design of an affinity chromatographic system lies in the appropriate selection and synthesis of an effective ligand. This ligand must display a unique affinity for the substance to be isolated. The ligand may be a reversible substrate analog inhibitor, an effector (a modulating molecule on allosteric enzymes), a cofactor, or a substrate. A substrate may function as a ligand under the following conditions:

(1) if the pH dependence of the K_m and the k_{cat} differ;

(2) if in metalloenzymes the substrate can be bound in the absence of the metal ion;

(3) if in a two-substrate system one substrate can be bound by the enzyme in the absence of the other substrate.

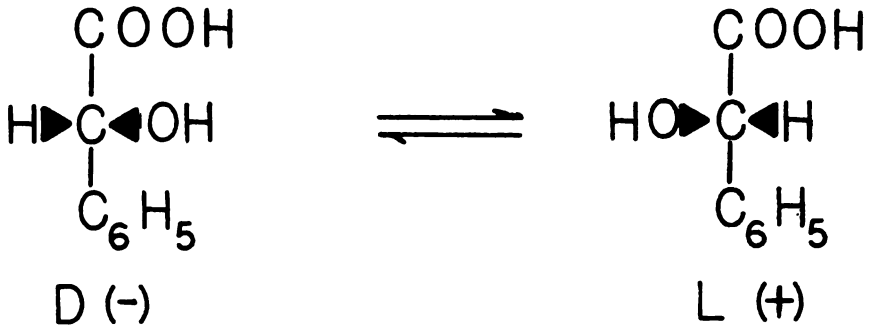
In the case of inhibitors, if the K_i is strong, i.e. approximately 1 mM or better for a free complex in solution, then a decrease in affinity of about three orders of magnitude after preparation of the insoluble derivative may still result in an effective and selective separation. It is difficult to prepare adsorbants when the K_i of the ligand is greater than 5 mM. Finally, the ligand must possess chemical groups which can be modified for coupling without loss of interaction with the protein.

C. Selection of a Ligand for the Multienzyme Complex

Selection of a ligand with affinity for the cross-linked multienzyme complex would at first glance appear to be a complicated task. If the complex indeed contained all the enzymes involved in the dissimilation of mandelic acid, then the researcher would have to choose among approximately eleven substrates as possible ligands, or even among four of the five common cofactors. The choices narrow considerably when substrate analog inhibitors are considered. Of the

five enzymes in the mandelate group and nine in the benzoate oxidase and β-ketoadipate groups of enzymes only mandelate racemase has been sufficiently studied to determine its mechanism of action and the nature of its active site (reviewed in Kenyon and Hegeman, 1979).

Mandelate racemase is an inducible enzyme which catalyzes the reversible inter-conversion of the D- and L-isomers of mandelic acid.



In addition to the D- or L-isomers of mandelic acid the enzyme accepts as substrates the p-hydroxy-, p-bromo-, p-chloro-, and p-methoxy-derivatives (Hegeman et al., 1970).

The more effective inhibitors of mandelate racemase are listed as follows (Hegeman et al., 1970; Fee, 1974):

Inhibitor	% Inhibition
1.) 1-hydroxy-2-naphthoic acid	87-90%
2.) 3-hydroxy-2-naphthoic acid	--"--
3.) 2-naphthoic acid	59.0%
4.) 4-methyl benzothiophene-2-carboxylic acid	63.5%
5.) coumarilic acid	38.5%
6.) salicylic acid	24.0%

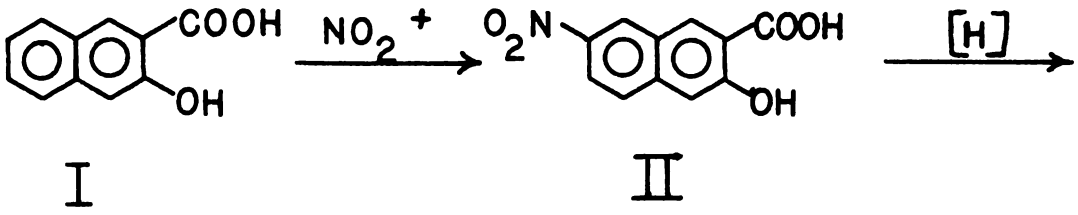
The K_i values for some of these inhibitors were determined by Maggio et al., (1975) in Hepes buffer (pH 7.0):

Inhibitor	K_m (M)	K_i (M)
1.) Mandelate	2×10^{-5}	-------
2.) salicylate		1.5×10^{-3}
3.) benzoate		2.7×10^{-3}
4.) 2-naphthoate		3.0×10^{-4}
5.) 3-hydroxy-2-naphthoate		3.8×10^{-4}

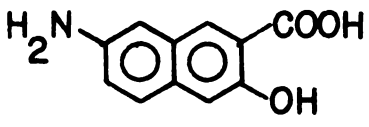
Studies of the reactivity of substrate analogs and the epoxide analogs of the active site-directed irreversible inhibitor, α-phenylglycidate, showed that an aromatic ring and the carboxylate group are important for activity, as is the distance between the aromatic ring and the carboxylate moiety, and the presence of the α-hydroxyl group (Fee, 1974).

From the above data, 2-naphthoate and its 1-hydroxy- and 3-hydroxy-derivatives appear to be the best choices as ligands for affinity chromatography. The 1-hydroxy-2-naphthoate was eliminated since its K_i value was not determined. Even though 2-naphthoate and 3-hydroxy-2-naphthoate have nearly similar K_i values, 3-hydroxy-2-naphthoate was chosen as the possible ligand because its ring system is more activated for chemical modification by electrophilic aromatic substitution than is the deactivated 2-naphthoate. Modification of 3-hydroxy-2-naphthoate, I, to 7-amino-3-hydroxy-2-naphthoate, III, via the 7-nitro- intermediate, II, would provide a ligand with a functional group at one end of the molecule by which it could be covalently attached to the spacer arm, leaving the active end of the inhibitor

available to the racemase as is shown in the following scheme.



I II



III

D. Results and Discussion

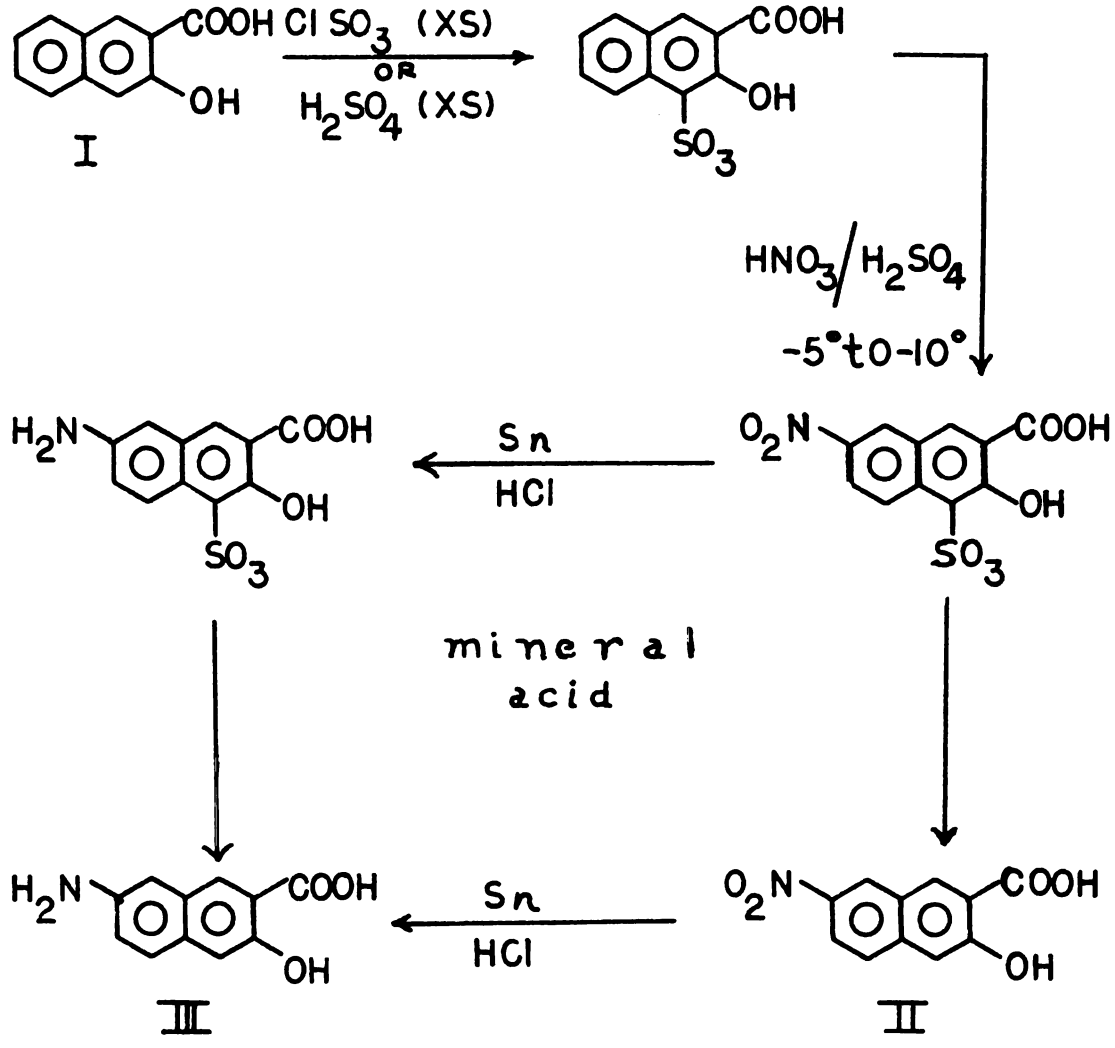
The synthesis of 7-amino-3-hydroxy-2-naphthoic acid was first reported by Cohen and Dudley (1910). The procedure involved protection of the hydroxyl group by methylation with CH_3I in the presence of NaOMe, followed by nitration with nitric acid in sulfuric acid, reduction of the nitro group to the amine with tin and HCl, and O-demethylation with 20% HI. The melting point of the isolated product was reported as 230-232° (dec).

Initial attempts to synthesize III by nitration but omitting the methylation step resulted in the formation of two products as observed by thin layer chromatography. The two compounds were not separable using silica gel column chromatography or preparative thin layer chromatography. The product was probably a mixture of the 4-, 5-, and 7-nitro- isomers. A repeat nitration involving two separate work-up procedures yielded a small amount of pure sample as determined by thin layer chromatography and by microanalysis, with mp 247-249° (dec). The compound isolated was most likely the 4-nitro-3-hydroxy-2-naphthoic acid. Protection of the 4-position would be necessary to eliminate formation of mixtures.

A second synthetic scheme to III beginning with 3-hydroxy-4-sulfo-2-naphthoic acid was reported by Hotz and Luce (1936). Synthesis of the 4-sulfo- derivative by

chlorosulfonation was reported by Dziewonski & Lowenhoef (1927). The 3-hydroxy-2-naphthoic acid could also be sulfonated by addition of excess sulfuric acid using procedures described by Adams et al., (1963) and Cason and Rapoport (1962).

The synthetic scheme is shown below.



The melting point of the final product III was reported to

be 298-299°. When compared with the melting point for the same compound as reported by Cohen and Dudley (230-232°, dec), it is obvious that the product of the earlier synthesis was not pure, or did not even have the same structure.

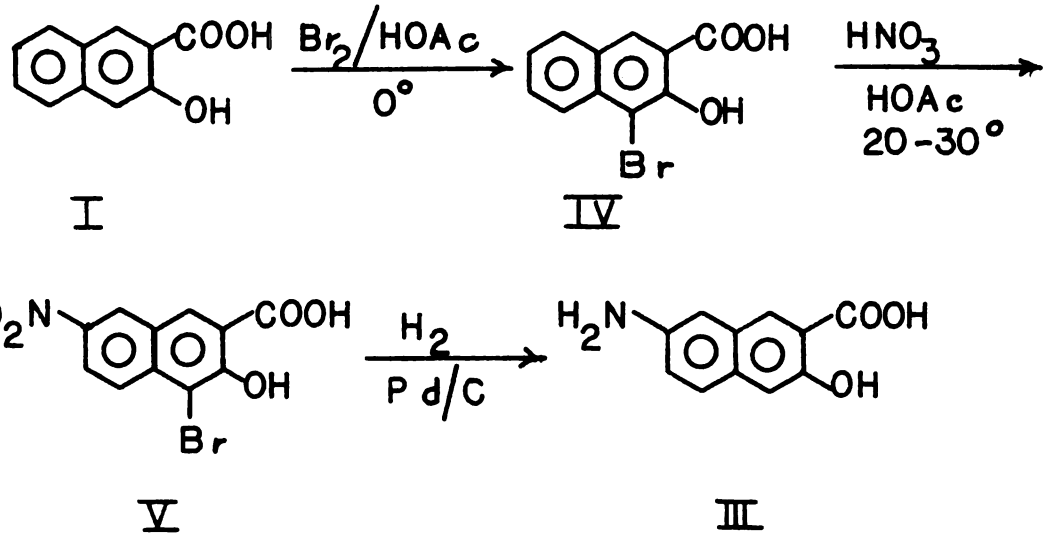
I was sulfonated in the presence of either a ten-fold excess of sulfuric acid or a six-fold excess of chlorosulfonic acid. The sodium sulfonate salt, isolated by a prolonged route and repeated recrystallization, was converted to the free acid and the melting point was determined to be 252-254° (dec). The value reported by Dziewonski for the 4-sulfo- compound was 182°. The melting points of the 5-sulfo- and 7-sulfo- isomers were not reported for the free acid but for the p-toluidine salts: 5-sulfo-3-hydroxy-2-naphthoate salt, mp 247° (dec); 7-sulfo-3-hydroxy-2-naphthoate salt, mp 268-269° (dec).

The synthesized sulfo-compound was converted from the free acid to the p-toluidine salt and the melting point was found to be 269-271° (dec). Therefore, the compound synthesized was 7-sulfo-3-hydroxy-2-naphthoic acid and not the desired 4-sulfo- isomer. It was discovered at a later date that the 7-sulfo- derivative could be converted to the sulfonamide, a compound which very likely could have been used as a ligand, but no further work was carried out on this compound.

Another electrophilic substitution reaction on aromatic

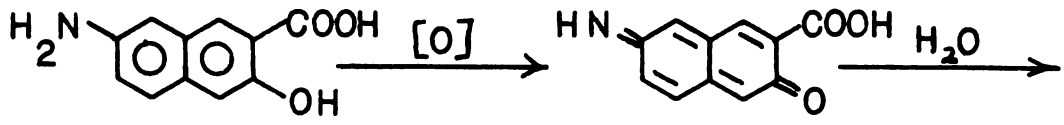
ring systems is bromination. The bromo-substituent can be easily removed by catalytic hydrogenation. Therefore, it should be possible to brominate the 4-position of I, followed by nitration in the 7-position and catalytic hydrogenation to debrominate the compound and to convert the nitro-group to the amine function.

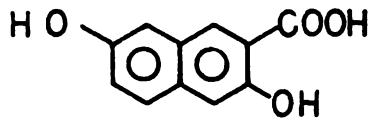
The proposed reaction pathway is shown below.



The 4-bromo- compound was first synthesized by Gradenwitz (1894), and the melting point was reported as 233-235°. The melting point of the readily synthesized 4-bromo-3-hydroxy-2-naphthoic acid was 234-237° (dec). The compound was treated with nitric acid in the presence of sulfuric acid at 0°. The isolated, crude 7-nitro- compound was hydrogenated in the presence of palladium on charcoal. Before the 7-amino- compound could be isolated, the solution underwent air oxidation; i.e. the yellow solution turned dark brown in air. Aromatic hydroxy-amines readily undergo

oxidation to iminoquinones which then hydrolyze to quinones.





Air oxidation could have been prevented by keeping the solution containing the desired product acidic, carrying out the reduction in acetic anhydride, or by alkylating the hydroxy-function. Any of these precautions would have made further experimentation with the compound as an affinity ligand difficult, if not impossible. A new functional group by which I could be attached to activated agarose had to be designed. The resultant, modified ligand would have to be stable under mild, biological conditions.

Attempts were made to attach an aminoalkyl group to IV by a variety of formylation techniques which were to be followed by reductive amination. The various formylation reactions used were the Vilsmeier-Haack and modifications of the Friedel-Crafts acylation. All these attempts resulted in either no reaction or in a mixture of products, including large amounts of starting material, which were not separated. It is likely that the ring system of 4-bromo-3-

hydroxy-2-naphthoic acid is too deactivated by the presence of the carboxylate and bromo- functions to undergo formylation.

No further work was undertaken to modify 3-hydroxy-2-naphthoic acid for affinity chromatography. Recent developments in the use of non-specific ligands such as the commercially available agarose-linked triazine dyes, Cibacron Blue and Procion Red, appear to be promising. Further studies for purification of mandelate racemase and the "crude" multienzyme complex could be attempted using affinity chromatography with these non-specific ligands or with an easily synthesized, stable, specific ligand.

E. Experimental

1. General Methods and Procedures.

a. Melting Points, Microanalyses, and Spectra.

All melting points or decomposition points were determined on a Thomas capillary melting point apparatus and were uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Infrared spectra were run as KBr pellets on a Perkin Elmer 157 spectrophotometer. ^{1}H NMR spectra were measured on a Perkin Elmer R12B 60 MHz spectrometer with samples dissolved in dimethyl sulfoxide (d_6) using tetramethylsilane (TMS) as reference.

b. Materials

Materials were obtained from the following sources: all commonly used inorganic salts and organic solvents were analytical grade and were obtained from Mallinckrodt or J.T. Baker; 3-hydroxy-2-naphthoic acid, chlorosulfonic acid, *p*-toluidine, and *o*-dianisidine were from Eastman; bromine was from Matheson-Coleman-Bell; palladium on activated carbon was from Aldrich.

c. Purification of 3-Hydroxy-2-naphthoic Acid.

The 3-hydroxy-2-naphthoic acid, technical grade, was usually purified in 50g batches (Klein & Benz, 1955). Approximately 1 to 1.5 L H_2O was placed in a 2L Erlenmeyer flask and heated. The naphthoic acid (50g) was added and stirred. Solid sodium bicarbonate (22g) was added in small aliquots (to prevent foaming) until the acid was dissolved. One to two grams of decolorizing carbon was added and the hot solution was filtered. Twenty-five mL glacial acetic acid was added to the hot filtrate with stirring. The compound precipitated as the free acid. After standing 1-2 h at room temperature the precipitate was filtered and recrystallized from hot ethanol with dropwise addition of water until the solution became slightly turbid. The soluton was allowed to stand for 8-12 h at 4°C. The crystals, which formed as light yellow, glistening plates, were filtered and dried in vacuo at 50°C giving approximately 35 to 40g product (70-80% yield), mp = 218-221°C.

d. Thin Layer Chromatography Conditions.

Thin layer plates, Silical Gel GF (250 microns) spread on glass, were obtained from Analtech. The 10 x 20 cm plates prescored to 2.5 x 10 cm were used. The solvent system was that given for the separation of phenol carboxylic acids (Kirchner, 1967). The system was as follows:

benzene:methanol:glacial acetic acid (90:16:8). A somewhat less polar system replacing methanol with dioxane was occasionally used - benzene:dioxane:acetic acid (90:25:4). When the 3-hydroxy-2-naphthoic acid was sulfonated, the products, which were water-soluble, would streak on the plate using the above solvents. The solvent system used for these compounds was the organic layer of n-butanol:glacial acetic acid:water (4:1:5).

The detection systems used were iodine vapors, ultraviolet light, and the Pauly spray reagent which detects phenol, aromatic amines, and sugar phenylosazones by diazotization (Kirchner, 1967). The Pauly reagent has two components: a.) 0.5% sulfanilic acid and 0.5% sodium nitrite in 1 N hydrochloric acid; b.) 1 N sodium hydroxide. The plate is sprayed with component a.) followed by component b.). The color of the spots ranges from yellow to red.

Other detection reagents used to follow formylation reactions were o-dianisidine (3-3'dimethoxybenzidine) as a saturated solution in glacial acetic acid, 5% ferric chloride in 0.5 N HCl, and 5% ferric chloride in water followed by 1 N HCl. The plates were heated after the ferric chloride spray (Stahl, 1969).

2. Chemical Syntheses

a. 4-Nitro-3-hydroxy-2-naphthoic acid was prepared by dissolving 3-hydroxy-2-naphthoic acid (5.0g, 0.0244 mole) in 40

mL glacial acetic acid. Nitric acid (1.69 mL, 0.0266 mole) in 15 mL glacial acetic acid was added dropwise over a period of 5-10 min. When addition was complete, the mixture was stirred for 5 min, and then cooled in an ice bath for 30 min. The mixture was poured into 200 mL ice/water from which a red-orange material precipitated. The entire mixture was left in the ice bath for 1 h and at room temperature for 12 h. The mixture was filtered and the brick-red precipitate was washed with a small amount of water. The work-up described below was carried out to isolate a pure compound for spectral analysis and microanalysis, not to optimize isolation conditions. The precipitate was dissolved in 70% ethanol and 1-2g decolorizing carbon was added. The mixture was heated and then filtered. The filtrate was divided into 20 mL- and 50 mL-aliquots which were subjected to base extraction and concentration, respectively. The 20 mL-aliquot was adjusted to pH 11 with 1 N NaOH and extracted with ether. The ether solution was dried with sodium sulfate and concentrated to induce crystallization. The solution was allowed to stand 12 h at 4°C during which time yellow needles formed. The crystals were filtered and recrystallized from hot dioxane to which water was added to turbidity. The solution was allowed to stand at 4°C for 12 h. The yellow needles which formed were filtered and dried, giving 0.8g, mp 247-249°. The 50 mL-aliquot was concentrated to dryness by lyophilization. The lyophilized

material was dissolved in ether and insoluble material was removed by filtration. The filtrate was concentrated to induce crystallization. The solution was left to stand at 4°C for 12 h during which time yellow needles appeared. The crystals were filtered and recrystallized from hot dioxane and water was added to turbidity. The yellow needles which formed were filtered and dried giving 0.245g, mp 247-250°. Total yield was 0.325g, (6.4% yield).

Anal. Calcd. for $C_{11}H_7NO_5$: C, 56.65; H,3.03; N, 6.01. Found: C, 56.75; H, 3.18; N, 5.74. IR: NO_2 stretch (asymmetric) 1515-1530cm^{-1}. ^1H NMR: δ 8.85 (1H,s), 7.4-8.3 (4H,m).

b. 7-Sulfo-3-hydroxy-2-naphthoic acid, sodium salt, was prepared by treating 3-hydroxy-2-naphthoic acid with either a six-fold excess of chlorosulfonic acid or a ten-fold excess of sulfuric acid.

(1.) Chlorosulfonation

3-Hydroxy-2-naphthoic acid (5.0g, 0.027 mole) was added slowly to chlorosulfonic acid (10 mL, 0.150 mole). The mixture was allowed to stir at room temperature for 15 min, and then was immersed in a 55°C water bath for 2 h after which the syrupy mixture was poured into 100g ice. The precipitate of unreacted starting material was removed by filtration followed by a wash with 100 mL cold water. The fil-

trate was allowed to stand at 4°C for 12 h to permit further precipitation of unreacted starting material which was removed by filtration. The pH of the filtrate was increased from approximately pH 0.5 to pH 2.0 with the slow addition of solid sodium bicarbonate (5-10g). Solid sodium chloride (6g) was added until the solution was slightly turbid. The solution was heated to dissolve the salt, allowed to cool slowly and to stand at 4°C for 12 h during which time pale yellow crystals formed. These crystals were filtered and dried, giving 0.90g. Concentration of the mother liquor by line vacuum resulted in further precipitation of two crops of crystals which were also filtered and dried, giving 0.08g and 0.18g, a total yield of 1.16g (23% yield). The fingerprint region of the infrared spectra showed each of these samples to be identical. The crystals of the 0.9g and 0.18g samples were dissolved in hot ethanol and insoluble, inorganic salts were removed by filtration. The ethanol solution was concentrated by heating to induce crystallization. The solution was allowed to stand at 4°C for 12 h during which time yellow, plate-like crystals formed. The crystals were filtered and dried, giving 0.65g (13% yield from startiing material, 60.5% yield from recrystallization). The crystals were isolated as the mono-sodium, mono-hydrate salt.

Anal. Calcd. for $C_{11}H_7O_6S \cdot Na \cdot H_2O$: C, 42.86%; H, 2.95%; S, 10.40%. Found: C, 42.48%; H, 3.15%; S, 10.5%.

^1H NMR: δ 8.7 (1H,s), 8.3 (1H,s), 7.9 (1H,s), 7.4 (1H,s).

(2.) Sulfonation

3-Hydroxy-2-naphthoic (20.06g, 0.106 mole) was added slowly to conc. sulfuric acid (50 mL, 0.917 mole) at room temperature. When the addition was complete, the temperature had increased to 30°C. The mixture was stirred for 30 min at this temperature and at 65°C for 8 h. The reaction was determined to be complete when no precipitaton of starting material was evident following addition of a small aliquot of reaction mixture to water. The reaction mixture was poured into 100g ice and allowed to stand at 4°C for 12 h during which time only a small amount of unreacted starting material formed. This starting material was removed by filtration. The pH of the filtrate was increased from 0 to pH 2 by careful addition of solid sodium bicarbonate (140g) followed by heating to 70°C and addition of solid sodium chloride (40g) until the solution became slightly turbid. The mixture was allowed to stand at 4°C for 12 h during which time a light yellow precipitate formed which was filtered and dried as crude product, giving 32.2g (104% yield), a mixture or sulfonate and inorganic salts. The crude product (6.0g) was dissolved in hot ethanol and the sand-like insoluble material was removed by decantation followed by

filtration of the ethanol solution. Three crops of crystals were isolated from repeated concentration of the ethanol solution by heating and allowing the solution to stand at 4°C for 12 h. Each crop of light yellow crystals was removed by filtration prior to further concentration of the ethanol solution giving a total of 3.65g (60.8% yield of amount recrystallized). The fingerprint region of the infrared spectrum of each sample was identical to that of the analytical sample obtained by chlorosulfonation. Thin layer chromatography in the n-butanol:HOAc:H_2O (4:1:5) system showed two spots for each sample when detected by ultraviolet light. In the presence of iodine vapors only the less polar (higher R_f) spot took up iodine, whereas the spot near the origin remained white. There was no evidence of other impurities. The contaminant at the origin appeared to be inorganic salts. To purify further the sulfonic acid salt by removing the inorganic salts, the salt (1.0g) obtained from the ethanol recrystallization was dissolved in boiling water. The solution was allowed to cool slowly at room temperature and then stand at 4°C for 12 h. The yellow needles which formed were filtered, washed with acetone then with ether and dried, giving 0.64g (64% yield of amount recrystallized). The fingerprint region of the infrared spectrum was identical to that of the analytical sample obtained by chlorosulfonation. Thin layer chromatography showed only a small amount of inorganic salt remaining.

c. 7-Sulfo-3-hydroxy-2-naphthoic acid was prepared by dissolving the sodium salt (0.5g) in a minimal amount of water and adding conc. HCl (75-80 mL) until the solution became slightly turbid. The mixture was allowed to stand at 4°C for 12 h during which time white crystals formed. The crystals were filtered and dried, giving 9.432g (93.4% yield), mp 252-254° (dec).

d. 7-Sulfo-3-hydroxy-2-naphthoic acid, p-toluidine salt was prepared by dissolving the free acid (prepared as above) (0.4g) in a minimum amount of hot water. p-Toluidine (0.2g) was added to conc. HCl (0.6 mL), and sufficient hot water was added to dissolve the salt. The sulfonic acid solution was added to the p-toluidine solution, and a white material precipitated upon cooling. The white precipitate was filtered and dried, giving 0.413g (81.6% yield), mp 269-271° (dec).

e. 4-Bromo-3-hydroxy-2-naphthoic acid was prepared from a solution of 3-hydroxy-2-naphthoic acid (10.0g, 0.053 mole) in 100 ml glacial acetic acid to which bromine (3.19 mL, 0.0584 mole) was added dropwise while the mixture was kept at 5°C. The bright yellow reaction mixture was stirred for 15 min and then filtered. The precipitate was washed with cold 50% (v/v) acetic acid, and then recrystallized by dissolving it in hot glacial acetic acid. The long yellow needles which formed were filtered, washed with cold 50% acetic

acid and dried *in vacuo*, giving 9.68g (68.4% yield), mp 235-238°C (dec). ^{1}H NMR: δ8.7 (1H,s), 7.4-8.2 (4H,m).

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