Journal of Industrial Microbiology & Biotechnology (1997) 19, 312–323

On learning to drive and being taken for a ride

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Learning to drive

I. Undergraduate and graduate studies at Leeds University (1957–1964)

In 1955 I was a student at Sir William Turner's Grammar School in Redcar, Yorkshire, UK. During that year I read Ernest Baldwin's book, 'Dynamic Aspects of Biochemistry.' This was my first introduction to the fields of thermodynamics and kinetics as they relate to living cells and led to my decision to study biochemistry at the University. With much excitement and great enthusiasm I applied for undergraduate studies to the Department of Biochemistry at the University of Leeds. Two weeks later I found out that excitement and enthusiasm can be fleeting, when a letter informed me in two short lines that my application was denied. The following year I again applied for entrance to the Leeds Biochemistry Department and started a journey that led me to this symposium today.

After 2 years of courses in physical, inorganic and organic chemistry, we were allowed to cross the threshold of the Biochemistry Department. Two years later I began studies for the PhD degree in the laboratory of the late Stanley Dagley. There were three reasons for choosing this career path. First, Stanley Dagley was the kindest and most understanding faculty member I had met during my 4 years at the university. Second, he was a magnificent teacher who could take any subject, reduce it to its most simple components and show its relationship to the biological world. Third, Dagley's work on the bacterial degradation of aromatic compounds was attracting national and international attention and seemed to be an ideal area for a PhD project.

I began research by trying to determine the structure of the ring-fission compound formed from 2,3-dihydroxyphenylpropionic acid by an *Achromobacter* sp. Crystals of the ring-fission compound were isolated by Peter Chapman prior to his departure for postdoctoral studies with Dr Gunsalus at the University of Illinois. I was not having much success with this first research project when Dr Dagley suggested that the pathway for the degradation of 2-hydroxymuconic semialdehyde might be more amenable to solution. The situation at that time is shown in Figure 1 which is a compilation of the results obtained by Dagley and his associates at Leeds, and Evans and Ribbons at the University of North Wales [6]. Dagley and Stopher showed that catechol was oxidized to 2-hydroxymuconic semialdehyde which was yellow at alkaline pH and colorless in acid. Identification of the analogous ring fission product formed from protocatechuic acid was a little more difficult until it was realized that the ring-fission product reacted rapidly with ammonium ions to form 2,4-lutidinic acid. The analogous reaction with 2-hydroxymuconic semialdehyde yielded picolinic acid at a much slower rate. These reactions are also shown in Figure 1.

Soon after I started studies on the degradation of 2-hydroxymuconic semialdehyde by *Pseudomonas* sp strain L, where L stands for Leeds, Osamu Hayaishi and his colleagues in Kyoto published a complete pathway for the degradation of catechol to acetate and pyruvate by a *Pseudomonas* sp. To put it mildly, I was dejected. However, Dagley encouraged me to see if I could confirm Hayaishi's pathway in *Pseudomonas* L. I couldn't. In Hayaishi's pathway an NAD^+-dependent dehydrogenase oxidized the aldehyde group of 2-hydroxymuconic semialdehyde to 4-oxalocrotonate which was then decarboxylated and the resulting product hydrated to yield 2-oxo-4-hydroxyvalerate. *Pseudomonas* sp strain L contained an inducible 4-oxalocrotonate decarboxylase but all attempts to show the NAD^+-dependent oxidation of 2-hydroxymuconic semialdehyde to 4-oxalocrotonate failed. Instead we were able to show that the C-1 fragment was liberated as formate. The further metabolism of 2-oxo-4-hydroxyvalerate by Hayaishi's organism involved oxidation to acetopyruvate followed by hydrolysis to give acetate and pyruvate. We were unable to show these reactions in *Pseudomonas* sp strain L. However, we did find that cell extracts, prepared from *Pseudomonas* sp strain U (see below) contained an inducible enantiospecific aldolase which cleaves 2-oxo-4-hydroxyvalerate to pyruvate and acetaldehyde. These products are converted to acetyl CoA and channeled into the TCA cycle [7].

Space does not permit me to describe the highs and lows of this period. Perhaps, however, one story will suffice. This, and other anecdotal references to the period covered by my graduate and postdoctoral studies were taken from Reference [12]. In 1962 Professor Hayaishi was in London and he called the Biochemistry Department at Leeds to see

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Received 25 July 1997; accepted 28 July 1997

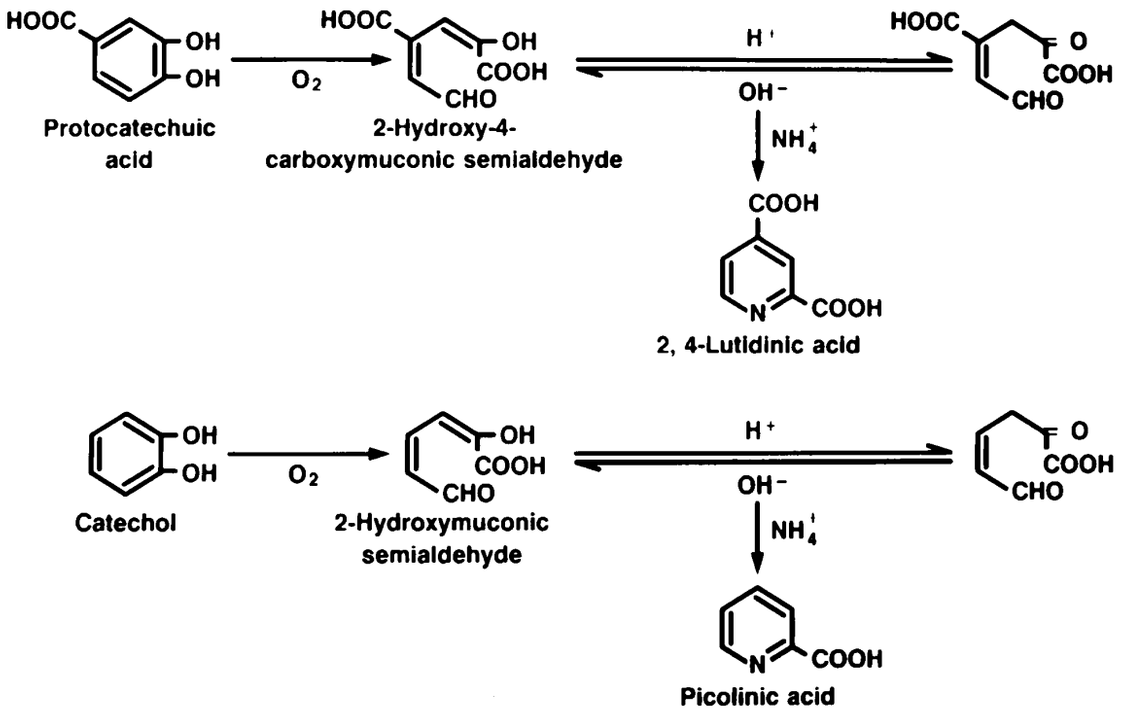

Figure 1 Ring fission reactions catalyzed by catechol 2,3-dioxygenase and protocatechuate 4,5-dioxygenase. The pyridine carboxylic acids, 2,4-lutidinic acid and picolinic acid are formed nonenzymatically in the presence of ammonium ions.

if Professor Dagley could come down for discussions on the *meta* pathway. Dagley was not feeling well at the time and he put a five pound note in my hand and sent me off to the big city. This was my first visit to London and the first time I was in such an ostentatious place as the Marble Arch Hotel. Professor Hayaishi was charming and we spent the whole afternoon discussing science in the hotel lounge where he graciously paid for the drinks. At least I thought we were discussing science. When I returned to Leeds, Dagley asked me what I had learned. It was only then that I realized that I had done most of the talking and Professor Hayaishi had been doing a lot of listening and nodding his head in encouragement. I spent the next 12 months in anguish fully expecting to see my dissertation published by another laboratory. Hayaishi was kind, it never happened, and I was able to thank him personally at the time of his retirement in 1981 [18].

In 1963 Dagley spent a sabbatical leave in Dr Gunsalus' laboratory at the University of Illinois. A polluted stream, aptly named Boneyard Creek, flowed beneath his rented house and from it he was able to isolate an organism which was given the designation *Pseudomonas* strain U (U for Urbana). Dagley showed that cell extracts prepared from *meta* cresol-grown cells of *Pseudomonas* strain U rapidly oxidized 4-methylcatechol to propionaldehyde and pyruvate. I was in the last year of my PhD studies at the time when I received Dr Dagley's letter from America describing these observations. I well remember the last part of that letter which contained a general metabolic scheme that accounted for my results with catechol, John Wood's demonstration of the formation of pyruvate from protocatechuate and Peter Chapman's earlier studies on the formation of succinate from 2,3-dihydroxyphenylpropionate. This general pathway is shown in modified form in Figure 2 and has been used to predict the pathways used by bacteria for the degradation of many substituted catechols [5]. However, I should also point out that Sala-Trepat and Evans showed that 2-hydroxymuconic semialdehyde is oxidized to oxalocrotonate by an *Azotobacter* species and this has been observed with several other bacterial strains. Thus the *meta* ring fission pathway has a split personality. The hydrolytic pathway is generally used by bacteria when ring fission leads to the formation of a ketone and the oxalocrotonate pathway is used when ring fission yields an aldehyde. Both pathways are shown in Figure 2.

I left the Department of Biochemistry in 1964 with mixed emotions. It had been my home for 5 wonderful years and there was a strong sense of family. A photograph taken in 1963 (Figure 3) shows the whole department, and includes faculty, undergraduate and graduate students, technicians and secretaries. There are some distinguished individuals on the front row. The fourth gentleman from the right is Bernard Kilby, who in 1947 emerged triumphantly from a cold room where he had spent several hours extacting and isolating β-ketoadipate from culture filtrates of *Vibrio* 01 that had been grown with phenol. The cold room was necessary due to the instability of β-ketoadipate which, like all β-ketoacids, decarboxylates very easily. *Vibrio* 01 was identified 36 years later by Paul Bauman in Stanier's laboratory as *Acinetobacter calcoaceticus*. This may be one of the first times, but by no means the last, that biochemists in the field of aromatic metabolism have shown a *laissé faire* attitude to bacterial taxonomy. The conventional wisdom throughout the years has always been, if it is Gram-negative and swims, it must be a *Pseudomonas* species. Next to Kilby is a young Stanley Dagley and the man in

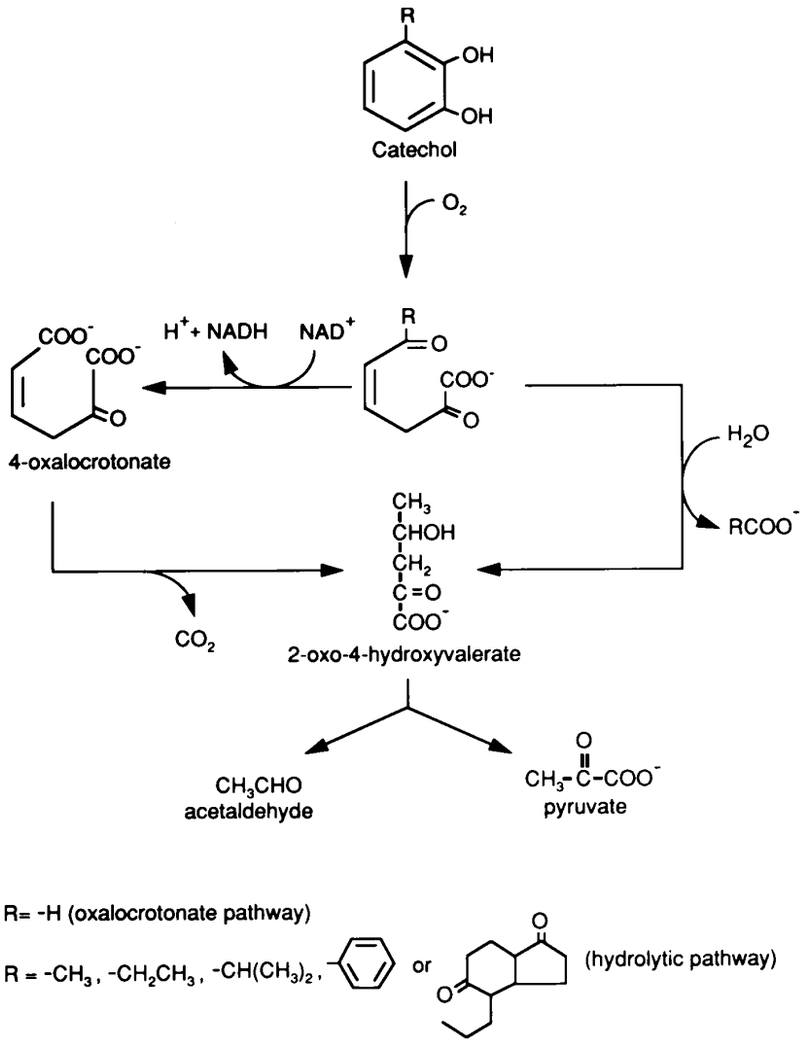

Figure 2 *meta*-Ring cleavage pathways for the degradation of catechol and its substituted derivatives.

the white suit is Professor Frank C Happold, the first Head of Biochemistry at the University of Leeds.

II. Postdoctoral studies at the Universities of Wisconsin and Illinois (1964–1967)

I joined Dr Charles Sih's laboratory in the College of Pharmacy at the University of Wisconsin at Madison in 1964, where I was assigned the task of elucidating the reactions used by *Nocardia restrictus* for the degradation of the steroid A ring. This fell into line rather nicely since the A ring was converted to a catechol and the experimental results confirmed the predicted pathway. The R substituent is shown at the bottom of Figure 2.

It was in the Dagley and Sih laboratories that I learned to drive in a metaphorical sense. Dagley's philosophy was to let his students design and carry out their own experiments. I cannot remember him ever directly suggesting an experiment or a technique. However, he always had time to discuss the results of any aspect of my research project. Dr Sih's approach was much more direct. He wanted to be involved in the details of each experiment and it was in his laboratory that I learned to appreciate the latest techniques of analytical chemistry and how to use them to solve problems in biodegradation and biotransformation. I also learned to drive in a more literal sense at Madison. Dr Sih loaned me his Rambler station wagon and instructed his new graduate student, George Peruzotti, to teach me how to drive. George was a man of great courage, and also a good teacher. One week later I had a license, we borrowed $500 from the bank and became the proud owners of a 1958 Chevrolet Biscayne.

In retrospect, I now realize that the education I received at Leeds and Madison was unique and prepared me for my next position as a postdoctoral student with Dr Reino Kallio in the Department of Microbiology at the University of Illinois at Urbana. Dr Kallio had just joined the University as Director of Life Sciences. He gave me two grant numbers, told me to equip his laboratory and do research on the bacterial oxidation of hydrocarbons. He did suggest that before he retired he would like to prove that the initial reaction in the bacterial oxidation of hydrocarbons is catalyzed by an oxygenase. It was a wonderful opportunity, and exciting things were happening in the Microbiology Department at Urbana. Leon Campbell was the new editor of the *Journal*

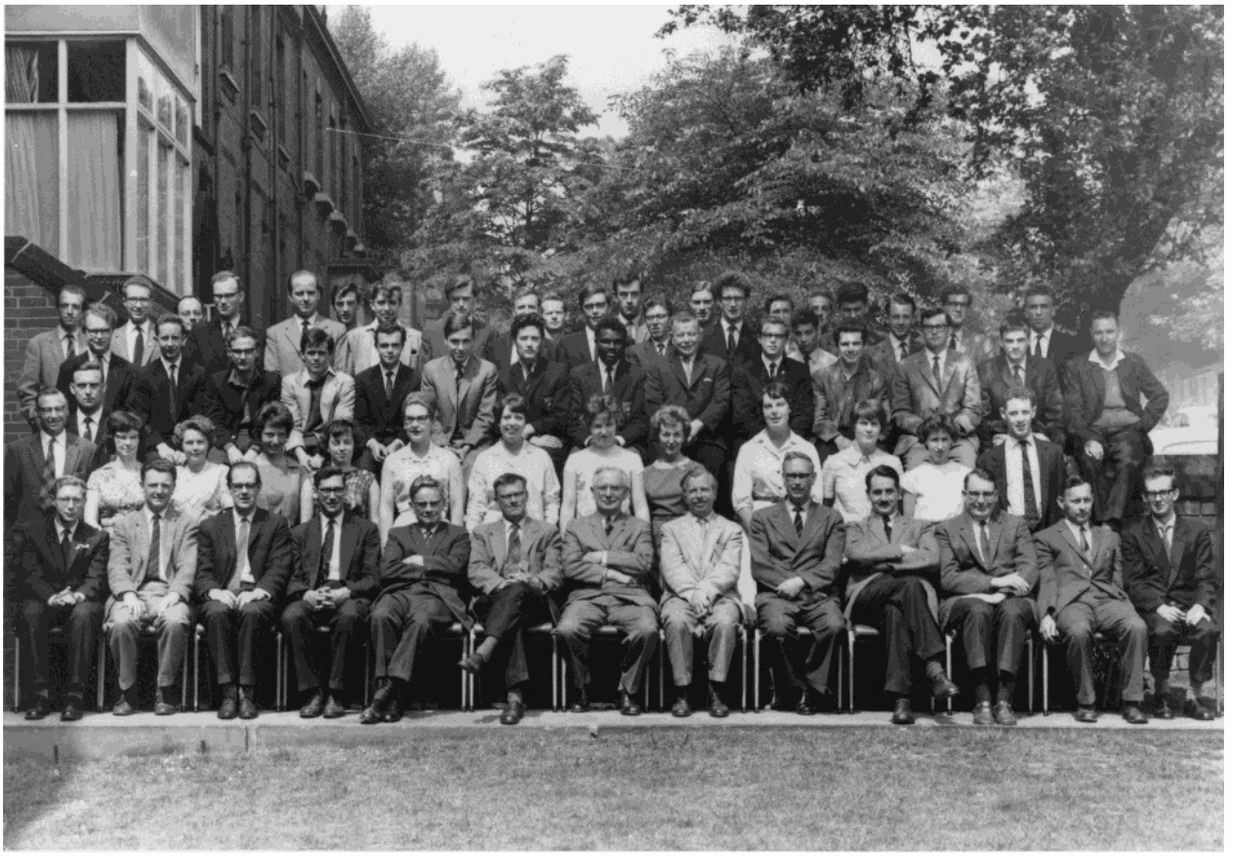

Figure 3 Biochemistry Department, University of Leeds, 1963. Front row, right to left: PW Trudgill, RP Hullin, DG Wild, B Kilby, S Dagley, FC Happold.

of Bacteriology, Sol Speigelman had produced conditions for the replication of a fragment of DNA, Ralph Wolfe was making great progress on the enzymology of methanogenesis, and Carol Woese, was thinking about the third kingdom.

Exciting things were also happening outside of the Microbiology Department. Two blocks up the road Dr Gunsalus and his students were dissecting the camphor methylene hydroxylase system. Omura and Sato in Japan demonstrated the presence of a unique cytochrome in liver microsomes that gave a characteristic absorption maximum at 450 nanometers when reduced in the presence of carbon monoxide. And at the National Institutes of Health, studies on the mechanism of action of phenylalanine hydroxylase and the NIH shift were in full swing. Little did we know that in these three laboratories we were witnessing the birth of the cytochrome P450 field and its relationship to drug metabolism, chemical carcinogenesis, and oxygen fixation. At that time I had an empty laboratory, grant money to spend, and a mandate to demonstrate the involvement of oxygen in hydrocarbon degradation. The choice of a project was not difficult. In 1965 all available evidence indicated that bacteria and mammals oxidize aromatic hydrocarbons to dihydrodiols in which the hydroxyl groups have a *trans* relative stereochemistry. Jerina and his associates showed that in the case of benzene and naphthalene, *trans*-dihydrodiols are formed by enzymatic hydrolysis of arene oxides. The latter are the initial products formed from aromatic hydrocarbons by mammalian microsomes (cytochrome P450). We isolated a strain of *Pseudomonas putida* from Boneyard Creek (the same source that produced *Pseudomonas* U) that would grow with ethylbenzene, toluene and benzene. Toluene was the best growth substrate and toluene-grown cells rapidly oxidized benzene, and *cis*-benzene dihydrodiol, *trans*-Benzene dihydrodiol, the expected metabolic intermediate, was not oxidized. Subsequent radioisotope trapping experiments confirmed that the *cis*-dihydrodiol was an intermediate in benzene oxidation [15]. The *P. putida* strain would not grow with *p*-chlorotoluene. Toluene-grown cells, however, oxidized *p*-chlorotoluene, and after extracting 25 liters of culture filtrate with ethyl acetate, silica gel column chromatography of the concentrated extract showed that the major product formed was the catechol derivative of *p*-chlorotoluene. The chromatographic procedure also yielded 38 mg of a compound that was identified as *cis*-2,3-dihydroxy-2,3-dihydro-4-chlorotoluene [16]. This was an exciting result since it represented the first report of the isolation of a *cis*-dihydrodiol and the first indication that *cis*-dihydrodiols may play an important role in the bacterial oxidation of aromatic hydrocarbons other than benzene. I also remember my aching arms after extracting 25 liters of culture filtrate and making a mental note to investigate the possibility of isolating mutants blocked in the enzyme following the dihydrodiol.

In 1996 I set out to try and obtain cell extracts that would oxidize benzene. The only enzyme assays available were

the stimulation of NADH oxidation or oxygen consumption in the presence of benzene. Cell extracts prepared by all known conventional methods were inactive and I began to realize why all of the previous work on aromatic metabolism had focused at the level of ring-fission enzymes which were always very active in cell extracts. I observed that crude cell extracts prepared from toluene-grown cells of *P. putida* contained an active ethanol dehydrogenase and I tried adding benzene dissolved in ethanol to cell extracts containing NAD^+. However, this NADH-generating system, first reported for benzoate and anthranilate hydroxylases [25], did not lead to benzene oxidation. In one last attempt to prepare active cell extracts, I lyophilized toluene-grown cells and ground the resulting dry powder with buffer in a mortar cooled by dry ice. The buffer froze during grinding and the mortar was allowed to warm to yield a viscous suspension which was centrifuged at 4°C. The straw-colored supernatant solution was tested in a Warburg respirometer for its ability to oxidize benzene. I remember preparing to add the benzene in ethanol solution from the side arm of the Warburg flask and noticing that the cell extract solution in the main compartment was a familiar yellow color. Some of the benzene had volatilized during the equilibration period and was converted to α-hydroxymuconic semialdehyde by the cell extract. I was ecstatic; after almost 10 months of negative results the road was open. By the time we were ready to leave Illinois we knew that benzene (toluene) hydroxylase consisted of at least two proteins, required NADH, ferrous iron and a sulfhydryl reagent for optimal activity, and oxidized benzene through *cis*-benzene dihydrodiol to catechol [30].

III. Faculty studies at the University of Texas at Austin (1967–1988)

(a) cis-Dihydrodiols: In 1967 I began my academic career as an assistant professor in the Department of Microbiology. The beautiful laboratory that I had been shown during my interview had disappeared and I and another beginning assistant professor were assigned temporary bench space in a teaching laboratory. Although this situation left much to be desired, it turned out to be one of those serendipitous situations that permeate the field of science. I had a limited amount of glassware, a box of petri plates and a paper by Nick Ornston on how to isolate mutants of *P. putida*. Within 2 weeks I had a collection of plates with brown colonies (catechol mutants), yellow colonies (ring-fission mutants) and more than 50 small colorless colonies that could no longer grow with benzene, toluene or ethylbenzene. Each of these colorless mutants was analyzed by thin-layer chromatography for the ability to accumulate neutral products from benzene. The 39th colony tested accumulated *cis*-benzene dihydrodiol when grown with glucose in the presence of benzene. The strain was designated 39/D and it is still the source of intensive investigation today. However, in 1967 the first direct demonstration of the enzymatic oxygen incorporation into a hydrocarbon occupied our attention. Toluene-induced cells were incubated with benzene in the presence of $^{18}O_2$ and the isolated *cis*-benzene dihydrodiol was sent to the mass spectrometry facility for analysis. Three days later we received a classical mass spectrum of [^{18}O]-phenol. A heated discussion with the head of the mass spectrometry laboratory centered on his conviction that the dihydrodiol was so labile it would never give the required parent ion. After much pleading and a little bribery, we persuaded him to lower the inlet temperature of the mass spectrometer. Ten minutes later he emerged from the dark room with a dripping freshly developed chart of the mass spectrum and the parent ion was seen as m/z 116, indicating the addition of two atoms of oxygen to the benzene nucleus [13]. Our exhilaration was somewhat muted by the realization that we should have conducted the experiment in a 50 : 50 atmosphere of $^{16}O_2$:$^{18}O_2$ and shown that both atoms of oxygen in *cis*-benzene dihydrodiol were derived from a single oxygen molecule.

Strain 39/D also oxidized toluene to a neutral compound which was isolated and crystallized.* The crystals were carefully placed in a desiccator and we left to celebrate at a local hostelry. When we returned the crystals had disappeared and were replaced with a pale yellow oil. Removal of the lid of the desiccator released the powerful fumes of a phenol which was quickly identified as *ortho*-cresol by infrared spectrophotometry. We crystallized more of the toluene metabolite, made a Diels–Alder derivative to stabilize the putative cyclohexadiene ring and obtained an NMR spectrum. Application of the Karplus equation led us to believe that the product was *cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) [14]. However, we were unable to convince our chemistry colleagues that the Karplus equation could be used to predict the stereochemistry of the hydroxyl groups in substituted cyclohexadiene diols. This led us to the laboratory of RE Davis in the chemistry department at Austin who determined the structure of a *p*-bromo triazoline dione derivative of the metabolite by X-ray crystallography [30]. This experiment confirmed our earlier structural identification and also showed that the dihydrodiol was a single enantiomer in which the hydroxyl groups had a 1*S*,2*R* configuration. The significance of this result in terms of asymmetric synthesis remained dormant for almost 20 years (see below). At the time we were sufficiently excited to know that we had discovered a new reaction in the microbial degradation of aromatic hydrocarbons. John Rogers purified *cis*-toluene dihydrodiol dehydrogenase and thus firmly established the initial reactions in the bacterial oxidation of toluene by our strain of *P. putida* as those shown in Figure 4.

It seemed logical at the time to reinvestigate the initial reactions involved in the bacterial oxidation of naphthalene. Several papers in the literature suggested that this compound was oxidized by bacteria to *trans*-1,2-dihydroxy-1,2-dihydronaphthalene which is the same metabolite formed by mammals. I was teaching a course in microbiology for engineering students that semester and fortuitously one of the laboratory experiments was the use of the enrichment culture technique to isolate bacteria that could grow with naphthalene as the sole source of carbon and energy. Luxuriant growth was obtained in liquid cultures. However, the

* No one ever left Dr Sih's laboratory without expertise in the crystallization of organic compounds.

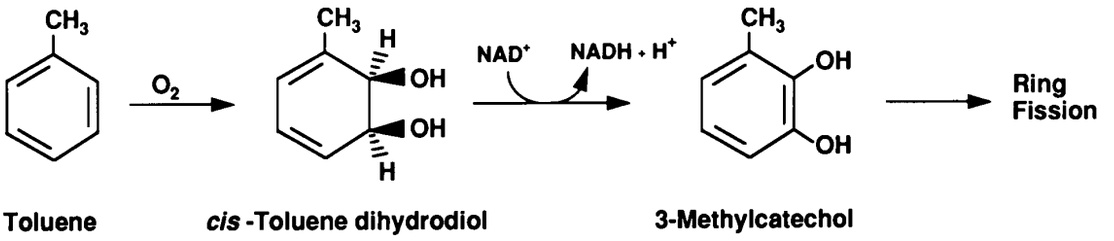

Figure 4 Initial reactions in the oxidation of toluene by *Pseudomonas putida*.

plates left much to be desired in terms of isolated colonies. Nevertheless, one pure culture was obtained and given the designation *Pseudomonas* sp strain NP. The Ornston mutagenesis procedure yielded many small colonies that could grow well with glucose but not naphthalene. The 119th colony tested oxidized naphthalene to a dihydrodiol which readily dehydrated to yield α-naphthol when treated with acid. The metabolite was subsequently identified as *cis*-(1*S*,2*R*)-dihydroxy-1,2-dihydro-naphthalene (*cis*-naphthalene dihydrodiol) [27]. The latter studies were conducted in collaboration with Donald Jerina and his colleagues at the National Institutes of Health. Thakor Patel purified a dehydrogenase from strain NP which oxidized *cis*-naphthalene dihydrodiol to 1,2-dihydroxynaphthalene [39] and the initial reactions in the bacterial oxidation of naphthalene were thus shown to be analogous to those observed for toluene (as shown in Figure 4).

We were unable to isolate bacteria that could grow with polycyclic hydrocarbons larger than anthracene. This led us to isolate a strain of *Beijerinckia* which could grow with biphenyl. The rationale for this approach was based on the fact that biphenyl has a 'bay region' similar to that found in polycyclic aromatic hydrocarbons. We thought that enzymes induced to oxidize biphenyl might also oxidize polycyclic hydrocarbons at the 'bay-region.' Martha Cone-Wells and Rowena Roberts isolated a mutant (strain B8/36) of the *Beijerinckia* sp (now known as *Sphingomonas yanoikuyae*) that oxidized biphenyl to *cis*-2,3-dihydroxy-2,3-dihydrobiphenyl (*cis*-biphenyl dihydrodiol) and showed that a dehydrogenase present in the parent organism would oxidize the dihydrodiol to 2,3-dihydroxybiphenyl [17]. The absolute stereochemistry of *cis*-biphenyl dihydrodiol and several other diols produced by *P. putida* 39/D was determined in collaboration with Herman Ziffer and Donald Jerina at NIH. Our subsequent studies, several of them in collaboration with Donald Jerina and his associates, showed that *cis*-dihydrodiols were the initial oxidation products formed from substrates that ranged in size from benzene to benzo[*a*]pyrene. These results clearly showed that *cis*-dihydroxylation is an important reaction in the bacterial oxidation of aromatic hydrocarbons.

(b) Fungal and algal oxidation of aromatic hydrocarbons: Carl Cerniglia joined our laboratory after completing his PhD degree with JJ Perry at North Carolina State University. Carl brought with him the filamentous fungus *Cunninghamella elegans* and a basic knowledge of aromatic metabolism. I knew much less about fungi. However, we were soon able to show that *C. elegans* oxidizes naphthalene to *trans*-naphthalene dihydrodiol, the same metabolite formed from this substrate by mammals. We then set our eyes on benzo[*a*]pyrene. At that time in the 1970s, studies on the mammalian oxidation of carcinogenic hydrocarbons were at their peak. Much of the activity focused on benzo[*a*]pyrene, the flagship molecule for studies on chemical carcinogenesis. It was known that the carcinogenic activity of this polycyclic aromatic hydrocarbon was due to the formation of an electrophilic metabolite. Subsequent studies showed that cytochrome P450 and epoxide hydrolase convert benzo[*a*]pyrene to the (–) enantiomer of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and that further oxidation yields (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-diol epoxide-2]. *C. elegans* oxidized benzo[*a*]pyrene to almost the same spectrum of metabolites formed by mammals and in an elegant series of experiments Carl was able to provide firm evidence for the formation of (+)-diol epoxide-2, the ultimate carcinogenic metabolite of benzo[*a*]pyrene [3]. In collaborative studies with Paul Szaniszlo, Carl showed that *C. elegans* and other members of the *Mucorales* family oxidize polycylic aromatic hydrocarbons by similar reactions to those used by mammals [4]. These studies and those on the bacterial oxidation of aromatic hydrocarbons suggest that major differences exist between eukaryotic and prokaryotic organisms in terms of the initial reactions used to oxidize these substrates (Figure 5). Eukaryotic organisms utilize a cytochrome P450 enzyme system to catalyze the addition of one atom of molecular oxygen to the aromatic nucleus to form arene oxides as the first detectable products. The oxides are reactive electrophiles which can undergo several different reactions, one of which is the enzymatic addition of water to form *trans*-dihydrodiols. In contrast, bacteria usually add both atoms of oxygen to the aromatic ring and *cis*-dihydrodiols are the first detectable products. The differences in oxygen activation and incorporation probably reflect the functions of the pathways observed. Eukaryotic organisms need to convert lipophilic xenobiotic compounds and many natural products to water-soluble derivatives for excretion to the external environment, while bacteria need to utilize these substrates as sources of carbon and energy for growth.

The above studies led to questions about the ability of cyanobacteria and eukaryotic algae to oxidize aromatic hydrocarbons. Many delightful days were spent at the University of Texas Marine Science Institute at Port Aransas where we collaborated with the late Chase Van Baalen. Carl, Chase and Martha Narro were able to show that marine cyanobacteria have the ability to form hydroxylated metabolites similar to those formed by mammals and bacteria. Chase was a unique individual with a genuine love

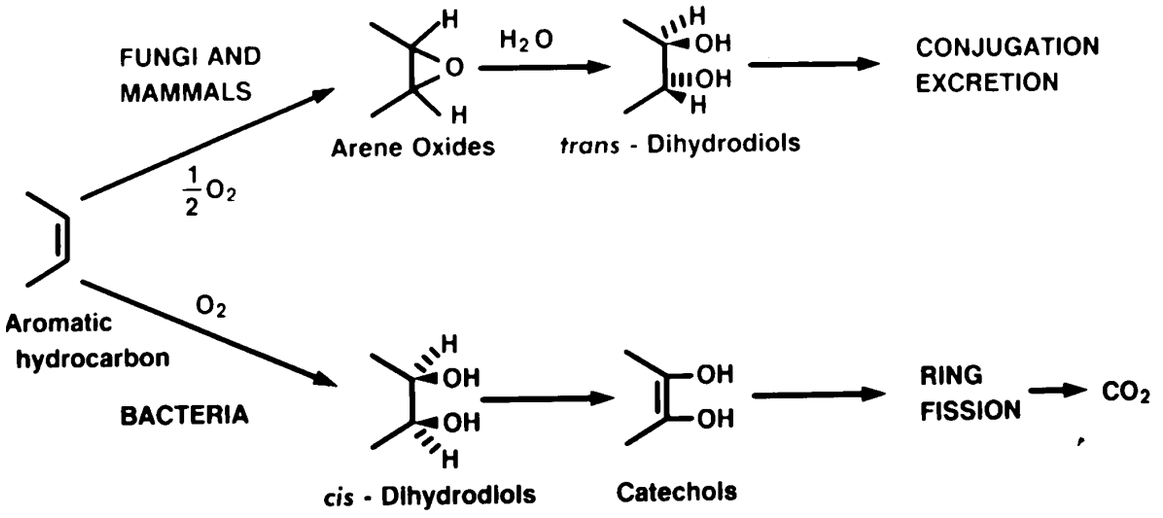

Figure 5 Initial reactions in the oxidation of aromatic hydrocarbons by bacteria, fungi and mammals.

for research, fishing and poker. He excelled at the first two activities and never once admitted that he lost at the third. He passed away too soon and we miss him.

(c) Toluene and naphthalene dioxygenases: Soon after our arrival at Texas we recommenced studies on the enzyme we eventually called toluene dioxygenase. Wu-Kuang Yeh made a major breakthrough when he showed that three, not two, proteins were required for enzyme activity [53]. Subsequent studies were facilitated by the development of a radioactive assay by John Rogers and purification of the oxygenase component by an affinity chromatography procedure developed by Mani Subramanian [45]. Mani joined our laboratory in 1978 after obtaining his PhD with Professor CS Vaidyanathan at the Indian Institute of Technology at Bangalore. Mani's knowledge and expertise in protein purification and microbial chemistry, coupled with his willingness to help others with problems, large and small, endeared him to everyone in the laboratory. The friendships he developed at that time are still ongoing today. During this period Alice Laborde began purifying naphthalene dioxygenase from *Pseudomonas* sp 9816-4 and the oxygenase component was purified by Burt Ensley. This was a period of exciting research activity. The purification and characterization of the components of toluene (Mani Subramanian, Martha Narro, Larry Wackett, Ernie Liu and Cuneyt Serdar) and naphthalene (Alice Laborde, Burt Ensley and Billy Haigler) dioxygenases showed that they are organized as shown in Figures 6 and 7. Electrons are passed from NADH to a flavoprotein (reductase) which reduces the Rieske [2Fe-2S] center in a ferredoxin. Electrons from the latter are passed to a Rieske [2Fe-2S] center in the terminal oxygenase component (ISP) and then to mononuclear iron at the active site of the enzyme. Both oxygenase components were shown to have an $\alpha_2\beta_2$ subunit composition and it was believed but not proven at the time that the Rieske [2Fe-2S] center and mononuclear iron were located on the α subunit in each enzyme.

(d) Initial studies on the regulation of toluene dioxygenase: Barry Finette chose to study the genetic aspects of toluene dioxygenase in *P. putida*, which was designated as the parent F1 strain as the number of its mutant derivatives began to grow. Barry developed a novel procedure, based on the use of redox dyes, for the identification and isolation of mutants defective in each structural gene in the toluene dixoygenase complex [11]. He then went on to utilize transposon mutagenesis to determine the gene order in the toluene dioxygenase (*tod*) operon. Dick McCombie came from Ron Olsen's laboratory and made a major contribution by cloning the genes for the first three enzymes in the toluene catabolic pathway.

(e) Biodegradation of aromatic compounds: A diverse range of substrates were shown to be oxidized to *cis*-dihydrodiols by bacteria. Some of the more interesting products were those formed from dibenzo[1,4]dioxan (Gary Klecka) [29], dibenzothiophene (Alice Laborde) [31], acenaphthene and acenaphthylene (Mark Schocken) [42], and benz[*a*]anthracene (Bill Mahaffey and Carl Cerniglia) [35]. Other products included the identification of acid diols formed by enzymes encoded by a TOL plasmid (Gregg Whited, Larry Kwart and Dick McCombie) [52] and Jim Spain's identification of hydroquinone as an intermediate in the enzymatic oxidation of *p*-nitrophenol by a *Moraxella* sp [44]. In fact Jim's work was sufficiently exciting to warrant publication word for word under the title, 'The metabolism of *p*-nitrophenol by a *Penicillium chrysogenum*' [26].

It has always been my belief that students trained in analytical chemistry and reaction mechanisms have much to offer students in microbial physiology and metabolism. The reverse is also true. In 1978, Larry Kwart joined our group after obtaining his PhD in organic chemistry. His gentle personality enabled him to work with all members of our laboratory. He was constantly sought out as a source of organic syntheses and reaction mechanisms. At the time, Larry Wackett was probing the substrate specificity of toluene dioxygenase. He noted that styrene was hydroxylated

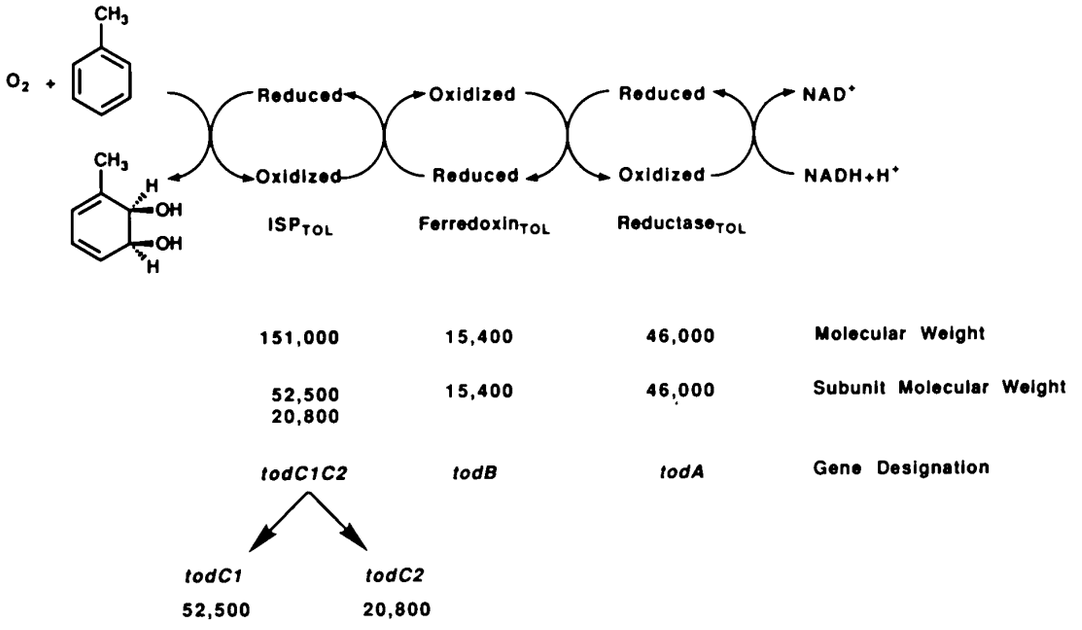

Figure 6 Organization of the toluene dioxygenase complex from *Pseudomonas putida* F1 [45–47,53].

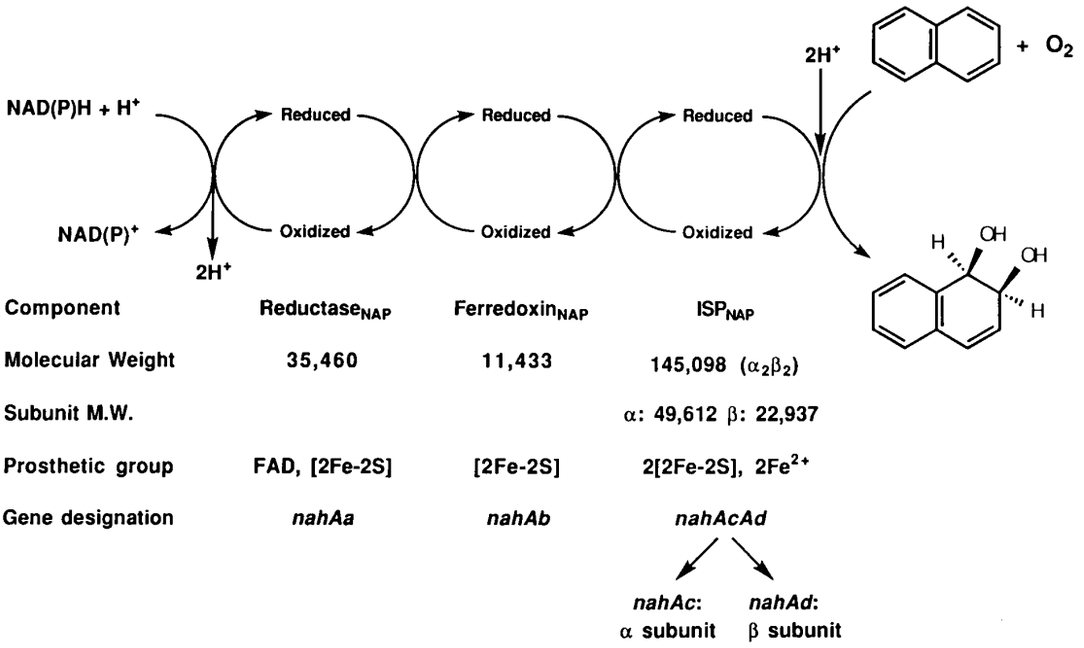

Figure 7 Organization of the naphthalene dioxygenase complex from *Pseudomonas* sp NCIB 9816 [8,9,22,23].

predominantly on the aromatic ring whereas indene and indan were oxidized to 1-indenol and 1-indanol as the major products. He and Larry Kwart determined that the benzylic hydroxylation products were formed in high enantiomeric excess and that toluene dioxygenase oxidized indan to (–)-(1*R*)-indanol whereas the naphthalene dioxygenase formed the (+)-(1*S*)-enantiomer [49]. These initial studies exposed the tip of the iceberg with respect to the substrate specificity of the aromatic hydrocarbon dioxygenases and several tables in Larry Wackett's dissertation continue to provide a fertile ground for future studies.

In 1981 I was a member of the Scientific Advisory Board of AMGEN, a fledgling biotechnology company in Thousand Oaks, California. One of the products AMGEN wanted to make was 1-naphthol which is the only product formed when *cis*-naphthalene dihydrodiol is treated with acid. The following year Burt Ensley accepted a position at AMGEN to lead a specialty chemicals group. He and his associates cloned the genes for naphthalene degradation from *P. putida* G7. Colonies of the recombinant strains when grown in rich media turned blue. Sidney Brenner suggested that the blue color reminded him of indigo. The fol-

lowing week we received a sample of the blue compound from Burt and Larry Wackett quickly identified it as indigo. Larry had also observed indigo formation from indole during his studies on the substrate specificity of toluene dioxygenase. This provided an explanation for indigo formation by naphthalene dioxygenase in the recombinant strain. *Escherichia coli* contains the enzyme tryptophanase which cleaves tryptophan to indole and serine. Indole is then oxidized to indigo by naphthalene dioxygenase [10]. Subsequent studies have shown that several, but not all aromatic hydroxylases, oxidize indole to indigo. AMGEN subsequently divested itself from the specialty chemicals field and the development of a 'green chemistry' procedure for the commercial production of indigo dye is currently being developed by Genencor International Company, South San Francisco, CA, USA.

In 1983, at the height of the diverse and exciting activities in the laboratory, I took my family to the Texas Gulf Coast to see the few remaining whooping cranes in their winter refuge. During that time I developed an unidentified illness which kept me out of the laboratory for almost 9 months. I returned to work expecting to find our research program in disarray. I was wrong.

Being taken for a ride (1983–1988)

During my absence from the laboratory, research progress had continued unabated as evidenced by the drafts of manuscripts on my desk and red ink in our research financial statements. The following 5 years were a special time when my students, a few faculty colleagues, and my own family helped me adjust to a new lifestyle.

I did note some diversions from the pre-1983 objectives. For example, Cuneyt Serdar cloned and expressed the parathion hydrolase gene from *Pseudomonas diminuta* and was in the process of cloning the genes for naphthalene dioxygenase from *Pseudomonas* sp NCIB 9816-4. Gregg Whited had commenced studies on toluene metabolism by *Pseudomonas mendocina* KR1 (KR for Ken Richardson, the student who isolated the organism). Strain KR1 initiated the degradation of toluene by adding one atom of oxygen to the aromatic nucleus to form *p*-cresol. Gregg's subsequent studies showed that *p*-cresol was oxidized to *p*-hydroxybenzoic acid and then to protocatechuic acid [51]. The latter compound was further metabolized by the *ortho* ring fission pathway. In this sequence of reactions *p*-cresol is first oxidized to *p*-hydroxybenzyl alcohol by a multicomponent enzyme system. Water is the source of the hydroxyl group in *p*-hydroxybenzyl alcohol since the reaction proceeds through a quinone methide intermediate. This reaction mechanism was first reported by Hopper in his studies on the bacterial oxidation of *p*-cresol. Gregg's work led to a subsequent collaboration with Lily Young and Inger Bossert on the anaerobic degradation of *p*-cresol [2].

In 1986 I received a phone call from Michael Nelson at the EPA research laboratories in Gulf Breeze, Florida. Mike had isolated an organism from a polluted pond that would degrade trichloroethylene (TCE). The organism would not grow with TCE, and only degraded TCE when filter-sterilized water from the pond was added to defined growth media. Samples of the defined growth medium, including the pond supplement before and after growth of the organism, were collected and sent to our laboratory for analysis. Bill Mahaffey, who was our GC/MS expert at the time, took on the project and within 2 days appeared in my office with the statement, 'My God, Gibson, it's phenol.' Bill's chromatograms were exceedingly complex but the major peak in the control sample was absent from the medium after growth of the organism. Subsequent studies at the EPA laboratories showed that phenol- and toluene-induced cells of their isolate would also degrade TCE [37]. This work spawned numerous papers on TCE degradation, most notably Larry Wackett's more recent imaginative construction of a recombinant strain that utilizes cytochrome $P450_{CAM}$ and toluene dioxygenase to degrade pentachloroethane through TCE to glyoxylate and formate [50].

In 1984 Donna Bedard invited me to give a seminar at General Electric Company's Research Laboratories in Schenectady, New York. It was a memorable visit which led to a productive collaboration with Herman Finkbeiner's research group, a distinct, but not impossible chance of breeding a winner of the Kentucky Derby, and a position as the Principal Investigator on a cooperative agreement between the University of Texas and GE on the degradation of polychlorinated biphenyls. This work resulted in Mark Schocken's demonstration that *Alcaligenes eutrophus* H850 catalyzed *cis*-hydroxylation reactions at both open 3,4-positions in 2,5,2′,5′-tetrachloro-biphenyl [36].

Being taken for a ride (1988–present)

In 1988 I was offered a position in the Department of Microbiology at the University of Iowa. The opportunity to interact with Jack Rosazza, Irving Crawford, Alan Markovetz and an interdisciplinary group known as the 'Biocats' was more than welcome. The transition was made much easier by Gerben Zylstra, Sadhana Chauhan, Shir-Ly Huang, Wen-Chen Suen and Fu-Min Menn, all of whom left Texas for the frigid winters of Iowa and helped to get our new laboratory designed and operational. Gerben joined our laboratory in 1986 after obtaining his PhD with Ron Olsen. In a relatively short period of time he sequenced all of the structural genes (*todABC1C2DE*) for the conversion of toluene to 2-hydroxy-6-oxo-hepta-2,4-dienoate (Figure 8) [54]. Subsequent studies by Fu-Min Menn and Gerben led to the sequence of *todF*, the gene encoding 2-hydroxy-6-oxo-hepta-2,4-dienoate hydrolase which was located as shown in Figure 8, the position predicted by Barry Finette's previous transposon studies. Gerben also constructed expression clones (*E. coli* JM109[pDTG601]) for toluene dioxygenase (*todC1C2BA*), toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase (*todC1C2BAD*) and toluene dioxygenase, *cis*-toluene dihydrodiol dehydrogenase and catechol 2,3-dioxygenase (*todC1C2BADE*) [54,55]. In addition we were able to develop excellent expression clones for $ferredoxin_{TOL}$ (Shir-Ly Huang) [24] and all of the naphthalene dioxygenase components (*nahAaAbAcAd*, Wen-Chen Suen and Diana Cruden) [43,48]. Wen also cloned the α and β subunits of the terminal oxygenase, ISP_{NAP} (*NahAc* and *NahAd*) and was able to show for the first time that extracts prepared from

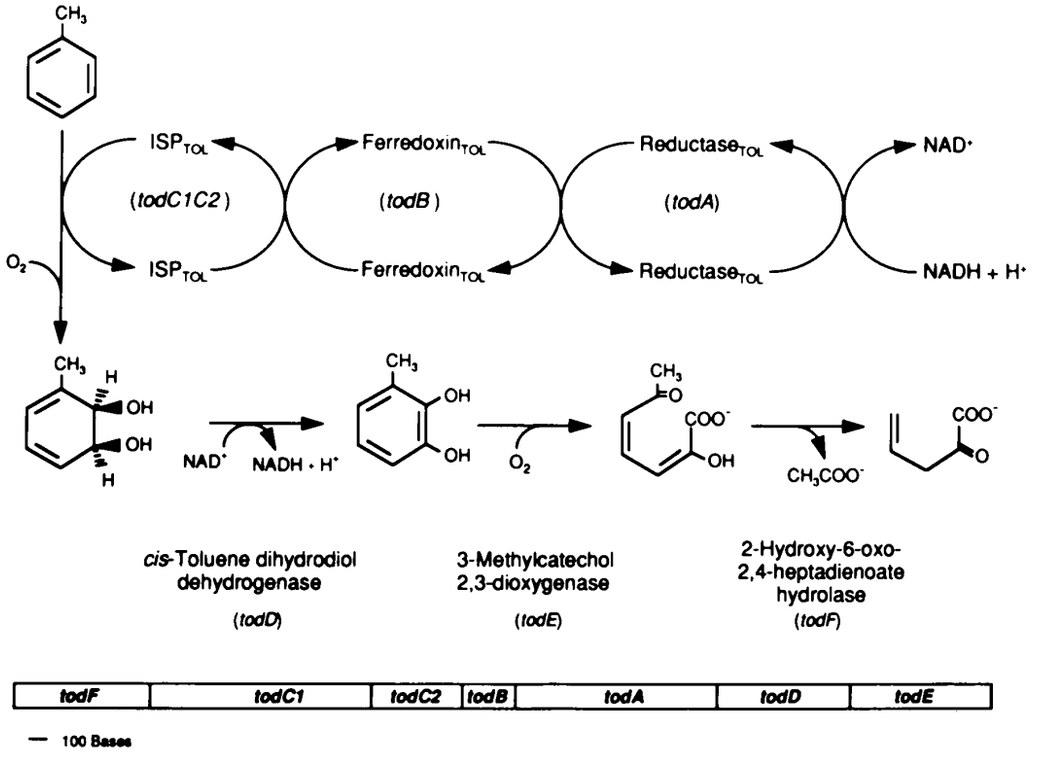

Figure 8 First four reactions in toluene degradation catalyzed by enzymes encoded by the *tod* operon [54,56].

induced strains could be combined to give naphthalene dioxygenase activity [48].

These beginning years at Iowa were enjoyable and hectic. My research group, capably led by Gerben, was driving me into uncharted waters in terms of recombinant DNA technology and it became very clear to me that the groundwork laid by this group of students was paving the way for future exciting research in enzymology and biotransformations. The Biocatalysis and Bioprocessing Center led by Jack Rosazza was attracting local, state and national attention and my interaction with the faculty members in my own department and those from the six departments associated with the Center were truly collegial. For the first time I felt part of a genuine academic environment. This period however, was not without its low points and the untimely passing of Irving Crawford, Diana Cruden and Al Markovetz left a void that could never be replaced and, in fact, marked the end of an era in the Microbiology Department at the University of Iowa.

New students who joined the laboratory took full advantage of the efforts of their predecessors. Sol Resnick followed up on Larry Wackett's and Billy Haigler's observations that toluene dioxygenase would catalyze the oxidation of indan to (1*R*)-indanol whereas naphthalene dioxygenase forms (1*S*)-indanol and also appeared to catalyze the desaturation of indan to indene. It did not take Sol long to recognize that his major interests were in the stereochemistry of the different products formed by aromatic hydrocarbon dioxygenases. In his early studies he was ably assisted by John Brand, a visiting professor from South Africa, and Dan Torok, who joined our group after obtaining his PhD in organic chemistry at Iowa. On his own initiative Sol developed his expertise in biotransformations and analytical chemistry to the stage where he published an elegant NMR method for determining the absolute stereochemistry of chiral diols [41]. Kyoung Lee focused his attention on the properties of purified naphthalene dioxygenase. The pure enzyme proved invaluable in clarifying ambiguities in substrate specificity studies with mutants and recombinant strains and also identified reactions that were not detected by studies with intact cells. The oxidation of toluene to benzyl alcohol and benzaldehyde is one example [33]. Additional studies showed that naphthalene dioxygenase also catalyzed stereospecific sulfoxidation reactions [32]. A recent review summarizes all of the known reactions of naphthalene dioxygenase [40] and shows that the enzyme can catalyze all of the known reactions of mammalian cytochrome P450 with the only difference being that naphthalene dioxygenase oxidizes double bonds to *cis*-dihydrodiols whereas cytochrome P450 forms epoxides from the same substrates.

Other enzymes that were investigated during this period were biphenyl 2,3-dioxygenase and 2-nitrotoluene dioxygenase. John Haddock extended preliminary results obtained by Louise Nadim in Texas to show that biphenyl 2,3-dioxygenase from *Pseudomonas* sp LB400 was a three-component system [21]. John purified and characterized the oxygenase and showed that it was another non-heme iron sulfur protein with a Rieske [2Fe-2S] center and mononuclear iron at the active site [19]. He was able to show that the purified enzyme oxidized 2,5,2′,5′-tetrachlorobiphenyl at the 3,4-positions [20] and thus laid to rest the idea that a different oxygenase in LB400 is responsible for the formation of the 3,4-diol.

Rose An showed that a similar multicomponent non-heme iron dioxygenase oxidized 2-nitrotoluene to 3-methylcatechol and thus purified the first enzyme to oxidatively remove a nitro substituent from an aromatic hydrocarbon [1]. This work is currently being continued by Becky and Juan Parales in Iowa City and the complete nucleotide sequence of all of the 2-nitrotoluene dioxygenase components has recently been reported [38].

Studies on toluene dioxygenase were given a strong boost by the isolation of a monoclonal antibody to the small subunit by Nancy Lynch. The antibody was used to prepare an affinity column which yielded pure ISP_{TOL} in a single step [34]. This work, carried out by Nancy and Haiyan Jiang, also led to the isolation of a pure sample of the β subunit of ISP_{TOL} and paved the way for ongoing studies on interactions between the large and small subunits of the oxygenase. More recently Haiyan and Becky have used site-directed mutagenesis to tentatively identify the amino acids responsible for chelating iron at the active sight of ISP_{TOL} [28].

My ride continues today through the efforts of Becky and Juan Parales, Kyoung Lee, Haiyan Jiang and Sol Resnick. New avenues have been opened with the identification of domains responsible for the regiospecificity and enantiospecificity of 2-nitrotoluene dioxygenase, the first demonstration of carbazole oxidation by a multicomponent enzyme system and the crystallization of the oxygenase component of naphthalene dioxygenase. In addition, our collaborations with Jim Spain (Florida), Derek Boyd (Belfast), S Ramaswamy and Björn Kauppi (Sweden) and Andy Robertson (Iowa) are productive and enjoyable.

If the foregoing pages seem a little turgid it is due to my wish to acknowledge as many people as possible who have played a role in my academic career. It is difficult to convey the warmth and friendships developed over the past 30 years with students, colleagues and collaborators. Perhaps the symposia and posters presented at this meeting say it best. The ride continues and the horizon, although much brighter, still reminds me that there are many roads yet to be explored and enjoyed.

Acknowledgements

Much of the work described in this article was supported by US PHS grant GM29909 from the National Institute of General Medical Sciences and the Air Force Office of Scientific Research grant No. F49620-96-1-0015. I gratefully acknowledge the efforts of Allen Laskin and Carl Cerniglia for organizing the symposium and Jim Spain and Gerben Zylstra for organizing the poster sessions that led to this Special Issue and all former students and collaborators who are not cited in the text. I thank Julie Nealson for her preparation of this manuscript and her unfailing attention to all of the other tasks that frees me to enjoy science and students alike. Finally I thank my wife Janet and daughters Karen and Christine for their love, support and encouragement through good times and bad.

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