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# Biodegradation, biotransformation and the Belmont*

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I would like to share with you some thoughts on biodegradation and biotransformation. My interests in these areas were generated and fueled by the late Stanley Dagley. Much of what follows has already been said by Dr Dagley in essays, reviews, and his autobiography, 'Lessons From Biodegradation' published in the 1987 volume of the *Annual Review of Microbiology* [6]. However, I do not apologize for repeating some of his philosophies and statements. He was the most scholarly and kindest person I have ever known and I know that he would approve. Few people know that Dr Dagley obtained his undergraduate degree in physical chemistry and that he began his research career with Sir Cyril Hinshelwood at Trinity College, Oxford. He wanted to conduct research in chemical kinetics and was dismayed to learn that Dr Hinshelwood would accept him as a student only if he would study the kinetics of bacterial growth. It wasn't until Sir Cyril showed him that if the sign of the exponential term is changed from negative to positive, the rate of increase of a growing bacterial population can be expressed by a familiar equation that describes the rate of a unimolecular gas reaction. Much relieved by this revelation Dagley began his career as a microbiologist. His studies with bacteria showed that the size of a crop of bacteria was a linear function of the amount of glucose provided for growth. This simple concept was not readily accepted in the 1930s when it was generally believed that bacteria stopped growing when they had filled all of the 'available biological space'. A few years later Monod described the kinetics of bacterial growth with mixed sugars when the concentrations of the sugars were present in limiting amounts. This work led to the concepts of diauxie, catabolite repression, allosteric regulation, the discovery of the operon, and a Nobel Prize. Since many of the results obtained from studies with bacteria are relevant to cellular activities in higher organisms, this was a period when *Escherichia coli* was raised to the status of 'honorary mammal' [5]. However, I can never look at a textbook, or paper on molecular biology, without thinking of Dr Dagley in his tattered white lab. coat and sandals, bent over a microscope, conducting careful hemocytometer counts and plotting growth versus substrate concentration, and I wonder what would have happened if he had grown his organism on a mixture of glucose and galactose. Two things are certain: I would not be giving this address and many molecular biology students would have been exposed to the concepts of thermodynamics as they apply to biological systems. He was a magnificent teacher who could take any subject, reduce it to its most simple components, and then reconstruct it and show its relationship to the biological world. He abhorred cartoons in textbooks, and the squiggle used in many texts to signify a high energy phosphate bond was one of several pictures that could cause him to raise his voice. Others were drawings of water wheels and pulleys that depicted 'streams of electrons cascading over jutting rocks of cytochrome' [6]. However, he was a true physical chemist and he always managed to correlate his opinions of such textbooks with the role of the Thatcher Government in dismantling academic research in the English universities. In other words, like the bacteria he loved so much, he knew how to use free energy for the maximum benefit.

The title of my presentation; 'Biodegradation, biotransformation and the Belmont', covers my scientific and some of my recreational interests. I hope that I will have time to talk about the Belmont since it is a subject that is near and dear to my heart, but for the present I would like to point out that just as thoroughbreds represent the evolution of one branch of the horse, there are concepts in microbiology that have undergone an analogous evolutionary process; and, just as certain sire lines in thoroughbreds die out, there are certain concepts in microbiology that have outlived their usefulness. I would like to start with the concept of metabolism which was defined by Foster in his book *A Textbook of Physiology* in 1888 as 'the biological transformation of substrates into products' [13].

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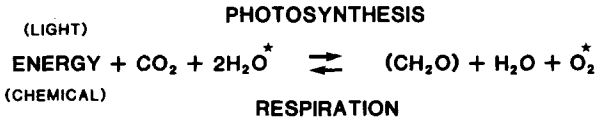
Foster then went on to define anabolism as constructive metabolism, and catabolism as destructive metabolism. These simple definitions were elegant and powerful because they suggested that anabolism and catabolism were inextricably linked and that together they could account for the total activities of a cell.

The relationship between anabolism and catabolism is seen clearly in the cycle of carbon in nature. Light energy from the sun is harnessed by the photosynthetic apparatus of plants, algae and certain bacteria and used to drive the synthesis of the biochemicals necessary for cellular growth from carbon dioxide and an appropriate electron donor. The carbon locked in these molecules is eventually released back to the atmosphere as carbon dioxide, mainly by activities of microorganisms. Thus, the carbon cycle represents an equilibrium between the processes of photosynthesis and respiration as shown below (Scheme 1).

The forward reaction led Lord Kelvin to exclude 'animal agencies' from his second law of thermodynamics, and it is only in the past 50 years that we have learned how living cells use acetyl-coA, ATP and similar compounds to circumnavigate the second law.

We now have a reasonable understanding of the catabolic processes that are involved in microbial metabolism as shown in Fig. 1. Sugars are broken down by the enzymes of the Embden–Meyerhoff pathway. ATP for biosynthesis is produced by substrate-level phosphorylation and oxidation reactions are mediated by the transfer of hydride ions to pyridine nucleotides. It is clear that the breakdown of glucose will cease unless the cell can find some way to regenerate oxidized NAD. This is accomplished by fermentative organisms by transferring electrons to organic compounds. Thus yeast metabolizes glucose by transferring reducing equivalents to acetaldehyde and ethanol accumulates as an end product. The same principle is observed in mammals when oxygen is in short supply. Reduced NAD transfers hydrogen and electrons to pyruvate to form lactic acid which accumulates in muscles and causes fatigue. I have often wondered what would have happened if evolution had led humans to use acetaldehyde as an electron acceptor.

The organic acids, aldehydes and alcohols produced by fermentation serve as carbon and energy sources for other groups of microorganisms which convert them to carbon dioxide, hydrogen, and acetate. These compounds, in anaerobic environments, serve as substrates for the growth of methanogenic bacteria which reduce carbon dioxide to methane. In contrast, aerobic organisms oxidize the pyruvate formed from glucose to carbon dioxide and water, as shown in Fig. 2. Again we see oxidation reactions that involve the removal of hydrogen and electrons which are transferred down the respiratory chain with the concomitant synthesis of ATP and the eventual four-electron reduction of oxygen to water.




Scheme 1

Oxygen is a water-soluble gas that exists in the triplet state with unpaired electrons. Organic compounds exist in the singlet state and reactions with oxygen are spin-forbidden. It is the low reactivity, or sluggishness of oxygen that enabled aerobic life to emerge from the primeval anerobic swamps about two billion years ago. Organisms that can take advantage of the energy gradient between organic compounds and oxygen do so by controlling the active forms of oxygen that pose a threat to cellular constituents. These include the superoxide anion and hydrogen peroxide which are formed by the sequential addition of single electrons to the oxygen molecule. We know that aerobic organisms have evolved enzymes such as superoxide dismutase, catalase and other peroxidases to minimize the destruction of cellular components by these reactive oxygen species. On the other hand obligate anerobic bacteria, such as the methanogens, do not possess superoxide dismutase or peroxidases and are killed in the presence of oxygen. An observation that led Dagley to conclude that 'the anerobes are the conservatives of the microbial world because they lurk in the mud and cannot tolerate radicals' [4].

There is very little indication in most contemporary biochemistry texts to suggest that oxygen has any other role in metabolism than to serve as an electron acceptor at the bottom of an energy gradient. However, the importance of oxygenases, enzymes that incorporate molecular oxygen into organic compounds, cannot be underestimated. For example, methane, the most reduced form of carbon, can exist in the presence of oxygen for years without anything happening. However, one would be ill-advised to use a match to search for a gas leak. Ruminants can belch 60–80 liters of methane a day, leading to the suggestion that the legendary dragon must have been a ruminant with an interesting ignition mechanism, and it is the biochemistry of the ignition mechanism that we seek to understand in the enzymatic incorporation of oxygen into methane by methanotrophic bacteria.

How do the pathways of intermediary metabolism and the biological activation of oxygen relate to biodegradation and biotransformation? From my own perspective I regard biodegradation as the catabolism of a compound into molecules that can enter the pathways of intermediary or central metabolism, for example, the bacterial oxidation of benzene to acetyl-coA and succinate. I believe that knowledge of the reactions leading to the formation of succinate and acetyl-coA are implied by the term biodegradation.

Biotransformation, on the other hand, can be considered as a modern term for metabolism. That is, the biological conversion of substrates into products. Biotransformations can thus be regarded as catabolic, as in the microbial conversion of glucose to ethanol, or anabolic as in the biosynthesis of antibiotics. However, biotransformation is often used in a more restrictive sense that relates to the conversion of compound A by one or more enzymatic steps

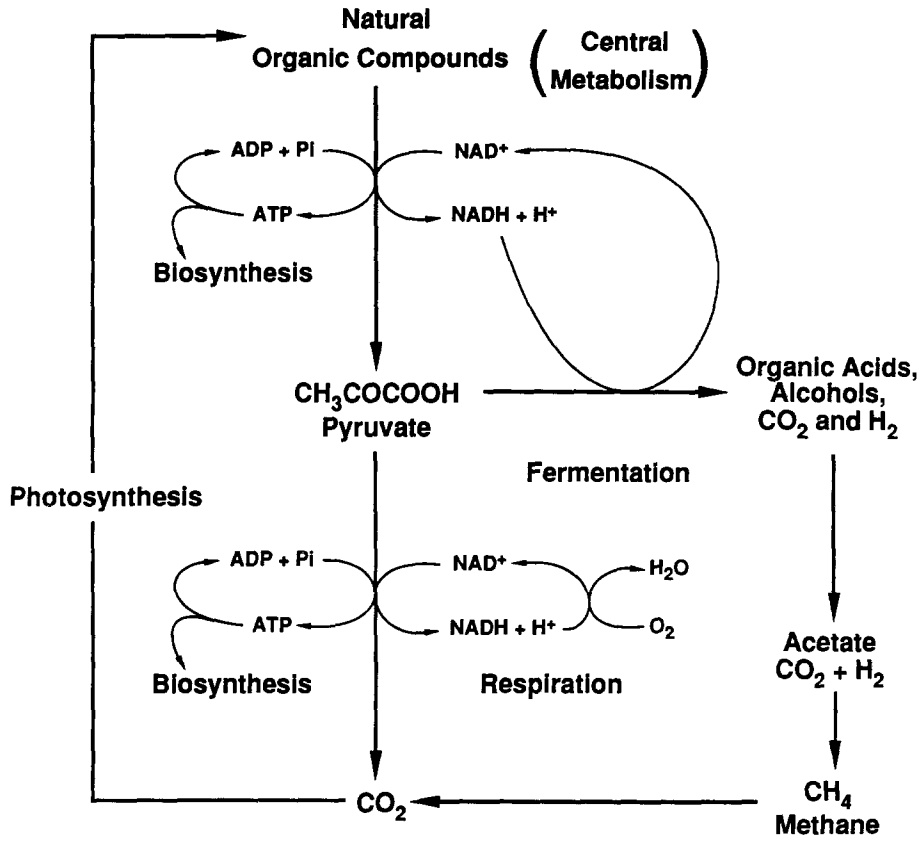



Fig. 1. Central catabolic pathways involved in bacterial metabolism.

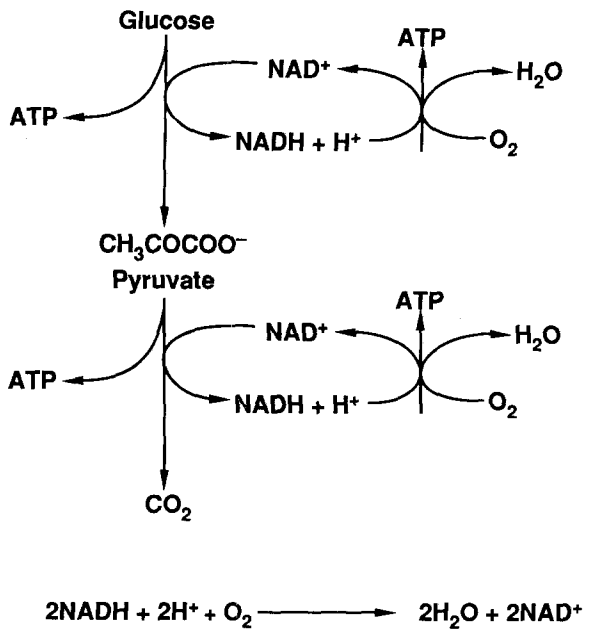



Fig. 2. Synthesis of ATP during aerobic metabolism.

to compound B, where B may be a desirable or undesirable product, for example, the transformation of Reichstein's substance S to cortisone and the transformation of trichloroethylene to the carcinogen vinyl chloride.

It is important to recognize that catabolism and anabolism refer to the fundamental activities of living cells, whereas biodegradation and biotransformation are really terms that define our objectives within the area encompassed by metabolism. However, there are dangers in generating new terms to define who we are and what we do as practicing microbiologists. The danger lies in the invention of new biological phenomena where none exist. I believe that the term cometabolism falls into this category, and I think that it is worth a few minutes to look at the evolution and use of cometabolism because it is a seductive term. Many people use cometabolism to refer to the degradation of a compound in the environment where one or more reactions are carried out by several different microorganisms. This is acceptable, although it lacks the rigor that one would wish to see in scientific terminology. However, this is not the description of cometabolism that we find in the literature.

In 1959 Leadbetter and Foster at the University of Texas at Austin described a new technique, eventually called cooxidation, for biochemical studies on the bacterial oxidation of hydrocarbons [25,26]. Foster described cooxidation as

'the oxidation of non-growth hydrocarbons when present in a medium where one or more different hydrocarbons are furnished for growth' [14]. Raymond, Jamison and their colleagues at Sun Oil Company [28] made extensive use of the cooxidation technique in their studies on the biological activities of *Nocardia*, and two examples are shown in Fig. 3.

In the first example hexadecane serves as the growth substrate and the organism converts the non-growth substrate, ethylbenzene, to phenylacetate. It would be reasonable to suggest that the enzyme responsible for the oxidation of the methyl group of hexadecane is also responsible for the oxidation of the methyl group of ethylbenzene. In the second case one could suggest that *p*-xylene induces the enzymes responsible for its own oxidation to dimethyl-*cis*,*cis*-muconate. In both cases, these are examples of biotransformation that can be tested experimentally.

In 1977 Horvath introduced the term cometabolism to include cooxidation, and stated that, 'Cometabolism refers to any oxidation of substances without utilization of the energy derived from the oxidation to support microbial growth and does not infer presence or absence of growth substrate during the oxidation' [20]. In other words, from the very beginning the prefix co was not regarded as essential for the phenomenon of cometabolism. Horvath then went on to say that, 'The process of cometabolism represented to the microbial physiologist a new oxidative mechanism for which there was no adequate explanation'. This statement identified cometabolism as a fundamental property of the bacterial cell on a par with DNA replication and, like DNA replication, it required experiments to determine the mechanisms involved. For example, 'The actual mechanism of cometabolism of *m*-chlorobenzoate by *Arthrobacter* sp. was elucidated by Horvath and Alexander [21] using whole-cell suspensions and cell-free enzyme preparations. Benzoate-grown cells oxidized *m*-chlorobenzoate without a lag and preincubation of cells with the halogenated analog induced the cells to metabolize both benzoate and *m*-chlorobenzoate' [20]. The product formed from *m*-chlorobenzoate was identified as 4-chlorocatechol (Fig. 4). These observations led to the conclusion that 'the relatively unspecific nature

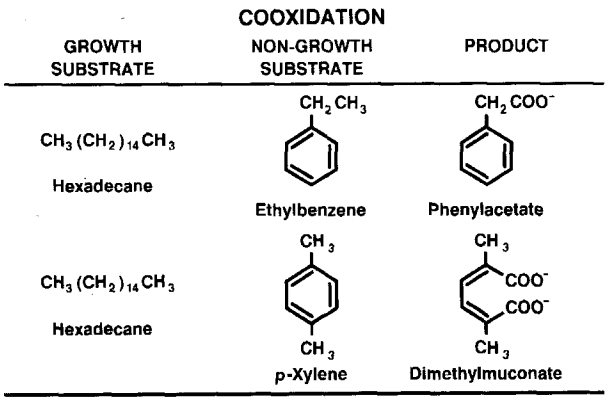



Fig. 3. Examples of cooxidation by bacteria. Adapted from Raymond and Jamison [28].

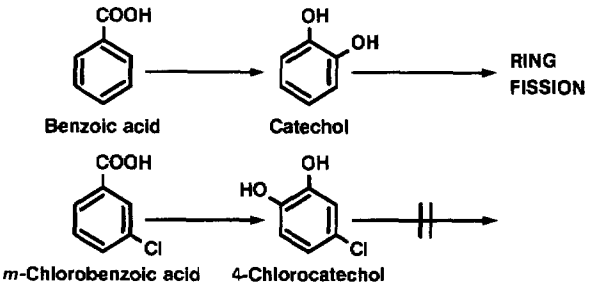



Fig. 4. Bacterial cometabolism. Oxidation of benzoic and *m*-chlorobenzoic acids by bacteria [20, 21].

of the benzoate oxidase enzyme and the specificity of the ring-cleaving enzyme for an unsubstituted catechol appeared to account for cometabolism' [20]. Let us look at this situation a little more closely. In 1957 Dagley and Patel [11] showed that *p*-xylene-grown cells of a *Pseudomonas* species oxidized xylenols to substituted *p*-hydroxybenzoates, which were not further metabolized as shown in Fig. 5.

Dagley and Patel concluded that the enzymes responsible for the oxidation of *p*-cresol to *p*-hydroxybenzoic acid have a relaxed substrate specificity that permits the oxidation of 3,4-xylenol to 2-methyl-4-hydoxybenzoic acid. This acid accumulates because it is not a substrate for the next enzyme in the *p*-cresol pathway. The same situation and interpretation applies to the oxidation of *m*-chlorobenzoic acid to 4-chlorocatechol. The only difference between the two experiments is that Dagley and Patel did not claim to have discovered an explanation for a new phenomenon in microbial physiology. To quote Dagley, 'There is more at stake here than semantics or definitions. It is a matter of stimulating or discouraging careful scientific investigation. The disappearance of a compound from a system may require a complex analysis before we can understand precisely what has happened. To say that its disappearance is due to the 'phenomenon of cometabolism' is about as helpful as saying that Beethoven's fifth symphony is due to the 'phenomenon of sound' [4].

Finally, in a more serious vein I draw your attention to a paper published in the *Journal of Theoretical Biology*,

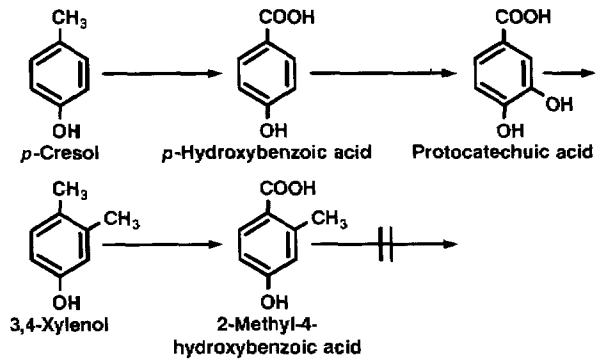



Fig. 5. Bacterial catabolism. Oxidation of *p*-cresol and xylenols by bacteria [11].

entitled 'Cometabolism: a critique', by Hulbert and Krawiec [23]. The authors conclude that 'None of the demonstrated features of cometabolism differ from those of ordinary catabolism or anabolism', and state that, 'Cometabolism falsely suggests that a previously unrecognized capacity for the transformation of substrates exists in some cells'. They proposed that use of the word cometabolism be abandoned.

Although cometabolism has been a thorn in my side for many years, this is not the case with biodegradation and biotransformation, especially as these terms relate to aromatic compounds. About 10 or 12 years ago Dr Allen Laskin convened a symposium at the National Meeting of the American Society for Microbiology on the Bacterial Degradation of Hydrocarbons. All but one of the speakers originated from the University of Leeds, England, or the University of North Wales at Bangor and were introduced collectively as the British Mafia. What Dr Laskin failed to mention was the existence of at least four other families involved in aromatic metabolism and these are shown in Fig. 6.

I thought that it might be of interest to go back and look at some of those early pioneers of the field and their contributions to the field of aromatic metabolism and microbial physiology in general.

We know that there are vast amounts of aromatic compounds in nature in the form of lignin, and that fungi, such as *Phanerochaete chrysosporium*, play an essential role in the carbon cycle by depolymerizing lignin to monomers that can serve as substrates for fungi and bacteria. We also know that many of these smaller aromatic molecules are converted to catechol or protocatechuic acid and that these compounds serve as the starting points for two major metabolic pathways known as the β-ketoadipate and *meta*-fission pathways respectively.

The β-ketoadipate pathway is a product of research conducted in England, the USA and Japan. The English contribution was initiated by Frank C. Happold who was the first Professor and Head of the Department of Biochemistry at the University of Leeds.

The American branch of the β-ketoadipate pathway was founded by Roger Stanier at the University of California at Berkeley. Stanier's childhood was much bleaker and more miserable than anything found in a Charles Dicken's novel, nevertheless he survived and went on to become a major influence in the development of microbiology in the USA. Stanier regarded himself as 'woefully inadequate' in his knowledge of chemistry and biochemistry, but this self-assessment is hard to believe when one considers his contributions to the fields of carotenoid biosynthesis and aromatic metabolism [29].

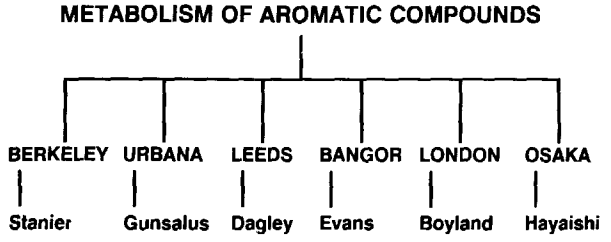



Fig. 6. Scientists who pioneered studies on the metabolism of aromatic compounds.

The third contributor to the β-ketoadipate pathway was Osamu Hayaishi who graduated from Osaka University School of Medicine in 1942 and served as a medical officer in the Japanese Navy until the end of the Second World War. He returned to Osaka and joined the laboratory of Professor Yashiro Kotake who was well known for his studies on the metabolism of tryptophan. At that time research funds were almost nonexistent, the facilities were poor, and there were no laboratory animals. Faced with this situation Hayaishi decided to study tryptophan metabolism by bacteria, and started a chain of events that led him to Berkeley, the National Institutes of Health and the β-ketoadipate and *meta*-fission pathways [19].

The story of the β-ketoadipate pathway began in 1932 at the University of Leeds when Happold and Key isolated an organism from gasworks effluent that would grow with phenol as the sole source of carbon and energy. The organism was identified as a strain of *Vibrio* and given the designation 01. Some 36 years later, Paul Bauman in Stanier's laboratory at Berkeley identified *Vibrio* 01 as *Acinetobacter calcoaceticus*. This may have been the first, but by no means the last, time that biochemists in the field of aromatic metabolism have shown a laissez-faire attitude to bacterial taxonomy. The conventional wisdom throughout the years has always been, if it is Gram negative and motile, it must be a *Pseudomonas* species. In 1947, W. C. Evans, who was later to become professor of biochemistry at the University of North Wales, isolated catechol and protocatechuic acid from culture filtrates of *Vibrio* 01 that had been grown with benzoate and *p*-hydroxybenzoate respectively, and in the following year Bernard Kilby emerged triumphantly from a cold room, where he had spent several hours extracting and isolating β-ketoadipate from culture filtrates of *Vibrio* 01. The cold room was necessary because of the instability of β-ketoadipate, which, like all β-ketoacids easily undergoes decarboxylation.

In 1950 studies on the β-ketoadipate pathway entered log phase. Stanier's research group showed that catechol and protocatechuate were converted to β-ketoadipate by two independent pathways. In the same year, Hayaishi and Suda partially purified an enzyme they called pyrocatechase and showed that it oxidized catechol to *cis,cis*-muconic acid. Stanier invited Hayaishi to spend a sabbatical leave in Berkeley to work on the pathway. The situation at that time is shown in Fig. 7.

Stanier was convinced that *cis,cis*-muconate would be converted to β-ketoadipate by cell extracts. Hayaishi was not so sure. However, some time after Hayaishi joined the laboratory Stanier was delighted to find that his visitor had a secret cache of *cis,cis*-muconate. I am sure that Hayaishi did not share the same feeling, since Stanier persuaded him to use all of his muconate, all five milligrams of it, in one big experiment which was designed to show that *cis,cis*-muconate is an intermediate in the oxidation of catechol to

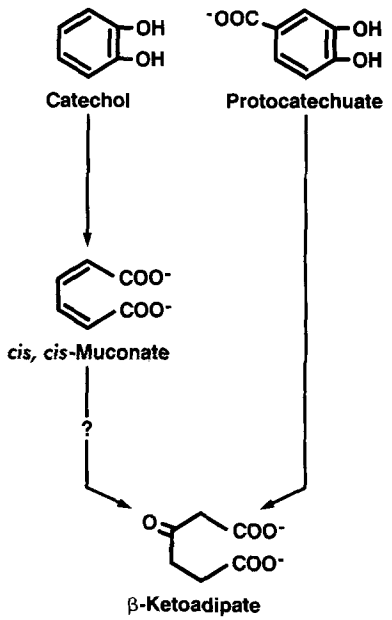



Fig. 7. The β-ketoadipate pathway 1950.

β-ketoadipate. The experiment failed, and the relationship between the two became strained [29].

It wasn't until the following year that the reason for the failure of the experiment was explained. Evans and Smith at Bangor synthesized *cis,cis-*, *cis,trans-* and *trans,trans-*muconate and showed that *cis,cis*-muconate was unstable in aqueous solution and readily isomerized to the *cis,trans-*form. This was the isomer that Hayaishi had isolated and it was not a substrate for enzymes of the β-ketoadipate pathway.

Another intermediate was added to the pathway when Stanier and Macdonald showed that protocatechuic acid was converted to *cis,cis*-β-carboxymuconate in an analogous reaction to that catalyzed by pyrocatechase. The final steps of the β-ketoadipate pathway were elucidated by Dr L. N. Ornston who was one of Stanier's last graduate students. His dissertation resulted in four consecutive papers in the *Journal of Biological Chemistry* in 1961 (summarized in reference [13]). This work showed that the catechol and protocatechuic acid branches of the β-ketoadipate pathway converge not at β-ketoadipate, but at β-ketoadipate enol lactone as shown in Fig. 8. Ornston identified the lactone preceding β-ketoadipate enol lactone in the protocatechuic acid branch of the pathway, and purified the enzymes which converted both lactones to the enol-lactone. After postdoctoral positions with Hans Kornberg in England and I. C. Gunsalus in Urbana, Ornston joined the biology department at Yale University where to this day he conducts elegant studies on the regulation and evolution of the β-ketoadipate pathway in *Pseudomonas, Acinetobacter* and other genera.

Before 1957 it was assumed that the bacterial degradation of catechol and protocatechuic acid was always initiated by cleavage between the hydroxyl groups to give muconic acids. However, in that year Dagley and Patel showed that

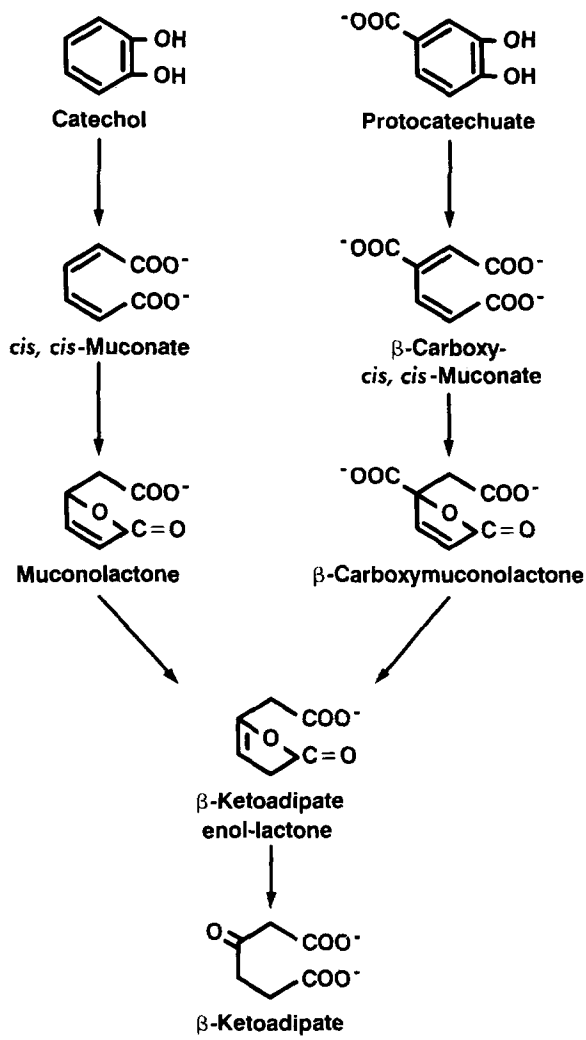



Fig. 8. The β-ketoadipate pathway 1965.

ammonium sulfate-treated cell extracts, prepared from *p*-cresol-grown cells of a pseudomonad, oxidized protocatechuic acid to a compound that resisted all attempts to determine its structure. The reaction occurred with the uptake of one mole of oxygen per mole of substrate and the product was a dicarboxylic acid not a tricarboxylic acid which would have been formed if fission had occurred between the hydroxyl groups. Eventually, elemental analysis showed that the metabolite contained nitrogen, and soon after the compound was identified as 2,4-lutidinic acid. This unexpected product was shown to be formed in a nonenzymatic reaction between a new ring-cleavage product and ammonium ions present in the cell extract. Prior to that time Dagley and Stopher had shown that cell extracts prepared from *o*-cresol-grown cells of *Pseudomonas* L (L for Leeds) oxidized catechol to a yellow acid which was identified as 2-hydroxymuconic semialdehyde and now they were able to show that 2-hydroxymuconic semialdehyde reacted, albeit more slowly, with ammonium ions to form picolinic acid [8]. These reactions are shown in Fig. 9.

In 1961 I joined Professor Dagley's research group at the

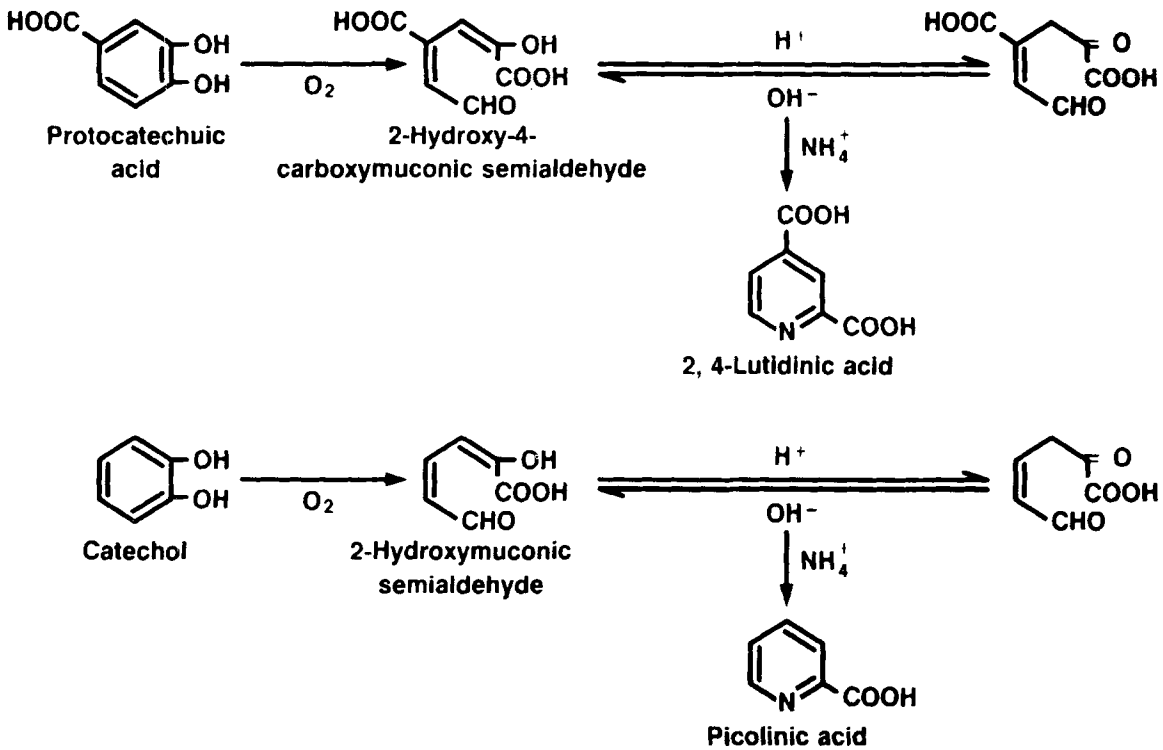



Fig. 9. Nonenzymatic formation of pyridine carboxylic acids from catechol and protocatechuic acid ring-fission products.

University of Leeds and it was decided that I should work on the degradation of 2-hydroxymuconic semialdehyde, the *meta*-ring fission compound formed from catechol by *Pseudomonas* L. Soon after I started the project, Hayaishi and his colleagues in Kyoto published a pathway for the degradation of 2-hydroxymuconic semialdehyde to acetate and pyruvate [27]. I was crushed. However, Dagley encouraged me to see if I could show the same reactions in *Pseudomonas* L. I couldn't. Our work with *Pseudomonas* L led to the elucidation of a different pathway and this is shown in Fig. 10. Hayaishi's pathway is shown on the left. The initial reactions involved the NAD-dependent oxidation of 2-hydroxymuconic semialdehyde to 4-oxalocrotonate which was then decarboxylated to 2-keto-4-hydroxyvalerate. We were able to show that 2-hydroxymuconic semialdehyde was converted to 2-keto-4-hydroxyvalerate. However, all attempts to demonstrate the formation of 4-oxalocrotonate were unsuccessful. Instead we were able to show that formate, not carbon dioxide, was formed from the ring-fission compound. The bottom part of Hayaishi's pathway involved the oxidation of 2-keto-4-hydroxyvalerate to acetopyruvate followed by hydrolysis to give acetate and pyruvate. We were unable to show these reactions in *Pseudomonas* L. However, we did find that cell extracts, prepared from *Pseudomonas* L, contained an inducible enantiospecific aldolase which converted 2-keto-4-hydroxyvalerate to pyruvate and acetaldehyde as shown on the right-hand side of Fig. 10 [9]. Time does not permit me to describe the highs and lows of this period. However, perhaps one story will suffice. In 1962 when Professor Hayaishi was in London, he called the Biochemistry Department at Leeds to see if Professor Dagley could come to London for discussions on the ring-fission pathway. Dagley was not feeling well at the time and asked me to go in his place. This was my first visit to London and my first time to be in such an ostentatious place as the Marble Arch Hotel. Professor Hayaishi was charming and we spent the afternoon discussing science in the hotel lounge where he graciously paid for the drinks. When I returned to Leeds Dr Dagley asked me what I had learned about Hayaishi's results. It was only then that I realized that I had spent the whole afternoon describing our own work. I spent the next 12 months in anguish fully expecting to see my dissertation project published from another laboratory. Professor Hayaishi was kind, it never happened.

In 1963 Dr Dagley spent a sabbatical leave in Dr I. C. Gunsalus's laboratory at the University of Illinois. A polluted stream, known as Boneyard Creek, flowed beneath his rented house and from it he was able to isolate an organism which he called *Pseudomonas* U (U for Urbana) which was much more efficient than *Pseudomonas* L. in converting catechol to pyruvate and acetaldehyde. He showed that cell extracts prepared from *m*-cresol-grown cells of *Pseudomonas* U oxidized 4-methylcatechol to pyruvate and propionaldehyde. I was in the last year of my PhD studies at the time when I received Dr Dagley's letter concerning these new observations. I well remember the last part of the letter which contained a general metabolic scheme that accounted for my results with catechol, John Wood's demonstration of the formation of pyruvate from protocatechuic acid and Peter Chapman's earlier studies on the formation of succinate from 2,3-dihydroxyphenylpropionate. This general scheme

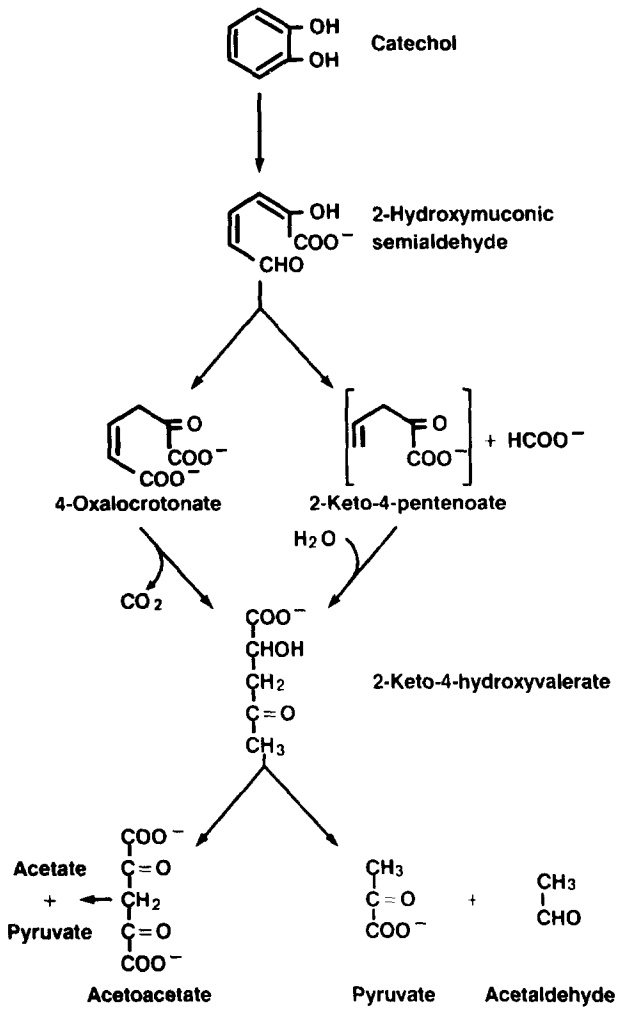



Fig. 10. Proposed pathways for the degradation of catechol by Nishizuka et al. [27] and Dagley and Gibson [9].

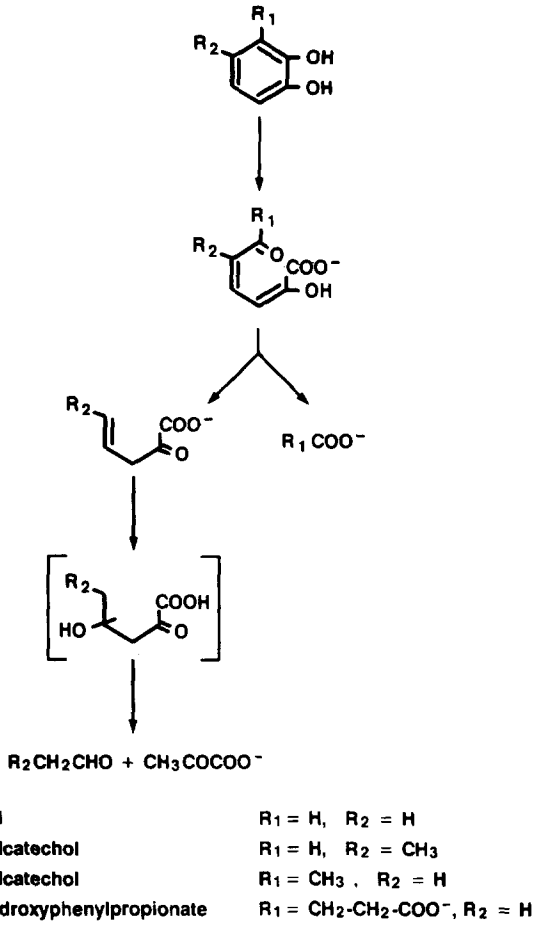



Fig. 11. General scheme for the degradation of substituted catechols [7].

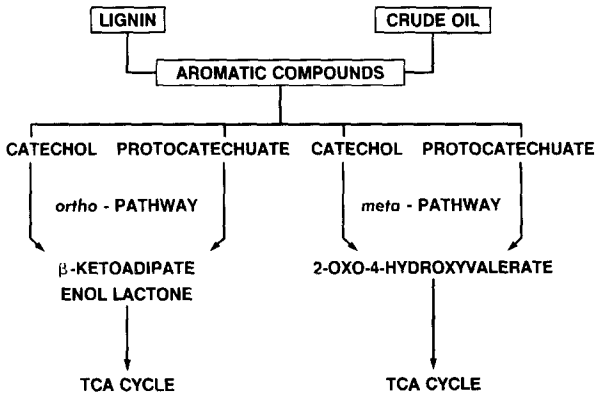



Fig. 12. Convergent pathways for the bacterial degradation of aromatic compounds. [1]The compound formed from protocatechuate via the *meta*-pathway is the carboxyl substituted 2-oxo-4-hydroxy-valerate derivative (2-oxo-4-hydroxy-4-carboxyvalerate).

which is shown in Fig. 11 has been used to predict the pathways used by bacteria for the degradation of substituted catechols [7]. For example, the scheme proved accurate in predicting the reactions involved in the degradation of the steroid aromatic A-ring. This work was conducted in Dr Charles Sih's laboratory at the University of Wisconsin [17]. The pathway has also been used quite often to explain the formation of chlorinated benzoates from polychlorinated biphenyls, although, as Bedard has shown [1], in the absence of more experimental data this is a practice that should not be encouraged.

Lignin and crude oil are the major sources of numerous aromatic compounds that are found in the environment and many of these compounds are degraded to catechol or protocatechuate and then through β-ketoadipate or 2-oxo-4-hydroxyvalerate to intermediates that can enter the tricarboxylic acid cycle (Fig. 12). It was these pathways of metabolic convergence that led Dr I. C. Gunsalus to explore the organization, expression and selectivity of the genes involved in aromatic metabolism, for he regarded metabolic convergence as a mechanism for reducing the total genetic load of the cell [18]. At that time little was known about *Pseudomonas* genetics. The first breakthrough came in 1967 when Gunsalus and Chakrabarty developed transducing and conjugation systems for *Pseudomonas*. However, they were unable to link mutations in aromatic metabolism with

auxotrophic markers. All of the evidence accumulated by Chakrabarty indicated the presence of catabolic plasmids, but the isolation of plasmid DNA resisted all of his best efforts and it was left to Jim Johnston, a later Gunsalus student, to develop the procedures for the isolation of the NAH and SAL plasmids which carry the genes for naphthalene and salycylate degradation respectively. These pioneering experiments led to an explosion in research on catabolic plasmids and their role in the regulation of aromatic metabolism as evidenced by publications from the laboratories of Gunsalus, Chakrabarty, Olsen, Timmis, Williams, Bayly, Bagdasarian, Harayama, Nakazawa, Schell, and many others.

Boyland is not a household name in the field of microbial aromatic metabolism. His studies at the Chester Beatty Research Institute in London began in 1935 and focused on polycyclic aromatic hydrocarbons and their role in carcinogenesis. His experimental protocol was quite simple, feed hydrocarbon x to rats or rabbits and identify the metabolites excreted in the urine. This was the biochemist's animated version of the engineer's black box. However, it was Boyland's remarkable ability to interpret the activities within the box that set him apart from his contemporaries.

Although only small amounts of material were isolated, Boyland and his colleagues were able to show that polycyclic aromatic hydrocarbons are oxidized to optically active *trans*-dihydrodiols and phenols by independent pathways. He also showed that these hydroxylated metabolites were excreted as glucuronide and sulfate conjugates. However, none of these metabolites had the reactivity that was required to explain how polycyclic hydrocarbons cause cancer. Boyland then suggested that epoxides could be the precursors of *trans*-dihydrodiols as shown in Fig. 13. The footnote, which is taken from Boyland's address to the Royal Society in 1950 [2], turned out to be prophetic. Some 20 years later Jerina and his colleagues at the National Instiutes of Health showed that arene oxides are the first detectable products in the oxidation of aromatic hydrocarbons by mammals [10], and subsequent studies have shown that enantiomers of polycyclic diol epoxides are the reactive metabolites involved in the initiation of tumors in experimental animals [31].

It was Boyland's hypothesis that stimulated our own interest in the metabolism of aromatic hydrocarbons. In 1965 I actually wanted to study the mammalian oxidation of these compounds. However, I was offered a position in the Microbiology Department at the University of Texas and my future chairman made it very clear to me that this would not be possible. This did not cause me too much concern because there were suggestions in the literature that bacteria use the same reactions as mammals to initiate the degradation of aromatic hydrocarbons, and the thought of developing a microbial model for aromatic hydrocarbon metabolism seemed quite attractive. We isolated a strain of *Pseudomonas putida* that would grow with toluene and began experiments designed to show that the organism would oxidize toluene to a *trans*-dihydrodiol, the expected mammalian metabolite. To our surprize we found that the first detectable product in toluene metabolism was a *cis*-dihydrodiol [15]. Subsequent studies showed that *cis*-hydroxylation is a common reaction in the bacterial oxidation of aromatic hydrocarbons that range in size from benzene to benz[*a*]anthracene (Fig. 14) [16].

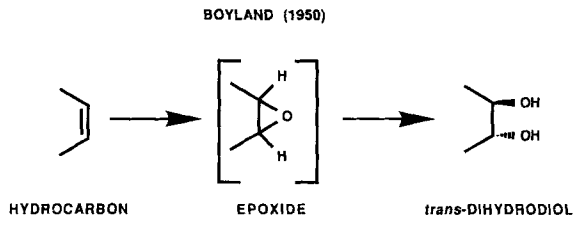



Fig. 13. Proposed initial reactions in the oxidation of aromatic hydrocarbons by mammals [2].

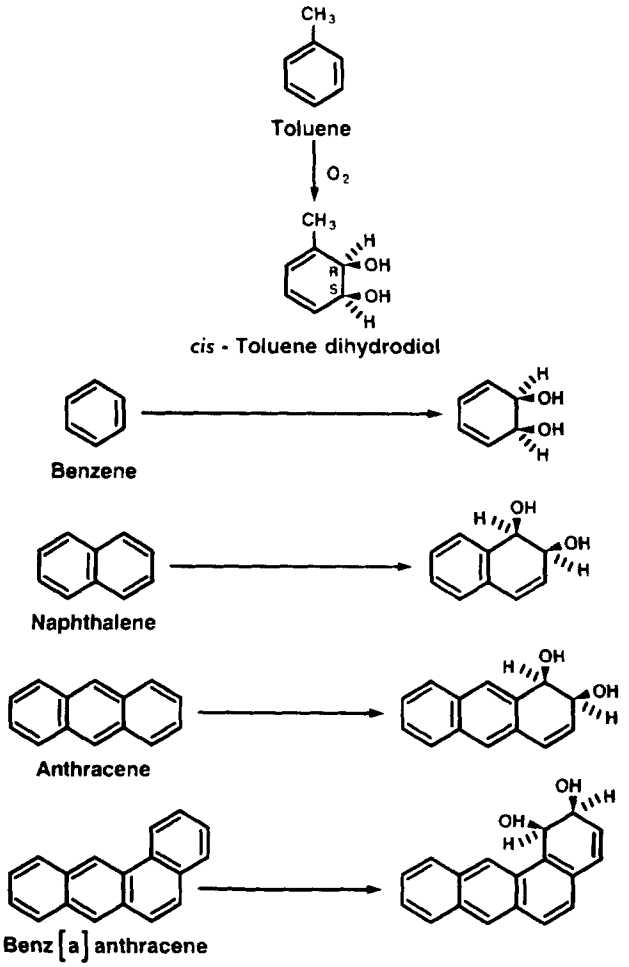



Fig. 14. *cis*-Dihydrodiols formed from aromatic hydrocarbons by bacteria.

When Carl Cerniglia joined our laboratory he conducted an elegant series of experiments to show that fungi oxidize several aromatic hydrocarbons to a similar spectrum of metabolites as those formed by mammals (summarized in references [3] and [16]). Although there are some exceptions, we have suggested that bacteria and higher organisms use different reactions to initiate the oxidation of aromatic hydrocarbons as shown in Fig. 15. Thus, bacteria incorporate both atoms of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols as the first detectable products. Subsequent oxidation of the dihydrodiols yields catechols which can enter the β-ketoadipate pathway or the *meta*-fission pathway depending on the bacterial strain being studied. In contrast, higher organisms oxidize aromatic hydrocarbons by incorporating one atom of oxygen into the aromatic nucleus to form reactive arene oxides which can undergo enzymatic hydration to *trans*-dihydrodiols, isomerize to phenols, or react with cell nucleophiles such as DNA to initiate a chain of events that can, in some cases, lead to tumor formation.

The bacterial degradation of aromatic hydrocarbons can be considered biodegradation because we would like to know how these compounds are converted to carbon dioxide and small molecules that can enter the tricarboxylic acid cycle. The fungal and mammalian oxidation of aromatic hydrocarbons can be considered biotransformation because the substrates are converted into products which are excreted by the cell, but there is really little difference between the two terms. The information gained from studies on the biodegradation of aromatic hydrocarbons can easily be turned into a study of biotransformation as shown by the demonstration of indigo formation by the cloned naphthalene dioxygenase genes [12], and the oxidation of toluene to *cis*-toluene dihydrodiol which can be used as a chiral synthon for the production of prostaglandin $E_{2\alpha}$ [24] as shown in Fig. 16. Since our first report on the formation *cis*-toluene dihydriol by *Pseudomonas putida* F1 in 1970, more than 100 different arene *cis*-diols have been reported in the literature. Interest in this class of compounds is due to the fact that they are usually single enantiomers that can serve as chiral reagents for a wide variety of potential pharmaceutical products [24]. The elegant work of Hudlicky and his students has focused on the incorporation of symmetry rules into the protocols for the synthesis of a variety of natural products. This imaginative approach has led to the stereocontrolled and enantiodivergent syntheses of a large number of carbohydrates, hydroxylated terpenes and alkaloids [22].

Marjorie Stephenson in her book *Bacterial Metabolism* published in 1949 [30], states that 'Biochemical studies on bacteria pay the highest dividend', leading Dagley to wonder if she would have used the word dividend if she could have foreseen the current develòpments in the pharmaceutical and biotechnology industries [5].

It was at this point in the address that I intended to give the lineage of the scientific children and grandchildren of the pioneers of aromatic metabolism. This is a research area that is very much alive; so much so, that any attempt to recognize the contribution of scientists that I have not mentioned would require several volumes. There has been a certain degree of inbreeding. However, the dangers inherent in such relationships have been reduced significantly by the introduction of new blood from other laboratories and institutions throughout the world. The resulting hybrid vigor ensures that new concepts and discoveries in the field of aromatic metabolism will continue for many years to come.

Hybrid vigor is not possible in thoroughbred race horses. All thoroughbreds can trace their pedigrees to three Arabian horses imported to England almost three hundred years ago. Thus all race horses are inbred and there are no statistical differences between the times recorded for ten furlong races in the 1930s and times recorded for the same distance today. The only exception is Secretariat who won the Triple Crown in 1973. The races were, the Kentucky Derby in a record time of under two minutes, the Preakness Stakes, in a record time that was not allowed because of a malfunction in the official clock, and the Belmont Stakes in a record time and by the almost unbelievable distance of thirty-one lengths. Secretariat's sire was Bold Ruler who was the best race horse of his day and the male-tail pedigree of Secretariat can be traced back through Bold Ruler to the Darley Arabian horse which was foaled in 1700 (Fig. 17). Note the unusual name of the sixth horse in the pedigree, POT-8-Os. According to legend, a stable hand was instructed to paint

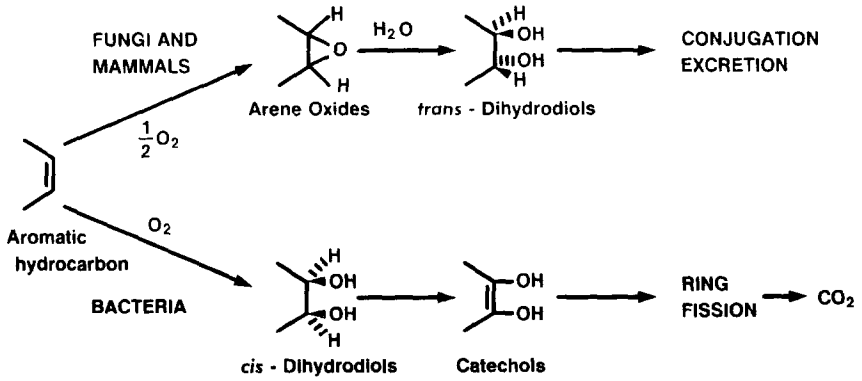



Fig. 15. Initial reactions in the oxidation of aromatic hydrocarbons by bacteria, fungi and mammals.

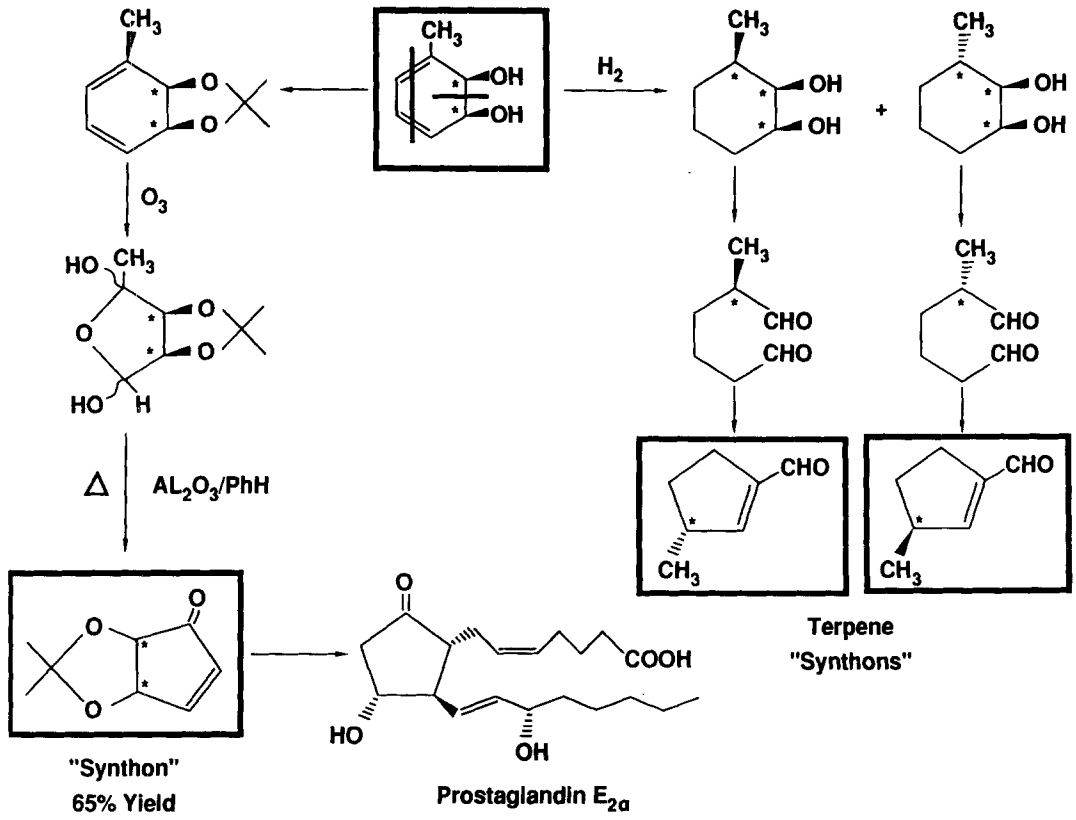



Fig. 16. Chemical synthesis of prostaglandin and terpene synthons from chiral *cis*-toluene dihydrodiol. Adapted from [12].

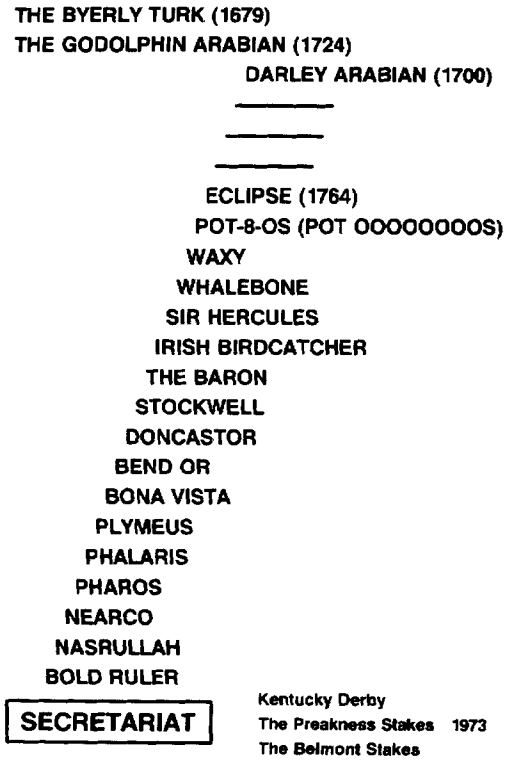



Fig. 17. Male-tail pedigree of Secretariat, winner of the Kentucky Derby, the Preakness Stakes and the Belmont Stakes in 1973.

the name of a horse on the stable door. The name of the horse was Potatoes. However, the lad did not know how to spell the word and painted POT followed by eight Os. The shortened version, POT-8-Os is certainly a more imaginative spelling of potatoes than that coined by Vice President Quayle earlier this year.

There were great hopes that Secretariat would sire a new class of race horse that could carry great speed over classic distances such as the Belmont. It never happened, and Bold Ruler's line will be carried on by his great grandson Seattle Slew who also won the Triple Crown and is the sire of this year's Belmont winner A.P. Indy.

In conclusion, the restricted gene pool of thoroughbred race horses makes it unlikely that we will see another Secretariat in the near future. However, bacteria do not suffer from the same constraints. They can exchange genetic information naturally and man can carry this further by 'targeting' specific gene acquisition and amplification. It is the techniques of recombinant DNA technology coupled with the capabilities of emerging young scientists that lead me to believe that we have only seen the tip of the iceberg in terms of the use of microorganisms for bioremediation processes, and the synthesis of specialty chemicals and chiral pharmaceutical products. The future success of biodegradation/biotransformation processes is assured. You could, and I would, bet on it.

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