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Biocatalysis, biodegradation and bioinformatics

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Biocatalysis, biodegradation and bioinformatics are prominent scientific fields in industrial microbiology and biotechnology. This paper describes developments in these fields with a focus on the role of David T Gibson as a researcher and mentor. He has pioneered studies on the mechanisms by which aerobic microorganisms transform aromatic hydrocarbons. In addition, his research has served as a model for further investigations into bacterial atrazine and dichloromethane catabolism described here. Microbial catabolism research requires information on organic chemistry, microorganisms, metabolic pathways, catabolic genes, and enzymes. These information needs are now being met more comprehensively by development of the University of Minnesota Biocatalysis/Biodegradation Database.

http://dragon.labmed.umn.edu/~lynda/index.html

The database is built on the ideas championed by David Gibson that a knowledge of microbial catabolic reactions should be organized in a mechanistic fashion and in a systematic format.

Keywords: biocatalysis; biodegradation; informatics; dehalogenation; oxygenases

Introduction

Sir Cyril Hinshelwood, a Nobel Prize winner in Chemistry, was the thesis advisor of Stanley Dagley who, in turn, directed David Gibson in his thesis research. In his book *The Structure of Physical Chemistry* [16], Hinshelwood stated, 'In its present stage of evolution, chemistry compromises between the abstract principle and the naive pictorial hypothesis.' This portrayed the state of chemistry earlier in this century, a period from which it has matured, in part due to the insights provided by Professor Hinshelwood. If he were alive today, Sir Cyril might offer a similar assessment regarding the state of knowledge about biodegradation, the area of research in which David Gibson and his colleagues currently work. We draw pictures of chemical compounds in various stages of catabolism and try to derive general principles that govern the enzymes and genes that underlie the process. But with over 8 million organic compounds known, it is essential, for clean commerce and academic scholarship, that we collectively learn to predict how new compounds will be biodegraded. To date, this goal is at best partly fulfilled.

Greater success in predicting biodegradation pathways for new compounds will require extending the knowledge that has accrued over the last decades. This paper describes some of the pioneering work of David Gibson and his coworkers that has begun to build the framework for a systematic organization of biodegradation information. It is organized as a series of general precepts for conducting research, as practised by David Gibson and his coworkers, that has guided the author's scientific career. Under each principle is given an example of some aspect of biodegradation that was elucidated by adhering to that princple. In this way, I acknowledge the many contributions of Professor Gibson to this area of science.

Principle 1: Acknowledge ignorance

To continually progress in science, it is important for the scientist to periodically confront his/her state of ignorance. The most exciting new discoveries lie at the fringe between knowlege and ignorance. So one must look into the abyss with one's feet planted firmly in the known, and know when to jump. Science seems, in this context, like a risky business!

But defining what isn't known, and what might never be accomplished, can help one define where to most productively focus one's efforts. For example, consider the biodegradation of organic compounds in its totality. Despite many elegant studies over the last half century, the surface has only been skimmed. Over 8 million organic compounds have been discovered or synthesized by organic chemists and the pace of new synthesis far exceeds the rate at which new bacterial catabolism is elucidated. Of the 8 million organic compounds, perhaps 10 000 have been studied in biodegradation research of any type, and there are approximately 100 compounds for which genes, enzymes, and detailed mechanisms for at least some part of the catabolic pathway are known.

A recognition of the 'Compound-Metabolism' gap, and thus the extent of our ignorance, leads microbiologists to the only practical method for closing the gap. It cannot be done by randomly obtaining information on the metabolism of organic compounds. Rather, it necessitates learning the biochemical strategies underlying the metabolism of particular organic functional groups. As this knowledge

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expands, it becomes increasingly possible to predict the metabolic fate of compounds for which laboratory research has yet to be conducted. David Gibson's laboratory has contributed significantly to our current understanding of the bacterial mechanisms of oxygen fixation into hydrocarbon substrates, particularly aromatic hydrocarbons. This focus on the mechanisms of biodegradation provides the organizational basis of the University of Minnesota Biocatalysis/Biodegradation Database on the World Wide Web [31] which will be discussed in more detail below. To develop such a database, it is critical to acknowledge that we will remain ignorant of biodegradation in its totality, yet we can progress by revealing underlying mechanisms.

Principle 2: Research deeply

In 1964, a mechanistic knowledge of bacterial aromatic hydrocarbon metabolism was limited and fragmentary. It was presumed that the initiating reactions in bacteria were the same as the well-established monooxygenase-catalyzed reactions observed to occur in mammalian systems [2]. Mammals oxidize benzene ring compounds to epoxides and subsequent hydration yields *trans*-diols. David Gibson's laboratory at the University of Texas at Austin firmly established that bacteria largely oxidize aromatic hydrocarbons to *cis*-diols via dioxygenase enzymes [13,15] This is the prelude to complete metabolism of the aromatic nucleus: oxidation of the *cis*-diol to a corresponding catechol, ring cleavage of the catechol, and metabolism of the resultant aliphatic intermediates via the tricarboxylic acid cycle [7,8,9,15].

Years of research by the Gibson laboratory established dioxygenation as the paradigm for aerobic bacterial aromatic ring metabolism. Fungi, by contrast, were shown to oxidize aromatic hydrocarbons via monooxygenation and derivitization reactions that were reminiscent of mammalian metabolism [4,5]. These observations led to the proposal of using fungi as models for mammalian metabolism of aromatic hydrocarbon carcinogens and drugs [26].

It is typical of research that extends broadly and deeply that the Gibson laboratory broke the paradigm they had established by discovering a bacterium, *Pseudomonas mendocina* KR1, that catalyzes the monooxygenation of toluene to yield *para*-cresol [34]. Subsequently, other bacterial monooxygenation reactions were discovered that transform toluene to *ortho*- and *meta*-cresol, respectively. Toluene 4-monooxygenase [25] and toluene 2-monooxygenase [24] have now been purified and shown to resemble soluble methane monooxygenase in that they contain a binuclear iron center. In the last 10 years, the versatility of aromatic hydrocarbon dioxygenases has become appreciated by Gibson and his coworkers to include the ability to catalyze monooxygenation [32] and desaturation [14] reactions. Fox and coworkers have shown that methane monooxygenase, xylene monooxygenase, and fatty acid desaturase contain structurally and spectroscopically similar binuclear iron centers [12]. Thus, the divisions between dioxygenases, monooxygenases, and desaturases come down to active-site details that were not even the subject of speculation 30 years ago.

In my laboratory at the University of Minnesota, we also follow the precept that research should go deeply and published work should be met with some skepticism. This has guided us in studies on bacterial metabolism of the herbicide atrazine. Thirty years of research publications had nearly enshrined the idea that bacteria metabolize atrazine via oxidative elimination of the alkyl side chains [11,29]. Analogous monooxygenase-catalyzed oxidative dealkylation was known to occur in the liver of mammals. Hydroxyatrazine had been observed in soils, but was attributed to soil-catalyzed abiotic dehalogenation [11,17]. *Pseudomonas* sp ADP, isolated from an atrazine spill site, does not produce dealkylated metabolites [23]. Molecular cloning of *Pseudomonas* atrazine genes in *E. coli* helped reveal that the organism initiated metabolism by a hydrolysis reaction yielding hydroxyatrazine (Figure 1) [10]. Since then, other atrazine-degrading bacteria have been identified as containing the gene responsible for hydroxyatrazine formation (deSouza, Sadowsky and Wackett, unpublished data) and we believe that this compound is largely formed in soil biologically. Further studies have elucidated the metabolic logic underlying bacterial atrazine metabolism that proceeds through cyanuric acid, a readily metabolizable compound for many soil bacteria [6].

Principle 3: Simplify the system

Or, as Arthur Kornberg would say, 'Purify, purify, purify' [19]. The biodegradation literature is full of *in vivo* studies that yielded highly equivocal results. In some of those cases, biochemical fractionation of the system under study would have allowed the experimentalist to simplify the system and obtain clear-cut results. This typically requires that the respective gene be identified and cloned or the responsible enzyme be purified to homogeneity. This has paid unexpected dividends in the case of atrazine chlorohydrolase (AtzA), the enzyme catalyzing atrazine hydrolysis to hydroxyatrazine (Figure 1).

The reaction catalyzed by AtzA has now been studied in some detail [10]. AtzA converts atrazine to hydroxyatrazine via a hydrolysis reaction, as demonstrated by showing incorporation of [^{18}O] from [^{18}]O-H_2O into the hydroxyl group of the product. The enzyme is relatively specific; only substrates containing a chlorine atom and an alkylamino side chain are hydrolyzed. Melamine and terbuthylazine are not substrates for AtzA. In steady-state kinetic studies with atrazine, the substrate saturation curve is hyperbolic and the derived K_M and V_{max} are calculated to be 150 μM and 2.6 μmol per min per mg protein, respectively. Based on a holoenzyme molecular weight of 245 000, the k_{cat} is 11 s^{-1}.

Studies on the properties of AtzA are relevant to its

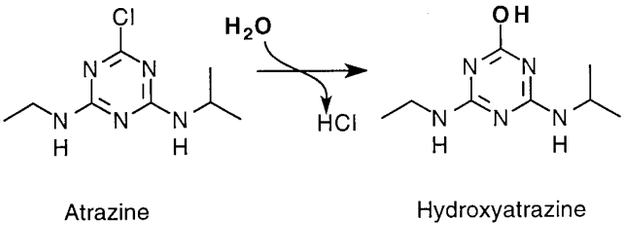

Figure 1 The hydrolysis reaction catalyzed by purified atrazine chlorohydrolase (AtzA).

potential application in treating atrazine-contaminated drinking water supplies. The product of the AtzA-catalyzed reaction, hydroxyatrazine, is non-phytotoxic and is not known to be toxic or carcinogenic to mammals. Thus, the first metabolic step carried out by *Pseudomonas* sp ADP represents the best possible step from an environmental remediation standpoint. While the intact organism will metabolize atrazine to carbon dioxide and ammonia, the enzyme could prove more efficacious for the following reasons: (i) *Pseudomonas* sp ADP makes only a modest level of AtzA because cell nitrogen needs are much lower than carbon requirements; (ii) *atzA* expression is down-regulated by inorganic nitrogen sources that are present in most contaminated waters; and (iii) enzyme can be produced cheaply and in large quantity by recombinant bacteria. There are no regulatory constraints against adding a small amount of proteinaceous, non-living material that effectively catalyzes the hydrolysis of a highly regulated compound (atrazine) to a non-regulated compound (hydroxyatrazine). Studies are ongoing to explore the use of highly active AtzA in the remediation of atrazine-contaminated water.

Principle 4: Apply organic chemistry liberally

Biodegradation, by definition, involves the transformation of organic compounds, typically yielding thermodynamically simpler compounds. Whether one focuses on enzymes, genes, or ecological interactions, it is nearly inescapable that one must, at some point, elucidate the structure of compounds generated by microbial metabolism. Because of this, the experimentalist who pays attention to organic chemistry will typically be able to conduct biodegradation research more broadly and deeply. Below is an example of how a knowledge of organic chemistry was instrumental in revealing details of bacterial growth on the solvent dichloromethane.

All well-studied aerobic dichloromethane-degrading bacteria are methylotrophic bacteria which contain a glutathione-dependent dichloromethane dehalogenase [21]. Dichloromethane dehalogenase converts CH_2Cl_2 to formaldehyde which the bacteria can assimilate for carbon and oxidize to carbon dioxide to generate ATP. It was established that dichloromethane dehalogenase requires glutathione for activity but that glutathione was not consumed in the reaction [18]. This observation was consistent with the intermediacy of *S*-chloromethylglutathione which undergoes hydrolysis to glutathione, formaldehyde and chloride (Figure 2). However, this intermediate is too unstable to observe directly and so the mechanism remained hypothetical. In fact, the Enzyme Commission had characterized the enzyme as a lyase rather than a glutathione *S*-transferase. Subsequently, sequence information indicated low but significant amino acid identity between dichloromethane dehalogenase and mammalian glutathione *S*-transferases [20]. Based on the prevailing knowledge of mammalian glutathione *S*-transferase reaction mechanisms, the hypothesized halomethylglutathione intermediate thus gained some experimental support.

The issue was resolved via experiments that were based on knowledge obtained from the organic chemical literature. It is known that fluoromethylthioethers are orders of

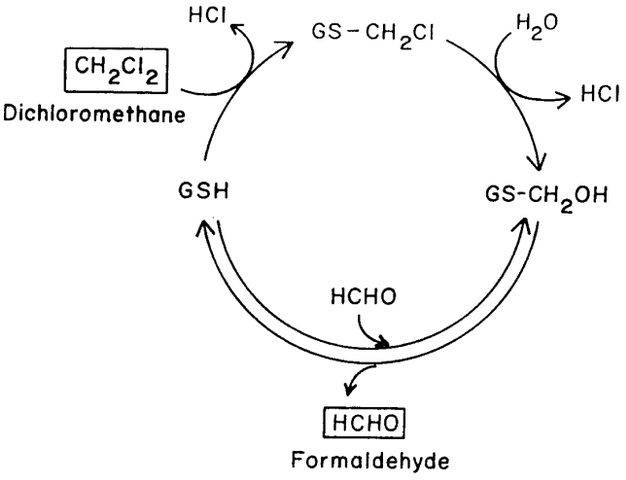

Figure 2 The mechanism of enzymatic dichloromethane dechlorination first proposed by Thomas Leisinger and his coworkers.

magnitude more stable than their chlorinated counterparts [28]. In this context, chlorofluoromethane was used as a substrate for dichloromethane to probe the proposed mechanism. The substrate modification had to be subtle because dichloromethane dehalogenase is active only with dihalomethanes. As shown in Figure 3, a fluoromethyl-enzyme intermediate may be stabilized, as would *S*-fluoromethylglutathione, but the direct hydrolysis product fluoromethyl alcohol would decompose very rapidly to formaldehyde and fluoride. Thus, with one substrate, one can test directly for three possible alternative intermediates along the enzyme reaction pathway. When the experiment was conducted, only *S*-fluoromethylglutathione was detected and it was shown to accumulate to a steady state level by ^{19}F-NMR [1]. It subsequently decomposed to formaldehyde and fluoride. All of the fluoride produced by the reaction could be accounted for by this reaction pathway. These data are completely consistent with the mechanism proposed by Kohler-Staub and Leisinger [18] in which the enzyme catalyzes a reaction between the glutathione sulfur atom and the carbon atom of dichloromethane. This was the first example of a bacterial glutathione *S*-transferase being involved in the metabolism of chlorinated compounds. More recently, they have been implicated in the metabolism of pentachlorophenol, polychlorinated biphenyls (PCBs), and the herbicide 2,4,5-trichlorophenoxyacetic acid [2,4,5-T) [30].

Principle 5: Always embrace new methods (or Biodegradation on the World Wide Web)

Methods of research are continually changing. However, the recent advent of the World Wide Web portends even

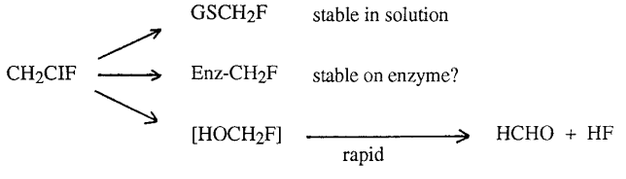

Figure 3 The respective reaction intermediates derived from CH_2C1F via different hypothetical mechanisms of dichloromethane dehalogenase.

greater changes in the way scientists share information. Electronic publishing can make information more accessible, one doesn't have to be at a center of learning to partake of the knowledge infrastructure. There is opportunity for more immediacy in communicating findings. This will be a positive development if the benefits of peer-review are retained in a faster electronic publishing process.

It is already apparent that the development of databases for sequence and protein structural information, and their availability via the World Wide Web, have accelerated the pace and quality of molecular biological research. In this context, the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD), freely available on the World Wide Web, was established as a resource for the international biodegradation research comunity [31].

http://dragon.labmed.umn.edu/~lynda/index.html

The UM-BBD contains information on chemical compounds, their respective microbial catabolic pathways, catabolic genes, catabolic enzymes, and microorganisms. The UM-BBD is both useful and used as indicated by the current monthly average of 37000 accesses. The users of the UM-BBD represent 75 countries on six continents.

Under Principle 1, it was pointed out that it is impossible for studies of microbial catabolism to keep pace with the discovery of new chemical compounds. Thus, there exists an ever-increasing Compound-Metabolism gap. This reality has important implications with regard to the development of environmentally-friendly industrial chemicals and that, in turn, requires a knowledge of their biodegradation. Furthermore, government regulatory agencies will increasingly be confronted with ruling on the environmental fate of chemicals for which direct biodegradation data are unavailable. This gap can only be filled by using a broad-based knowledge of biodegradation to predict the fate of new compounds for which direct data are missing. As discussed earlier, this necessitates an understanding of metabolic logic and the compilation of a wide information base on compounds for which microbial metabolism has been studied. The UM-BBD offers a mechanism for deciphering the patterns of bacterial catabolic metabolism that can be used for predicting the course of biodegradative metabolism.

The UM-BBD can aso contribute to solving problems in the metabolic engineering of microbial pathways for biotechnology. Metabolic engineering has become increasingly tractable because of new techniques in molecular biology but its ultimate success requires a workable metabolic blueprint. This requires an encyclopedic knowledge of microbial metabolism, or extensive library research. The UM-BBD can thus impact metabolic engineering by providing the information to construct many blueprints and to assess their feasibility. Using the information encoded in the UM-BBD, appropriate software could be used to ask questions of a computer as to how metabolic pathways might be constructed to go from starting compound X to end-product Y. One could further investigate the thermodynamic feasibility of that metabolism and whether genes encoding the appropriate enzymes have been cloned in a given host strain. It is likely that a computer could investigate many alternative pathways very rapidly ensuring that one has the best possible plan before heading to the laboratory.

As a relatively simple example, we constructed a two-step metabolic pathway for the metabolism of pentachloroethane to formate and glyoxylate to demonstrate the feasibility of transforming a highly chlorinated compound to non-chlorinated, non-toxic endproducts [33]. This required the simultaneous expression of seven genes encoding two functional enzyme systems. The plan was based on the knowledge that enzyme system 1, encoding cytochrome P450cam, was capable of reductive dechlorination of chlorinated alkane solvents [3], in this case reducing pentachloroethane to trichloroethylene. Enzyme system 2, toluene dioxygenase, that was first purified by Gibson and coworkers [27], oxidizes trichloroethylene to formate and glyoxylate [22]. The recruitment of these enzymes to construct a dechlorinating pathway required a knowledge of their fortuitous enzymatic reactions. This is precisely the type of information that can be, over time, compiled on a World Wide Web database and provide the building blocks for the next generation of novel metabolic pathways.

The compilation of information, and its dissemination, sounds a lot like education and the UM-BBD has been used instructionally in several courses at the University of Minnesota. This all important aspect of the UM-BBD is discussed below.

Principle 6: Always be a teacher

Aristotle said, 'Teaching is the highest form of understanding.' This sentiment provides the underpinnings of the modern research University where research and teaching go hand in hand.

At the University of Texas at Austin, David Gibson taught a course called Microbial Transformations. While it included some of the subjects of his laboratory's research, Microbial Transformations roamed broadly into many areas of science: the biogeochemical basis of fossil fuel biogenesis, microbial ecology, and molecular evolution. It allowed the student to see the interconnectedness of diverse disciplines and the richness of microbial molecular sciences.

The University of Minnesota has also had a strong tradition of teaching in Microbial Biochemistry. For many years, Regent Professor Stanley Dagley taught Minnesota students the metabolic *logic* of biodegradation. The word logic is emphasized because it was most important that the students learned to reason how microbes use chemical principles to their advantage for optimally obtaining carbon and energy via biodegradative metabolism.

A new course, 'Biocatalysis and Biodegradation', is now being offered by Professor Lynda Ellis and the author at the University of Minnesota.

http://biosci.cbs.umn.edu/class/bioc/5309/

It is being taught completely over the Internet to an international student body. The course draws on the extensive amount of information now available on the World Wide Web including, but not limited to, the University of Minnesota Biocatalysis/Biodegradation Database. Continuing in the Dagley tradition, 'Biocatalysis and Biodegradation' does not focus on merely presenting information on biodegradation. In fact, with the increasing availability of data,

easily obtained on the Web, information is increasingly cheap, but the skills of information processing are more at a premium than ever. A centerpiece of the course is a discussion of the principles of biodegradation reactions. The logic follows that of the Periodic Table of the Elements, which has served as the unifying scheme for understanding the reactivity of chemical compounds. Prior to the Periodic Table, discovery in chemistry was a mining operation; discovery had a strong component of chance. With the advent of the Periodic Table, chemistry had the theoretical underpinnings to develop much more predictive capabilities. This, without a doubt, was instrumental in accelerating the progress of chemistry.

Biodegradation is, in terms of maturity, somewhat like the discipline of chemistry one century ago. The compound-metabolism gap discussed above mandates that biodegradation research cannot profitably advance by only discovering new biodegradative reactions one at a time. Real progress in the discipline must come from elucidating the patterns of biodegradative reactions that provide the capability to predict the pathways for biodegradation of millions of organic compounds. In research and teaching, this must be our collective goal.

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