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MICROBIAL ADAPTATION TO PESTICIDES

Prepared for publication by

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Microbial adaptation to presticides

Abstract - Edaphic and genetic processes influence the rate of microbial adaptation to pesticides and subsequent metabolism of these pesticides in soils. Availability of the degradable molecule to soil microorganisms is determined by a number of competing sorption reactions with soil colloids. Kinetic studies on readily biodegradable herbicides exhibit an initial lag phase followed by a rapid decline in concentration. A number of genetic alterations can occur in bacteria responsible for degradative enzyme synthesis during the lag phase. In addition to specific internal DNA sequence changes and recombinations, external DNA additions can occur via plasmids between various soil bacteria. Degradative plasmids for the herbicides 2,4-D and dalapon have been isolated and characterized. There are interesting parallels between the development of antibiotic resistance in the clinical environment and enhanced pesticide metabolism in problem soils.

INTRODUCTION

Microbial metabolism is an important process for degrading pesticides in the soil environment. Biochemical research on pesticide metabolism over the last three decades has primarily been aimed at identifying the microorganisms, metabolites, and enzymes associated with a specific pesticidal compound. With advances in modern microbial genetics, some insight is developing into the evolutionary events that occur during the pesticide adaptation process. While the exact mechanisms for pesticide adaptation are not completely understood, a review of some of the current research on the genetic alterations that are known to occur with the introduction of pesticides and other xenobiotic substances into a soil microbial community should be informative to the pesticide chemist. The purpose of the review is to acquaint chemists primarily concerned with pesticide metabolic research with some of the concepts being developed on the role of DNA in regulating the metabolism of pesticides by soil microorganisms.

The current review is limited primarily to herbicides and will examine research on edaphic factors affecting biodegradation; the herbicide adaptation process in soils; cross adaptation and problem soils; microbial genetics and evolution with emphasis on the role of extrachromosomal elements, i.e., plasmids, viruses, transposons and insertion sequences; and adaptive evolution using herbicides, xenobiotics, and antibiotic resistance as examples.

Soil parameters affecting biodegradation

When a biodegradable herbicide moves into the soil environment, a number of competing reactions may alter the availability of the molecule in the solution phase. An understanding of these competing reactions is necessary since they will ultimately influence the concentration and consequently the microbial adaptation and metabolism processes. The rate of microbial breakdown of organic herbicides in soil-water environments has been shown to depend on its concentration (Ref. 1 & 2) and its availability (Ref. 3 & 4) to the microbial community. Soil organic matter is the principal component governing the availability of pesticides to microbial metabolism. Several mechanisms have been proposed for adsorption of pesticides to soil organic matter (Ref. 5). Two or more of the following mechanisms may occur simultaneously depending on the chemistry of the pesticide molecule and the organic matter surface: Van der Waals forces, hydrophobic bonding, hydrogen bonding, ion exchange, and ligand exchange. Many of the biodegradable herbicides are acidic and adsorption depends on the pH of the system. The magnitude of adsorption of acidic pesticides, such as 2,4-D, by soil organic matter, however, is much lower than that of cationic or basic pesticides (Ref. 6). More important are the organic matter-clay complexes in most mineral soils, and Khan (Ref. 5) points out that these complexes must be considered in evaluating pesticide adsorption. As a general rule, in a soil with an organic matter content up to about 6%, both mineral and organic surfaces are involved in adsorption, but at higher organic matter contents most adsorption will occur on organic surfaces (Ref. 7).

At very low concentrations, little mineralization of 2,4-D occurred when this herbicide was present in aqueous systems at initial concentrations of 2 to 3 ng/ml (2-3 ppb) or less, while 60% or more of the chemical initially present at higher concentrations was converted to CO_2 in 6 days (Ref. 1). Apparently microorganisms did not assimilate the carbon derived from metabolism of 2,4-D at low concentrations.

A study of the relationship between the availability of the herbicide diallate to organisms or enzyme systems responsible for metabolism, the number or biomass of microorganisms capable of degrading diallate, and the activity or physiological status of the organism has

appeared in the literature (Ref. 4). A direct correlation was observed between the soil biomass and rate of diallate degradation in 11 soils. Even in soils with a large biomass, however, the biodegradation of diallate was decreased when the microbial activity was lowered by reduced temperatures. The availability of diallate for metabolism was strongly affected by adsorption, measured as Freundlich K values in the same 11 soils. Rapid metabolism was observed only in situations of high biomass and high availability (low K value). This led to the conclusion that, for a biodegradable herbicide such as diallate, the rate of degradation was a complex function interrelating the size of the biomass in the soil, the biochemical activity of that biomass as affected by a number of physiological factors, i.e., temperature, moisture, nutrient status, and others, and the availability of the chemical to the biomass.

An appreciation of the complex physicochemical environment surrounding the biodegradable herbicide molecule in soils is necessary if one is really attempting to describe the metabolic process *in toto*. Sorption processes that tenaciously bind primary metabolites, such as the chlorinated anilines, can significantly alter the mineralization rate of these important products.

Herbicide adaptation processes in soils

Some of the earliest work on microbial adaptation to herbicide molecules was conducted on the phenoxyalkanoic acid herbicides, which include 2,4-D, 2,4,5-T, and MCPA. The early evidence that microbial detoxification in soils was a major degradative process for the phenoxyalkanoates has been reviewed by Loos (Ref. 8). Stimulation of 2,4-D decomposition by warm, moist soil conditions and by additions of organic matter; the correlation between the rate of 2,4-D degradation and buildup of aerobic soil bacteria; and the inhibition of degradation in air-dried and autoclaved soil all provided early evidence that the degradation process was mediated by soil microorganisms.

The now classic works of Audus (Ref. 9), with soil enrichment techniques using herbicide substrates as a nutrient source, did much to elucidate the kinetics of the adaptive process. The course of substrate disappearance was followed in soil perfusion experiments. Initially there occurred a small but fairly rapid decrease in the herbicide concentration due to soil sorption processes. This phase was followed by a lag phase, during which no appreciable change in herbicide concentration was observed. The length of this lag phase varied considerably between compounds. A period of rapid substrate disappearance followed the lag phase. Upon subsequent additions, the same substrate or a structurally related substrate disappeared rapidly without a lag phase and generally obeyed first-order kinetics. The changes in concentration of a biodegradable herbicide as a function of time are shown in Fig. 1.

Lag phases have been observed in enrichment cultures for several biodegradable herbicides, including dalapon (Ref. 10) and a number of aniline-based herbicides including propanil, propham, solan, and swep (Ref. 11). Soil type and pH were two edaphic factors that have been shown to influence the length of the dalapon lag phase, which varied from roughly 5 to 40 days. The lag phase for the aniline-based herbicides varied from only 2 to 5 days and was shown to depend on chemical structure and soil type.

These adapted soil microbial populations have two important agronomic implications. First, soil organisms induced to metabolize a single, biodegradable herbicide generally exhibit a fairly broad substrate specificity, as demonstrated with the phenoxys (Ref. 8), acetamides, acylanilides, carbamates, toluenes and phenylureas (Ref. 11), and halogenated aliphatic

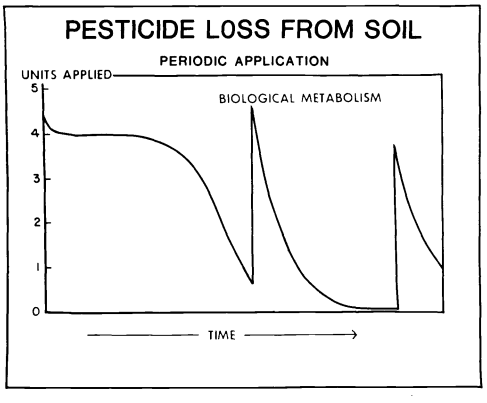

Fig. 1. Kinetics of pesticide biodegradation.

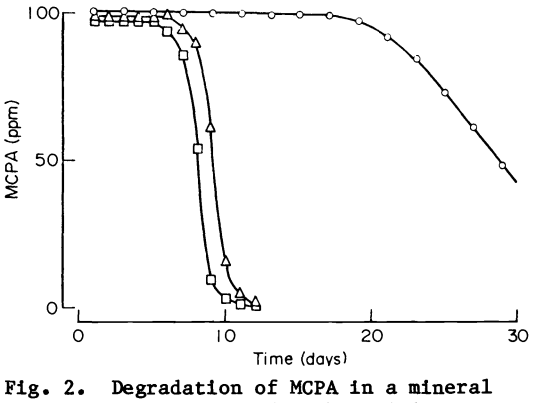

Fig. 2. Degradation of MCPA in a mineral salts medium inoculated with soil from the field experiment with annual application of MCPA for 0 (o), 1 (Δ), and 18 (□) years. Samples were taken 9 months after the preceding application.

acids which would include dalapon (Ref. 10 & 12). This modestly wide substrate specificity has important implications for "problem" soils discussed in a subsequent section. Second, soils that have been microbially enriched to decompose a particular phenoxyacetic acid retain this degradative ability for long periods (Ref. 8). For example, the ability to degrade 2,4-D rapidly without a lag period persists in soil in the absence of 2,4-D for at least a year after the initial 2,4-D treatment. Under field conditions, increased ability to degrade 2,4-D after previous treatment with the herbicide has been reported by Newman and Thomas (Ref. 13), Newman, Thomas and Walker (Ref. 14), Thornton (Ref. 15) and Hurle and Rademacher (Ref. 16). Similar field evidence exists for repeated applications of MCPA (Ref. 15, 17, 18, & 19). One field study in which 2,4-D and MCPA were applied to the same soil annually for 18 years clearly demonstrated the progressive metabolism of these two herbicides with time (Ref. 20). Repeated applications of the two herbicides resulted in a reduction in degradation time from 10 weeks for 2,4-D and 20 weeks for MCPA after one application to 4 to 7 weeks, respectively, after 19 years of annual application. An assay of field soils treated with MCPA from the same experiment is shown in Fig. 2. The persistent nature of the gene pools responsible for encoding degradative enzyme synthesis has important agronomic implications in the performance of the herbicide against its target host. This is another facet of the "problem" soils discussed in a subsequent section.

Biomass

Generally, large population increases in herbicide-degrading organisms occur in laboratory studies with treated soils during the lag phase. The number of dalapon (Ref. 21) and propanil (Ref. 22) metabolizing microorganisms increased significantly, as determined by the most-probable-number method, after incubation in five soils. Likewise, large population increases occurred in soil perfusion units incubated with the phenoxy herbicides (Ref. 9). It was also observed that untreated soils with high microbial biomasses almost always degraded the added herbicides more rapidly than untreated soil with low microbial activity.

In untreated field soils, the number of 2,4-D-degrading microorganisms was found to be low, ranging from 1 to 245 per gram of soil (Ref. 8). In contrast, the number of dalapon and propanil degraders in untreated soil was substantial, ranging from 1000 to 130,000 cells per gram for dalapon and, with one exception, ranged from 150 to 121,000 for propanil in five soils.

Regardless of the initial numbers, the population of these herbicide-degrading organisms in soil has to attain some critical size to degrade the herbicide at a reasonably rapid rate. At least part of the lag phase, then, in herbicide-amended soils, particularly in soil perfusion studies where massive substrate levels are initially present, would be the time required for the population to achieve this critical level. Once this level is obtained, subsequent additions of the same herbicide, or structurally related herbicides, are degraded without a detectable lag. Thus, the environmental flux caused by the selective pressure of the added chemical on the soil microbial population initially results in an increase in biomass of the degradative strains, which may have been previously triggered by some rapid genetic alteration at the DNA level.

If we reexamine Fig. 1 and consider only the initial lag phase and decline, an imaginary line can be drawn vertically through the graph, separating it into two periods (Fig. 3). The second period to the right of this imaginary line is the period of rapid metabolism, the area where most of the metabolic research has been conducted over the last 3 decades. Events during and after the rapid metabolism period are primarily where research on the identification of organisms, enzymes, and metabolites has occurred. The area to the left of the imaginary line is of primary concern to the present review. This period is characterized by rapid evolutionary events at the microbial level and the physicochemical events that affect the availability of the molecule. The section titled "Microbial Genetics and Evolution" will consider this period in greater detail.

Cross adaptation and problem soils

The adaptation process, as portrayed in Fig. 1, is a reproducible response that has held interest for the soil microbiologist trying to isolate pure cultures of soil organisms for a specific compound or class of compounds. The ability of adapted soil microorganisms to metabolize closely related structural analogues of the inducer compound was early recognized and referred to as cross adaptation (Ref. 9). There is now mounting evidence that cross adaptation is not only confined to idealized conditions usually encountered in soil perfusion columns under laboratory conditions, or to the more biodegradable herbicides found in certain soil situations, but may be an agronomic problem of yet unknown dimensions encompassing a large number of compounds previously not suspected of being involved in cross adaptative processes.

In certain geographic regions it has been observed that a number of soil-applied pesticides are failing to control their target pests. Soils in which this occurs are referred to as "problem" soils. The term "aggressive" soils or enhanced metabolism is also associated

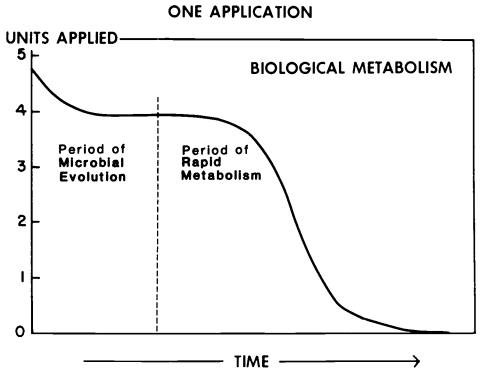

Fig. 3. Periods of evolution and rapid metabolism of a biodegradable pesticide.

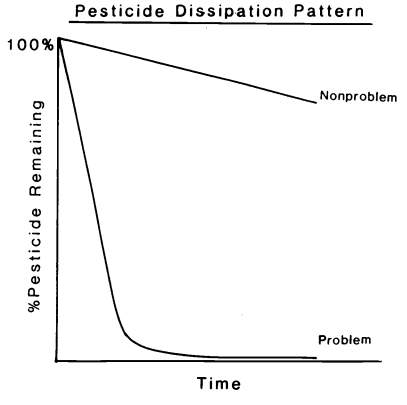

Fig. 4. Kinetics of pesticide biodegradation in problem and nonproblem soils.

with this process. All of the parameters responsible for the evolution of problem soils are not clearly understood, including pest resistance and population pressures, but the development of soil microbial populations extremely efficient in degrading these pesticides is now recognized as a major contributing factor. A typical and highly stylized response showing the kinetics of breakdown in problem and nonproblem soils is shown in Fig. 4.

A number of theories have been advanced to account for the problem soil phenomenon (Ref. 23) and include:

1. The repeated use of the same pesticide annually on the same soil.
2. The chemical similarities of several pesticides used on the same crop.

There is also speculation that the replacement of the more persistent, less degradable chlorinated hydrocarbon pesticides, in the case of the insecticides, for the more biodegradable alternatives may be a contributing factor.

Problem soils were first encountered with the thiocarbamate herbicides (Ref. 24 & 25). The similarity of many structurally related agricultural chemicals suggests that one pesticide may induce enzyme(s) capable of degrading a chemically related compound added simultaneously or subsequently to the same soil. The chemical linkages in pesticides that appear to be susceptible to cross adaptation in these problem soils include the carbamate -N-CO-O, urea N-CO-N, amide N-CO-C, ester COO-C, thiocarbamate N-CO-S, and dithiocarbamate N-CS-S bonds. The appearance of rapid microbial metabolism of these compounds in soils treated previously, and in some cases several years previously, again tends to emphasize the persistent nature of the responsible genetic elements in nature. Research is currently in progress to determine what agronomic practices or inhibitors are necessary to protect this potentially large number of pesticidal compounds from further losses in effectiveness.

MICROBIAL GENETICS AND EVOLUTION

Microbial studies in the past 3 decades have demonstrated the role of soil microorganisms in the degradation of herbicides. Biochemical genetics has shown that the code for degradative enzyme synthesis is contained on DNA molecules in soil organisms. The single cell or prokaryotic bacterium contains a single chromosome or genophore. Movable genetic elements may alter the DNA sequences and gene expression in prokaryotes. Genetic alterations (evolution) that occur in response to environmental flux, such as the introduction of a herbicide into the soil microbial community, take many forms. Unfortunately, the specific alterations that occur early in the herbicide adaptation process have not been examined *per se* and analogies must be drawn from other biochemical systems. In addition to specific internal DNA sequence changes (mutations) and recombinations, external DNA additions can occur, i.e., through gene duplications and extrachromosomal additions (Ref. 26, 27 & 28). It is this latter type that can account for very rapid genetic modification and response to environmental flux. These extrachromosomal alterations are discussed with respect to herbicide degradation.

Extrachromosomal elements

ECE's (extrachromosomal elements) are defined as additional genetic elements external to a cell's chromosome(s) (Ref. 27, 29, 30 & 31). ECE's contain peripheral genes (nonessential) while chromosome genes contain basic housekeeping genes (mostly essential), and this has proved to be a highly successful adaptive strategy amongst the prokaryotes.

Although ECE existence in eukaryotes has been demonstrated, e.g., control elements of maize (Ref. 32 & 33), 2 micron circle of yeast (Ref. 34 & 35), *Drosophila* elements (Ref. 36 & 37), and SV-40 virus in primates (Ref. 38), the genetic, ecological and evolutionary

importance in eukaryotes is less well understood. Many ECE's can be classified into insertion sequences, transposons, plasmids, bacteriocins, and viruses and are found widely (Ref. 39). Figure 5 illustrates these components with an example ECE, i.e., degradative plasmid.

Plasmids. Plasmids are small covalently closed circular DNA polymers, carrying genetic information and ranging in size from small 2250 nucleotide-long minicircles to larger complex structures that may contain 400,000 nucleotide pairs. They are independently replicating minichromosomes that are obligate intracellular ECE's, i.e., they cannot exist outside of the cell. Plasmids can be divided into conjugative or nonconjugative classes based upon their ability to transfer themselves at time of conjugation (cell-to-cell fusion). Additionally, plasmids can be classified as to functional gene ability, e.g., resistances (antibiotics, heavy metals, ions), pathogenicity (toxins, enzymes, cell surface binding molecules), metabolic ability (degradation of carbohydrates, xenobiotics, etc.), or genetic definitions (conjugal functions, fertility control, host range). Many plasmids are of potential agricultural importance, e.g., Agrobacterium Ti plasmid or nitrogen-fixing plasmids/transposons (Ref. 40 & 41).

Viruses. Viruses (especially microbial viruses) are ECE's that can maintain a stable extracellular existence, primarily due to a protective protein coat (capsid). In soil, phage (bacterial viruses) can be found in concentrations of 10^{11} phage/g soil and can persist for long periods of time, similar to bacterial or fungal spores. Additionally, the common spore-forming bacterial genus, Bacillus, uses phage as a primary ECE gene transfer system (Ref. 42). It is not known which (or whether all) variables found in Bacillus lead to phage as the dominant gene exchange mechanism. These variables are: Thick cell wall, which inhibits conjugation or plasmid transfer; a unique life cycle including a spore stage; a soil niche making conjugation difficult. These gram-positive associated variables would predict that other gram-positive soil organisms, such as Streptomyces, probably also use phage as a major genetic channel.

Transposons and insertion sequences. Insertion sequences (IS) are ECE's that contain no known genes unrelated to insertion function, while transposons are insertion sequences containing at least one expressible gene, e.g., antibiotic resistance (Ref. 43 & 44). Additionally, almost any gene can become transposable by incorporation into an appropriate IS. Transposons and insertion sequences are incapable of replicating autonomously and, thus, they are always found inserted into plasmid or chromosomal DNA. They encode unique transposase enzymes, which catalyze the insertion of copies of the transposon or insertion elements into other pieces of DNA. Thus, these discrete pieces of DNA are highly mobile and are found to pass freely between plasmids and chromosome in many species of bacteria. A representation of a transposon (TN) bounded by insertion sequences is shown in Fig. 6. Transposons and IS have been shown directly or indirectly to be responsible for the following chlorinated hydrocarbon degradations: MCA (monochloroacetic acid) (Ref. 45), 2-MCBA (2-chlorobutanoic acid) (Ref. 45), haloacetate (Ref. 46).

Several research projects are currently under way to determine whether the "problem" soil phenomenon discussed previously is mediated by ECE's. These studies are attempting to involve specific plasmid(s) with enhanced metabolism of selected thiocarbamate herbicides and methylcarbamate insecticides.

Extrachromosomal genetic elements and adaptive evolution in bacteria

Although evolution has been seen as primarily Darwinian, i.e., mutation and selection, for bacteria at least, adaptive evolution is generated as a response to environmental stress whereby rapid gene(s) alteration occurs via external gene recruitment (ECE's or gene duplication) and recombination. These processes can be viewed both at the macroevolutionary and microevolutionary levels.

Microevolution can be defined as rather small gene sequence changes while macroevolution is defined as large-scale evolutionary events that lead to insertion, deletion, or recombination of large DNA segments (Ref. 47 & 48). ECE's can affect evolution at the macro level via plasmids, viruses, and transposons and at the micro level via mutation, recombination, and IS and TN involvement, followed by subsequent selection. Macroevolution can be a faster response mechanism and also provides for larger DNA sequence alterations. The bulk of bacterial macroevolution appears to be due to ECE's (Ref. 27, 47, 49 & 50).

Positive microbial traits, such as pesticide degradation, most likely would come from ECE involvement due to both the short-term immediate stress (presence of toxic carbon source pesticide) as well as existent dissimilarity pathways (Ref. 51). The modular nature of ECE's provides an avenue for adaptive DNA experimentation, while the bulk of the cell's DNA remains conserved within the chromosome. Lastly, it should be pointed out that evolutionary alterations can occur both in regulatory sequences and in structural sequences. In fact, many genetic adaptations are initially regulatory, e.g., overproduction of a gene via regulation modification.

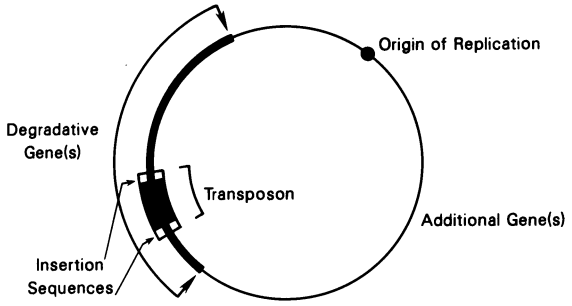

Fig. 5. A potential herbicide degradation plasmid.

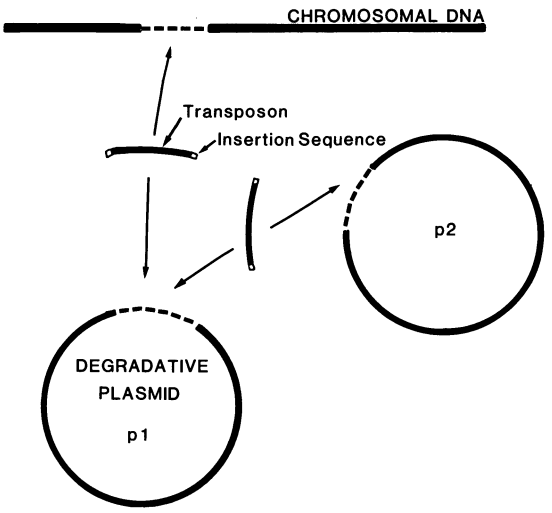

Fig. 6. Interrelationship between plasmids, chromosomes, transposons, and insertion sequences.

The amount of genetic information "present" in a species of bacterium exceeds that of a eukaryote (e.g., a yeast) since the ECE content or availability is very large. This means that a fast-growing bacterium can use advantageously both its simplicity and growth rate in the environment but at the same time call upon additional genes (via ECE and other microbial genetic reservoirs) to meet new selective pressures.

The exact mechanism underlying pesticide adaptive evolution is not known, but several opportunities exist for microorganisms. One, genetic alteration of the pesticide uptake mechanism (permease) would eliminate toxicity for sensitive strains; however, this would not enhance degradation unless the sensitive strains were involved in cometabolic events. Two, genetic changes would alter the toxic pesticide's targets within a cell and allow for tolerance to higher pesticide concentrations. Three, genetic changes would alter the specificities of existing enzyme systems so that the pesticide is degraded at a rapid rate and consequently the intracellular concentration does not reach toxic levels. This would probably occur within catabolic pathways that process pesticide analogs, e.g., aromatic catabolism for aromatic pesticides. This would lead to altered specificity of either a regulatory gene(s) or metabolic gene(s) or possible mutual alterations.

Pesticides not only present selective pressure via toxicity, but also offer a possible carbon and energy source to microorganisms (and in some cases sulfur, phosphorus, or nitrogen). Adaptive evolution of microorganisms probably combines both pressures, although the majority of reported work has been concerned with catabolic degradation rather than toxicity changes (Ref. 26, 51, 52 & 53).

Little will be said here about the impact of genetic engineering on pesticide chemistry; however, rather than relying on natural evolutionary events, directed evolution can now take place in the laboratory. By using recombinant DNA techniques and plasmids, e.g., plasmid-assisted molecular breeding (Ref. 54), modifications in existent pesticide genes can be performed as well as creating new pesticide genes. Techniques also are available for specifically changing the gene sequence of an isolated pesticide gene via <u>in vitro</u> mutagenesis.

The discovery of degradative plasmids for 2,4-D and dalapon (see section titled "Plasmid-associated herbicide enzymes") has important implications for the role of ECE's in the herbicide adaptative evolution process and the subsequent widespread dissemination of the responsible genes in the soil microbial community. Due to the dynamic nature and mobility of ECE's, large-scale DNA sequences can be lost, gained, transposed and inverted within and between bacterial cells. An example of this dynamic flux and mobility is shown in Fig. 6. Hypothetically, a gene originating as a segment of chromosomal DNA in one species of soil bacteria can translocate to a conjugative plasmid, and pass into the genetic material of a second species, where the gene may remain part of an ECE or migrate to a chromosomal locus. As pointed out by Davey and Reanney (Ref. 49), the number of permutations and combinations made possible by this blend of intermolecular and intercell transferability is virtually limitless. It is the integration of these mechanisms that forms an important component of the overall adaptative strategy of the bacterial cell.

The intermolecular-intercellular gene mobility provides one plausible explanation, in addition to natural gene longevity, for the persistent nature of the degradative capacity for certain herbicides in soils, as for example with MCPA in Fig. 2. It is possible that edaphic (e.g., pH or salinity) and climatic (thermal or moisture) stresses may suppress or virtually eliminate a selected degradative strain at a specific soil locale. The ability to transfer the degradative gene(s) to more resistant strains provides a mechanism for guaranteeing the survival of that degradative trait from year-to-year.

Antibiotic resistance: Important paradigms for pesticide science. After the initial findings of antibiotic resistance in clinical microorganisms in the period 1952-1967 (Ref. 55), several studies clearly demonstrated the evolution and spread of antibiotic resistance in clinical environments (Ref. 56-62). Within clinical environments, the nature of the selective stress is known, i.e., moderate to high doses of antibiotics. Additionally, the nature of the adaptive change can be seen where antibiotic resistance is encoded by one or more plasmids carrying one or more antibiotic resistance markers (consisting of either uptake modification or enzymatic detoxification of the antibiotic). Gene transfer of antibiotic genes has been demonstrated in clinical studies, laboratory studies, and even with *in vivo* studies, e.g., transfer from bacterium to bacterium on human skin at high frequencies within 24 hours (Ref. 56). It is interesting that the original recruitment source for these antibiotic resistance genes was from soil microorganisms, demonstrating the distance (phylogenetic and physical) that these ECE's can travel (Ref. 63).

With pesticide adaptive evolution, the selective pressure is negative (population elimination) since environmental concentrations are quite low, e.g., 1-100 ppm. However, this low concentration does offer a mild selective effect for microorganisms capable of utilizing pesticides as carbon and energy sources. Microorganisms that possess a moderate growth rate on many different carbon sources should be more successful in fluctuating environments (such as soil) compared to microorganisms that can catabolize fewer compounds but at greater rates, e.g., *Escherichia coli*, which is found in the more stable mammalian gut environment.

Enzyme evolution. Genetic experimentation at the enzyme level can be mediated by ECE's and a few examples will illustrate the principle.

Carbohydrate-utilizing enzyme evolution can be seen in the case of *E. coli*, where mutation of an enzyme (fucose isomerase) in the L-fucose pathway to constitutivity (not regulatory sensitive to pathway products) results in a new enzymatic capability, i.e., arabinose utilization (Ref. 64). This is a case of a small sequence change within a specific enzyme (microevolution).

With respect to pesticides, clear results by Knackmuss, Chakrabarty and colleagues show chlorobenzoate degradation evolutions, especially at the enzyme level (Ref. 65-68) where 3-chlorobenzoate pathway enzymes are altered such that other chlorobenzoates can be degraded, e.g., 4-chlorobenzoate, 3,5-dichlorobenzoate. This work is especially interesting here since it was performed with bacterial strains carrying plasmids coding for the genes involved in chlorobenzoate degradation. This is a case of DNA recruitment and relatively large rearrangements (macroevolution).

Plasmid-associated herbicide enzymes. Two recent reviews discuss in some detail the enzymes associated with pesticide metabolism (Ref. 69 & 70). It is beyond the scope of the current review to consider each of these enzymes in detail. The involvement of some of these enzymes with ECE's is now emerging. To date, two classes of enzymes that catalyze the initial reactions in herbicide biodegradative pathways are known to be encoded on plasmids. The herbicides include the halogenated aliphatic acids and the phenoxyalkanoic acids and the enzymes involved are the dehalogenases and oxygenases, respectively.

1 Dehalogenases

The dehalogenases are a unique, small group of enzymes that were first described about 10-15 years ago, but in which there has been considerable recent research interest. The enzymes are unique in that they catalyze the hydrolysis of halogen-substituted alkanoic acids, yielding either hydroxyalkanoic acids from monohalogenated acids or oxoalkanoic acids from dihalogenated compounds. Since halogenated compounds constitute a large number of biocides (although the alkanoic acids are a minor component of the herbicide family), they have generated interest among pesticide biochemists. For a general overview of this class of herbicides, the reader is referred to several reviews (Ref. 71 & 72). A more recent review on dehalogenases in soil bacteria has been prepared (Ref. 73).

The pesticides subject to catalysis by the dehalogenases are shown in Table 1.

1.1 Reaction mechanism. Two types of dehalogenases have been reported, haloacetic dehalogenase (EC 3.8.1.3) and the 2-haloacid dehalogenase (EC 3.8.1.2) (Ref. 74).

TABLE 1. Pesticides subject to dehalogenation

Common name	Type	Chemical name
Dalapon	H[1]	2,2-dichloropropionic acid
TCA	H	trichloroacetic acid
Compound 1080	R	sodium fluoroacetate

[1]In this and subsequent table, H = herbicide, R = rodenticide.

The haloacetate dehalogenase is an enzyme that catalyzes the following reaction:

$$XCH_2COO^- + OH^- \longrightarrow HOCH_2COO^- + X^-$$

(where X = a halogen). The enzymes are found in several Pseudomonas spp.; however, some of these enzymes are unable to hydrolyze fluoroacetate, due to the stability of this halogen-carbon bond. Consequently the haloacetic dehalogenases are classified into those that dehalogenate fluoroacetate (+) and those that do not (-).

The haloacid dehalogenases catalyze the following reaction:

$$CH_3CHXCOO^- + OH^- \longrightarrow CH_3CHOHCOO^- + X^-$$

The substrate includes the propionic and longer carbon chain aliphatic acids. The enzymes are subdivided into several types based on their ability to dehalogenate optical isomers. A DL-2-haloacid dehalogenase isolated from Pseudomonas sp. strain 113 catalyzed a non-stereospecific dehalogenation of both optical isomers of 2-chloropropionate. The reaction is catalyzed through a SN_2 type reaction, which leads to inversion of the products, i.e., L- and D-lactates were formed from D- and L-2-chloropropionate, respectively (Ref. 75). Different mechanisms have been elucidated for two dehalogenases isolated from Pseudomonas putida PP3 (Ref. 76). One enzyme (designated fraction I) dechlorinated both D- and L-2-monochloropropionate (2 MCPA), the rate of L-2 MCPA being 80% of the rate of D-2 MCPA, and yielded lactate with the same optical configuration as the substrate. A fraction II dehalogenase from P. putida PP3 dechlorinated D- and L-2 MCPA with the same isomer rates noted previously, but the lactates produced were of the opposite configuration to their precursors, i.e., enzymic dehalogenation with inversion of configuration.

1.2 Properties. Substrate specificity is the most commonly studied property of the various dehalogenases. The enzyme isolated by Motosugi et al. (Ref. 77) was specific for 2-haloalkanoic acids. Activity staining of disc-gels electrophoresed with a crude enzyme preparation exhibited a single protein with activity on D- and L-2-chloropropionates, monochloroacetate, dichloroacetate, 2,2-dichloropropionate and DL-2-chlorobutyrate and that activity was due to a single protein. The 60-fold purified DL-2-haloacid dehalogenase obtained from a crude extract of Pseudomonas sp. strain 113 by Motosugi et al. (Ref. 75) demonstrated a broad substrate specificity. The enzyme acted on 2-halogenated aliphatic carboxylic acids whose carbon chain lengths were less than five. It dechlorinated trichloroacetate to form oxalate.

The crystalline haloacetate halidohydrolase isolated by Kawasaki et al. (Ref. 78) showed the strongest affinity on monofluoroacetate, with a relative activity of 100 compared to 21 for monochloroacetate, 14 for monobromoacetate, and 0.03 for monoiodoacetate. It was slightly active on dichloroacetate, and 2-chloropropionate, but inactive on trichloroacetate, 3-chloropropionate, 2,1-dichloropropionate, 2-chlorobutyrate and chloroacetamide.

1.3 Plasmid involvement in dehalogenation. Kawasaki et al. (Ref. 79) have isolated and characterized a conjugative plasmid pUO1 from a Moraxella sp. strain B which encoded for the two haloacetic dehalogenases (EC 3.8.1.3) and a mercuric reductase enzyme. From a spontaneous mutant deficient in the (-) fluoroacetate ability, a plasmid (pUO11) which encoded for the fluoroacetate defluorination and mercuric reductase was isolated. The molecular sizes of pUO1 and pUO11 were estimated to be 43.7 $\pm$ 1.6 and 40.1 $\pm$ 1.3 megadaltons, respectively. This suggested that the pUO11 plasmid was a deletion mutant derived from pUO1, and that the deleted DNA segment had a molecular size of about 3.6 megadaltons. This fragment which encodes for the (-) fluoroacetate activity may well be a transposon.

2 Oxygenases

The oxygenase enzymes are generally associated with the early reaction of the phenoxyacetic herbicides. The most widely studied members of this herbicide family include

2,4-D, 2,4,5-T, and MCPA. The chemical names of these compounds are shown in Table 2. These enzymes catalyze cleavage of the side chain moiety from the substituted aromatic nucleus to yield the corresponding phenol and an oxidized side chain acid, presumably glyoxylate.

TABLE 2. Phenoxy herbicides subject to oxygenases

Common name	Type	Chemical name
2,4-D	H	(2,4-dichlorophenoxy)acetic acid
2,4,5-T	H	(2,4,5-trichlorophenoxy)acetic acid
MCPA	H	(2-methyl-4-chlorophenoxy)acetic acid

2.1 Reaction mechanism. The oxidative reaction proceeds by cleavage of the bond between the aliphatic acid side chain and the ether oxygen atom as demonstrated with phenoxy-^{18}O-acetic acid studies, which yielded the free phenol-^{18}O with cell-free extracts from a MCPA-adapted Arthrobacter sp. (Ref. 80).

An enzyme obtained from the same Arthrobacter sp., precultured on 2,4-D or MCPA, catalyzed the conversion of these two herbicides to 2,4-dichlorophenol and 2-methyl-4-chlorophenol, respectively (Ref. 81). A cell-free extract from Flavobacterium peregrinum has also been isolated which converts MCPA to the phenol (Ref. 82).

Another soluble enzyme preparation obtained from the same Arthrobacter sp. catalyzed the cleavage of the ether linkage of 2,4-D to liberate 2,4-dichlorophenol (Ref. 83). A proposed pathway involved oxidation of the methylene carbon to form the α-hydroxy-2,4-D derivative, which is presumably cleaved to 2,4-dichlorophenol and glyoxylate. This enzyme would be classified as a monooxygenase (EC 1.14.99) or possibly a hydrolase (EC 3.2.2).

The subsequent metabolism of chlorinated phenols has been extensively examined (Ref. 8 & 84). These reactions are summarized in Fig. 7. Ring cleavage appears to proceed through the intermediate formation of catechols from the corresponding phenols and subsequent ring opening to form a chloromuconic acid. The 2,4-dichlorophenol is hydroxylated to form the 3,5-dichlorocatechol by a mixed function oxidase (EC 1.14.13.7) which requires NADPH and oxygen (Ref. 85).

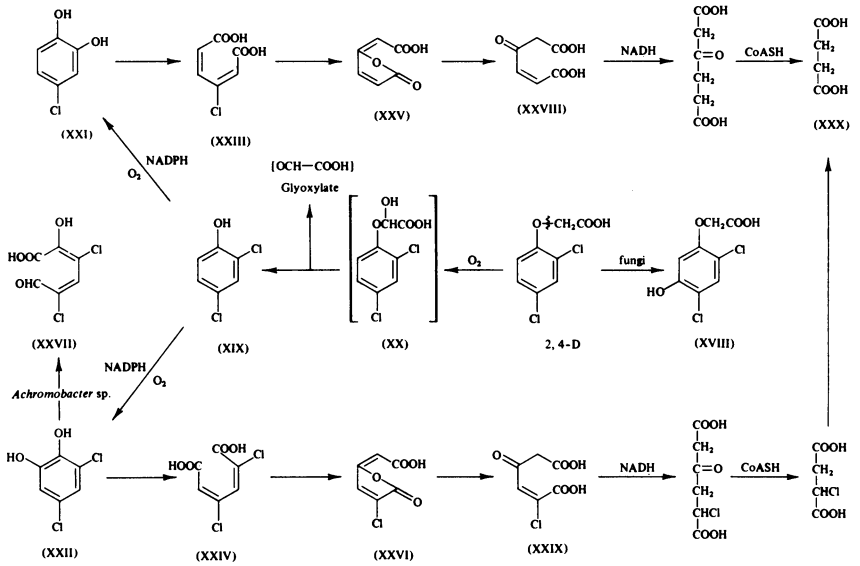

Fig. 7. 2,4-D metabolism by soil microorganisms.

2.2 Properties. The early bacterial enzyme extracts isolated from an Arthrobacter sp. exhibited a broad substrate specificity (Ref. 81). 2,4-Dichloro, 2-methyl-4-chloro, and 2- and 4-chlorophenoxyacetates were all dehalogenated, with evidence that the chlorinated phenols were intermediates prior to ring cleavage and chloride ion release into the medium. The enzyme activity (n moles of substrate transformed/min/mg protein) has been calculated to 0.1 for both 2,4-D and MCPA (Ref. 70).

2.3 Plasmid involvement. The first report of the involvement of plasmids encoding the degradation of 2,4-D appeared from Pemberton and Fisher (Ref. 86). In a subsequent publication (Ref. 87), a strain of Alcaligenes paradoxus was shown to contain a 58-megadalton conjugal plasmid, pJP1, encoding the enzymes required to degrade part or all of both 2,4-D and MCPA. The most recent report from the Australian group (Ref. 88) describes the biophysical and genetic properties of six independently isolated plasmids encoded for the degradation of 2,4-D and MCPA. These plasmids were isolated from Alcaligenes paradoxus and Alcaligenes eutrophus. Four of the plasmids, pJP3, pJP4, pJP5, and pJP7, had molecular masses of 51 megadaltons and transferred freely to a number of other bacteria.

Degradative plasmids and xenobiotics

Although ECE's can encode degradation of carbohydrates, e.g., lactose (Ref. 89 & 90), we are concerned here with plasmid ECE's encoding for hydrocarbon and xenobiotic compound degradations due to chemical relatedness, especially those with aromatic nuclei. Because aromatics are found naturally in decaying cellulosic materials or near crude oil deposits, it would be expected that microbial catabolic pathways could serve as wild type gene pools for gene recruitment in xenobiotic degradations, e.g., a newly introduced pesticide (Ref. 91).

Hydrocarbons. Several of the earliest reports of degradative plasmids came during the studies of hydrocarbon degradations, i.e., camphor (Ref. 92), octane (Ref. 93 & 94), salicylate (Ref. 95), naphthalene (Ref. 96), toluene (Ref. 97).

In addition to crude oil deposits, abundant related aromatics can be found in lignin and plasmids have been shown to code for these degradations (Ref. 98).

Pesticides. Although mixed microbial populations repeatedly have been shown to be capable of pesticide degradations (Ref. 45, 99-101), genetic studies are difficult to perform on mixed cultures. In addition to the two classes of pesticides discussed under the previous section, titled "Plasmid-associated herbicide enzymes," direct or indirect plasmid involvement has been shown for the following pesticide or pesticide-like compounds: 4-Chlorobiphenyl (Ref. 102), pyramin/antipyrin (Ref. 103), and parathion (Ref. 104).

Pesticide gene monitoring and evolutions

General phenomena that may indicate ECE involvement in pesticide degradation chemistries include:

1. Multiple degradations, especially for related pesticides and compounds.
2. Higher rate of pesticide degradations, e.g., are "problem" soils (Ref. 105) increasing in frequency?
3. Has pesticide degradation transfer to other species been noted, e.g., Pseudomonas to Alcaligenes?
4. Is there a rapid loss of degradation trait upon subculture in the absence of added pesticide, e.g., 10^{-5} or greater?

What specific observations or experiments in pesticide degradation can be used to understand the role of ECE's?

One possibility is to perform comparative pesticide degradation studies by microbial populations and(or) soils and compare the gene adaptation(s). Plasmid distributions in response to environmental flux are now starting to be pursued (Ref. 106).

Another possibility is to compare rapid pesticide degradation soils with slow soils. Fast soils should show a clear genetic potential for those pesticides. This genetic potential would probably reside in ECE's which could be examined in the following three manners.

(a) Confirm plasmid presence and mediation of degradation.
(b) Elucidate existence of possible multiple pesticide-specific plasmids.
(c) The DNA homology should be high if not identical among different isolates with the same pesticide degradation ability.

The kinetics of pesticide degradation (Ref. 107) should follow the kinetics of ECE creation and response (Ref. 47). For example, a "memory" (DNA sequences coding for pesticide degradation) should exist after initial exposures such that subsequent applications show a shorter lag time before degradation onset with possible enhanced rates. Pesticide-degrading

cell populations should also drop to very low (almost undetectable) levels after pesticide application cessation, but still be capable of a faster recovery response to subsequent pesticide applications.

In studying the above phenomena, several experimental techniques are now available. For example, genetic probes could be developed to study the adaptation and evolution of pesticide-degrading genes on plasmids within microbial populations in soil. Complementary radioactive (^{32}P) DNA probes can be made which can detect a specific degradative gene among total DNA from the host cell as well as all other DNA's obtained from a given soil sample.

It will be interesting to see what the above experiments yield in regard to pesticide-degrading genes. For example, what kinds of genetic changes are observable with low to high pesticide concentrations? Are these genetic changes regulatory, dose, specificity or activity gene changes?

Some important issues emerge from the previous discussion that have important implications for the role of ECE's and more specifically plasmids in microbial adaptation process. The number of permutations for ECE's and concomitant recombination produces a virtually unlimited arena for prokaryote evolution, and these advantages stem from certain intrinsic factors:

1. Plasmids are a highly successful survival strategy (even occurring in so-called ancient bacteria (archebacteria) or cyanobacteria (Ref. 108).
2. Plasmids' continued existence is not dependent on one cell line or species due to spread of evolutionary/ecological risk over many microbial populations.
3. There is no competitive handicap for ECE's, since transfer of encoded gene(s) via plasmid is done by transferring a DNA copy, rendering the original host cell still competitive.
4. Macroevolution is rapid compared to mutation or other microevolutionary adaptations since ECE's can rearrange genes in larger blocks more quickly.
5. ECE's provide a mechanism for attacking many substrates at a moderate rate, which is probably ecologically superior to attacking a few substrates rapidly within a high flux environment.

ECE studies hold many opportunities for pesticide science. Besides offering a chance to detect and monitor pesticide gene behavior, we should be able to illuminate specific evolutionary changes of pesticide-degrading organisms in the environment in order better to understand the fate of xenobiotics in that same environment.

STRATEGIES FOR FUTURE PESTICIDE TECHNOLOGIES IN AGRICULTURE

It has been demonstrated here that pesticide degradation evolutions may be occurring today and that they may have an impact on U.S. agriculture. Thus, initial strategies for pesticide technology should be explored to prevent potential problems. Some of these strategies might be:

1. Recognize that any chemical or pesticide used in the environment at less than an LD_{100} may inevitably result in resistant organisms, be they microbial, plant, or insect. We have already seen this occur with DDT-resistant insects, herbicide-resistant weeds, and to a certain extent with microorganisms (this may be more widespread than currently recognized).
2. Realize that there is a certain degree of regioselectivity in the enzymes responsible for pesticide degradations just as there is class specificity with antibiotic resistance enzymes, e.g., many penicillins are attacked by the same b-lactamase enzyme. It can be seen from the literature cited here that the same pesticide genes and enzymes can attack more than one pesticide and that they are potentially mobile genetic elements.
3. The shift from pesticide or any chemical sensitivity towards resistance is highly temporal. The development of resistance occurs only over a significant number of organism (insect, weed, microorganism) generation times. Thus, minimal pesticide contact time is highly desirable, assuming maximal efficiency in the smaller contact window.
4. Evolution of resistance or degradation genes in pests in also highly dose-dependent. A moderate to large environmental dose will yield maximal degradation and resistance evolution, while a very low dose may be beneath a threshold for evolution. This would argue for the development of higher activity pesticides.
5. Although economics will greatly dominate pesticide technology, a successful pesticide strategy will avoid selection pressures on pests.

Long-term success in pesticide technology will make every attempt to minimize the temporal application window to pest generation time as well as minimize the dose applied. This is especially important today since many groups are attempting to place chemical pesticide

resistance genes into crop plants with the intent of giving them herbicide resistance. This strategy will probably only result in a more rapid evolution of pesticide resistance and degradation in weeds and soil microorganisms, although temporary protection may result. Probably the most important aspect is that we now have new and more sophisticated research tools, e.g., genetic engineering and biochemistry, which will greatly aid in studying evolutionary patterns in pesticide science.

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