# Degradation of Synthetic Organic Molecules in the Biosphere

## NATURAL, PESTICIDAL, AND VARIOUS OTHER MAN-MADE COMPOUNDS

*Proceedings of a Conference*
SAN FRANCISCO, CALIFORNIA
June 12–13, 1971

NATIONAL ACADEMY OF SCIENCES
WASHINGTON, D.C. 1972

NOTICE: The symposium reported herein was undertaken under the aegis of the National Research Council with the approval of its Governing Board. Such approval indicated that the Board considered the problem to be of national significance, that its elucidation required scientific or technical competence, and that the resources of NRC were particularly suitable to the conduct of the project. The institutional responsibilities of the NRC were then discharged in the following manner:

The participants in the symposium were selected for their individual scholarly competence and judgment with due consideration for the balance and breadth of disciplines. Responsibility for all aspects of this report rests with the parent committee, to whom our sincere appreciation is expressed.

Although the proceedings of symposia are not submitted for approval to the Academy membership or to the Council, each is reviewed according to procedures established and monitored by the Academy's Report Review Committee. Such reviews are intended to determine, *inter alia*, whether the major questions and relevant points of view have been addressed and whether the reported findings arose from the available data and information. Distribution is approved, by the President, only after satisfactory completion of this review process.

This study was supported by the U.S. Department of Agriculture.

LIBRARY OF CONGRESS CATALOGING IN PUBLICATION DATA

Main entry under title:

Degradation of synthetic organic molecules in the biosphere.

"The symposium reported herein was undertaken under the aegis of the National Research Council."

Includes bibliographies.

1. Biodegradation–Congresses. 2. Pesticides–Biodegradation–Congresses. I. National Research Council.

QP517.B5D44 628.5 72-8844

ISBN 0-309-02046-8

*Available from:*

Printing and Publishing Office, National Academy of Sciences
2101 Constitution Avenue, N.W., Washington, D.C. 20418

Printed in the United States of America

COMMITTEE ON AGRICULTURE AND THE ENVIRONMENT

I. C. GUNSALUS, *Chairman*
ANSON R. BERTRAND
ARNE E. CARLSON
STANLEY DAGLEY
ALVIN C. DALE
HERRELL DeGRAFF
WENDELL W. KILGORE
ROBERT S. MORISON
DAVID PIMENTEL
JOHN F. TIMMONS
PAUL E. WAGGONER
ROBERT WHITE-STEVENS
A. RICHARD WILLIAMS

SUBCOMMITTEE ON CHEMICAL ADDITIVES

WENDELL W. KILGORE, *Chairman*
ARNE E. CARLSON
STANLEY DAGLEY
DAVID PIMENTEL

# Preface

In April 1970, the Secretary of Agriculture asked the President of the National Academy of Sciences for assistance in finding answers to the following questions:

- What strategies can be used to maintain a strong food and fiber industry in this country, while eliminating or minimizing environmental conflicts resulting from production practices?
- What can be done to make rural America a more pleasing place to live?

In response, a committee was formed within the Division of Biology and Agriculture to study issues posed by these questions and to prepare a report. These proceedings, prepared by the Subcommittee on Chemical Additives, are addressed to one aspect of the Secretary's request—disposal of wastes.

In developed countries, most people know that industrial, domestic, and agricultural wastes are disposed of in ways that insult the environment and that improved disposal methods are urgently needed. To some extent, accumulations of wastes can be disposed of by biological means, and some pesticides and other nonnatural compounds can be decomposed by microbial action. The possibilities of these approaches are explored in this report.

## COMMON AND CHEMICAL NAMES OF PESTICIDES AND OTHER CHEMICALS

| Common Name | Chemical Name |
|---|---|
| Aldrin | 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene |
| Amiben | 3-amino-2,5-dichlorobenzoic acid |
| Amitrole | 3-amino-*s*-triazole |
| Amobam | diammonium ethylene bis[dithiocarbamate] |
| AMP | adenosine monophosphate |
| BHC | benzene hexachloride |
| Cacodylic acid | hydroxydimethylarsine oxide |
| Captan | *N*-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide |
| Carbaryl | 1-naphthyl *N*-methylcarbamate |
| ChE | cholinesterase |
| Chlordane | 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene (principal constituent) |
| Chlorobenzilate | ethyl 4,4′-dichlorobenzilate |
| Chloropropylate | isopropyl 4,4′-dichlorobenzilate |
| Chloroxuron | 3-[4-(4-chlorophenoxy)phenyl]-1,1-dimethylurea |
| Chlorpropham | isopropyl *m*-chlorocarbanilate |
| CoA | coenzyme A |
| CPA | 4-chlorophenoxyacetate |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 2,4-DB | 4-(2,4-dichlorophenoxy)butyric acid |
| DBP | dibutyl phthalate |
| DDD | 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane |
| DDE | 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene |
| DDP | didecyl phthalate |
| *p,p′*-DDT | 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane |
| *o,p′*-DDT | 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane |
| 2,4-DEB | 2(2,4-dichlorophenoxyethyl)benzoate |
| 2,4-DEP | tris(2,4-dichlorophenoxyethyl)phosphite |
| Diazinon | *O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate |
| Dichlorprop | 2-(2,4-dichlorophenoxy)propionic acid |
| Dicryl | 3′,4′-dichloro-2-methylacrylanilide |
| Dieldrin | 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a,-octahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene |
| Difolatan | *N*-trichloroethylmercapto-4-cyclohexene-1,2-dicarboximide |
| Dimethoate | *O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl)phosphorodithioate |
| Dithane-45 | zinc salt of manganese ethylenebisdithiocarbamate |
| Diuron | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| DNA | deoxyribonucleic acid |
| DSMA | disodium methanearsonate |
| EDTA | ethylenediaminetetraacetic acid |
| Endrin | 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo-endo*-5,8-dimethanonaphthalene |
| EPN | *O*-ethyl *O*-*para*-nitrophenyl phenylphosphorothioate |
| EPR | ethylene propylene rubber |
| Erbon | 2-(2,4,5-trichlorophenoxy)ethyl 2,2-dichloropropionate |

## COMMON AND CHEMICAL NAMES OF PESTICIDES AND OTHER CHEMICALS (continued)

| Common Name | Chemical Name |
|---|---|
| FAD | flavin adenine dinucleotide |
| Ferbam | ferric dimethyldithiocarbamate |
| Folpet | *N*-trichloromethylthiophthalimide |
| Guthion | *O,O*-dimethyl *S*-4-oxo-1,2,3-benzotriazin-3(4H)-ylmethyl phosphorodithioate |
| Heptachlor | 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene |
| Ioxynil | 4-hydroxy-3,5-diiodobenzonitrile |
| Karsil | *N*-(3,4-dichlorophenyl)-2-methylpentamide |
| Lead arsenate | lead arsenate |
| Lindane (γ-BHC) | γ-1,2,3,4,5,6-hexachlorocyclohexane |
| Linuron | 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea |
| Malathion | *S*-[1,2-bis(ethoxycarbonyl)ethyl] *O,O*-diethyl phosphorodithioate |
| Maneb | manganese ethylenebisdithiocarbamate |
| MCPA | 4-chloro-2-methylphenoxyacetic acid |
| MCPB | 4-[4-chloro-*o*-tolyl)oxy] butyric acid |
| MCPES | 2-[(4-chloro-*o*-tolyl)oxy] ethyl sodium sulfate |
| MCPP | 2-(2-methyl-4-chlorophenoxy)propionic acid |
| Methoxychlor | 2,2-bis(*para*-methoxyphenyl)-1,1-trichloroethane |
| Metobromuron | 3-(*p*-bromophenyl)-1-methoxy-1-methylurea |
| Monolinuron | 3-(4-chlorophenyl)-1-methoxy-1-methylurea |
| Monuron | 3-(4-chlorophenyl)-1,1-dimethylurea |
| MSMA | monosodium methanearsonate |
| Mylone or Dazomet | tetrahydro-3,5-dimethyl-2*H*-1,3,5-thiadiazine-2-thione |
| Nabam | sodium ethylenebisdithiocarbamate |
| NAD | nicotinamide adenine dinucleotide |
| NADP | nicotinamide adenine dinucleotide phosphate |
| Paraoxon | diethyl *para*-nitrophenyl phosphate |
| Parathion | *O,O*-diethyl *O-para*-nitrophenyl phosphorothioate |
| PCP | pentachlorophenol |
| PMA | (acetato)phenylmercury |
| Polyram | zinc activated polyethylenethiuram disulfide |
| Prometryne | 2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine |
| Propanil | 3′,4′-dichloropropionanilide |
| RNA | ribonucleic acid |
| Sesone | sodium 2,4-dichlorophenoxyethyl sulfate |
| Silvex | 2-(2,4,5-trichlorophenoxy)propionic acid |
| 2,4,5-T | 2,4,5-trichlorophenoxyacetic acid |
| TDE | 1,1-dichloro-2,2-bis(*para*-chlorophenyl)ethane |
| Tepp | ethyl pyrophosphate |
| 2,4,5-TES | sodium 2,4,5-trichlorophenoxyethylsulfate |
| Thiram | tetramethylthiuram disulfide |
| TOTP | triorthotolylphosphate |
| TPN | triphosphopyridine nucleotide |
| Vapam (SMDC) | sodium *N*-methyldithiocarbamate |
| Zineb | zinc ethylene-1,2-bisdithiocarbamate |
| Ziram | zinc dimethyldithiocarbamate |

# Contents

STANLEY DAGLEY

# Microbial Degradation of Stable Chemical Structures: General Features of Metabolic Pathways

## THE RELEVANCE OF STUDIES WITH NATURALLY OCCURRING COMPOUNDS

Microbes are able to degrade chemical structures of many types, and it is probably true to say that *every* organic molecule that is synthesized by living matter may also be degraded, in turn, by microbes. The versatility of soil pseudomonads was documented as early as 1926 by den Dooren de Jong—observations that later formed the foundation for the outstanding taxonomic study undertaken by Stanier, Palleroni, and Doudoroff (1966). Unlike den Dooren de Jong and Stanier *et al.*, some microbiologists in the past have been tempted to subscribe to what Martin Alexander (1965) has called the illusion of microbial infallibility; that is, if a compound is capable of being oxidized at all, somewhere on earth one can find microbes capable of catalyzing its degradation. This is a false principle that might encourage industry and agriculture to believe that Nature—usually through what we now recognize to be microbial agencies—will continually and automatically cleanse herself of all the surplus compounds that chemical ingenuity presents. There is no sound reason for this hope. Although it will be demonstrated later that pseudomonads sometimes owe their versatility to the broadness of substrate specificities of the enzymes they

elaborate, the very word "specificity" loses its meaning unless some limits are set to enzymic activity. Furthermore, these enzymes are proteins that the organism must synthesize in response to inducers or derepressors, and the chemical structures of these compounds must, in turn, bear some resemblance to those of natural metabolites.

A man-made compound will be biodegradable only when the relevant microbes are able to use the enzymic apparatus acquired during evolution designed to exploit the diverse sources of energy found in nature. The study of biodegradability, therefore, is linked inevitably with the study of both the mechanisms of induction and the modes of action of those enzymes employed for degrading natural products. In any laboratory study of man-made molecules, there will come a point at which experimental findings must be interpreted within the framework of knowledge of microbial metabolism in general.

Chemical speculation, however, is not enough. To understand the limits of biodegradability, the enzymology involved must be studied. For example (see Goldman, "Enzymology of Carbon–Halogen Bonds," p. 147), the enzyme fumarase catalyzes the hydration of fluorofumarate to give $\alpha$-fluoromalate, from which $H^+$ and $F^-$ ions are immediately expelled to give oxaloacetate (Clarke *et al.*, 1968). This hydration is predicted on purely chemical grounds by Markownikoff's rule. However, chlorofumarate and other halogenofumarates are hydrated the other way round, yielding $\beta$-substituted malates, and the halogen is not expelled. The intervention of the enzyme gives an unexpected result—one that cannot be predicted from electron density distributions for molecules not bound to this enzyme (Teipel *et al.*, 1968).

## THE INITIATION OF MICROBIAL CATABOLIC SEQUENCES

To what metabolic features do soil microbes owe their ability to utilize compounds that are largely biochemically inert? Aerobic bacteria employ most of the sequences and cycles which occupy a central position in metabolic maps and which are generally found in other living forms. Their unique biochemical assets lie in their ability to catalyze early steps in degradation, which other organisms cannot accomplish, and to form metabolites that can enter the common pathways of metabolism, such as the Krebs cycle or the fatty acid "spiral." Thus, an alkane remains biochemically inert until a terminal carbon has been oxidized. The fatty acid so formed may then

enter a main channel of metabolism, namely, β-oxidation. A saturated ring-structure is also inert until a hydroxyl group is inserted, and, if this occurs at an appropriate point in the molecule, the possibility then arises that further oxidation may break the ring, providing fragments that can be degraded. The unsubstituted benzene nucleus is an inert resonance structure, but when suitably substituted with two hydroxyl groups, enzymic ring-fission may then occur.

The vast majority of biological oxidations in general metabolism, however, are not accomplished by the insertion of oxygen into molecules. Rather, hydrogen atoms are removed and are transferred to NAD or NADP. The reason for this may lie in the formation of the earth's atmosphere. Some 4 to 5 × $10^9$ years ago, the atmosphere of the earth probably contained very little gaseous oxygen or hydrogen. Earlier than this, hydrogen was abundant but was lost by diffusion to outer space; later, gaseous oxygen slowly accumulated to give the proportion that exists today. When neither gas was abundant, those compounds that we now identify as biochemicals were thermodynamically stable enough to persist for the long periods of time required for the inception of evolutionary processes. But as the proportion of gaseous oxygen in the atmosphere increased, these biochemicals represented an increasingly unstable type of molecule that contained carbon and hydrogen, of which the stable forms in excess oxygen are carbon dioxide and water, respectively. No wonder, then, that in general metabolism hydrogen atoms are transferred through the agency of specialized coenzymes and that oxygen gas is usually excluded from metabolic sequences until terminal oxidation to form water. By contrast, the biochemical successes of aerobic microbes are largely due to their ability to use molecular oxygen in a controlled manner at the *commencement* of a metabolic sequence. A little oxygen—sufficient to initiate catabolism—is incorporated into biochemically inert molecules.

## EXAMPLES OF REACTIONS THAT INITIATE PATHWAYS OF ENZYMIC DEGRADATION

### *Monooxygenases*

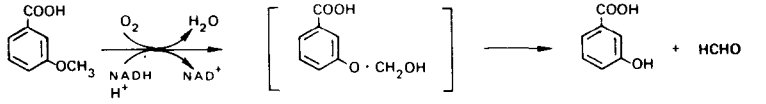
Equation (1) illustrates that the inser ion of one atom of $O_2$ into a substrate requires reducing power, since the other atom of the molecule is reduced to water. The need for a reducing agent, usually

$$RH + O_2 + [H] \longrightarrow ROH + H_2O \qquad (1)$$

NADH or NADPH, and also the incorporation of one atom of oxygen into the substrate, was established by Mason *et al.* (1955). A specific example of this type of reaction—catalyzed by enzymes that have been variously termed "hydroxylases," "mixed function oxidases (or oxygenases)," or "monooxygenases"—was provided by Gholson *et al.* (1963) and Baptist *et al.* (1963). These authors showed that cell-free extracts of *Pseudomonas oleovorans*, grown with *n*-hexane, required NADH to oxidize *n*-octane to *n*-octanol [see Eq. (2)]. Further reactions requiring NAD converted octanol to octanoic acid.

$$CH_3 - [CH_2]_6 - CH_3 + O_2 + NADH + H^+ \xrightarrow{Fe^{2+}} CH_3 - [CH_2]_6 - CH_2OH + NAD^+ + H_2O \qquad (2)$$

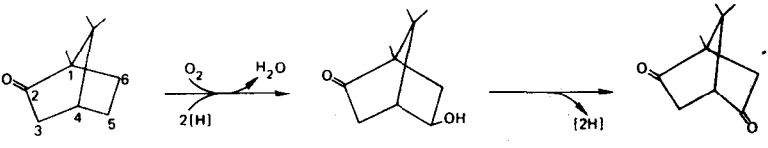
Methyl groups attached directly to the benzene nucleus are also attacked by $O_2$ in enzymic reactions requiring NADH (Hopper and Chapman, 1971; Chapman and Hopper, 1968). In Eq. (3), the




(3)

the methyl group, present in this case as methoxyl, was removed from 3-methoxybenzoate by extracts of *Pseudomonas aeruginosa*, which also showed a need for NADH. Ribbons (1970, 1971) established the stoichiometry for reactants and products in accordance with this equation and showed that a similar enzyme system from *Pseudomonas testosteroni* consists of a complex of at least two protein fractions. A large number of naturally occurring compounds contain methoxyl groups and are doubtless metabolized in this manner by soil microbes. It is also probable that the ether linkage of the herbicide 4-chloro-2-methylphenoxyacetate is cleaved by a similar mechanism to release the side chain as glyoxylate (Gamar and Gaunt, 1971).

The degradation of camphor [compound (a), Eq. (4)] by *Pseudo-*

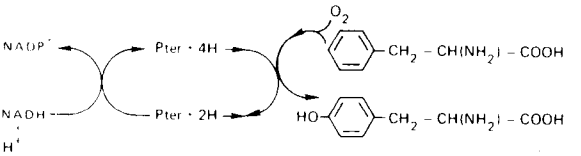



(a) Camphor (b) (c)

(4)

*monas putida* provides an example of a bicyclic ring system for which metabolism is initiated by a mixed function oxidase. The hydroxyl group, introduced at Carbon-5, is then oxidized to a keto group by a dehydrogenase that requires NAD (Hartline and Gunsalus, 1971).

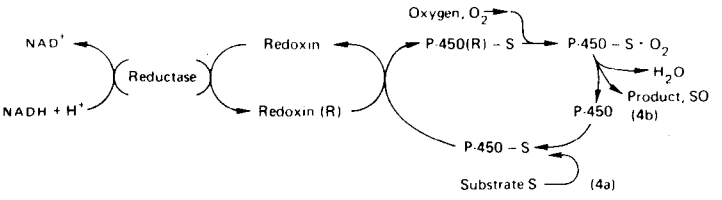
Currently, the main thrust of research in the area of enzymic hydroxylation is directed toward elucidating the mechanisms by which reducing power is harnessed and a single atom of molecular oxygen is inserted into the substrate molecule. It has become evident that this result may be accomplished in a variety of ways. Kaufman (1962) has reviewed work with mammalian systems that catalyze the conversion of phenylalanine into tyrosine. Equation (5), which omits




(5)

some details, shows that, although the primary source of reducing power is NADPH, the hydroxylation reaction receives the necessary hydrogen atoms from a reduced pteridine, 6-methyltetrahydropterin (Lloyd *et al.*, 1971). For a system from *Pseudomonas,* Guroff and Rhoads (1967) also showed that, in addition to NADH, a tetrahydropteridine was required for the oxidation of phenylalanine to tyrosine.

Details of the initial reaction in the microbial degradation of camphor [Eq. (4)] have been described by Katagiri *et al.* (1968). They found that three fractions were required: first, an iron–sulfur protein, putidaredoxin, which was reduced by NADH; this reaction required a second protein, namely, the reductase of Eq. (6); the

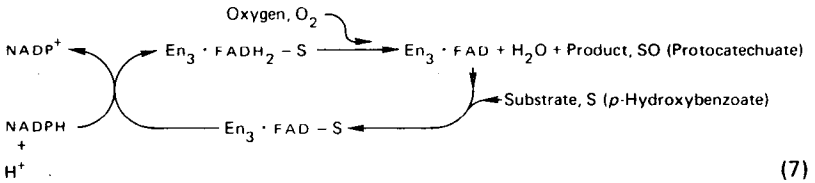



(6)

third fraction, the hydroxylase itself, was a cytochrome (P-450). Putidaredoxin, therefore, is an intermediary in transferring reducing power to the site of hydroxylation. At this site, a cycle of reactions occurs as shown in Eq. (6). First, cytochrome P-450 com-

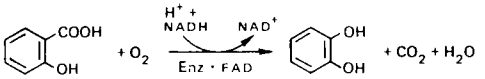
bines with the substrate S and undergoes reduction. This complex then takes up $O_2$, reducing one atom of oxygen and inserting the other into the substrate as a hydroxyl group. Oxidized cytochrome P-450 is now available to take another molecule of substrate through the cycle.

Mention will be made of two systems that catalyze similar reactions but differ from camphor hydroxylase in the management of reducing power. (a) A widely-used substrate for the growth of many soil pseudomonads is *p*-hydroxybenzoate; and before its nucleus can be cleaved, a second hydroxyl group must be inserted to give protocatechuate. One of the *p*-hydroxybenzoate hydroxylases studied by Hesp *et al.* (1969) is a FAD-containing protein that combines with substrate S and then reduced by NADPH as shown in Eq. (7). Molecular oxygen is then taken up and reduced. Flavin




(7)

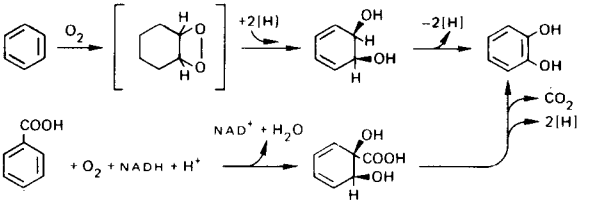
adenine dinucleotide (FAD) is re-oxidized as hydroxylation occurs. (b) Salicylate hydroxylase catalyzes Eq. (8) and is also a FAD enzyme (Takemori *et al.*, 1969) that uses NADH as the primary source of reducing power. In this reaction, hydroxylation proceeds with the simultaneous loss of carbon dioxide.




(8)

*Dihydroxylations*

In all the foregoing systems, one molecule of oxygen furnished one hydroxyl group. By contrast, the reaction sequences shown in Eq. (9) provide examples of reactions that utilize both atoms of $O_2$, thus inserting two hydroxyl groups into the substrate simultaneously. It appears that one molecule of oxygen is taken up by benzene to form a peroxide, and that the NADH required in the dihydroxylation is utilized for reducing the peroxide to a dihydrodiol. Gibson *et al.* (1970a) selected a mutant of *Pseudomonas putida* that accu-

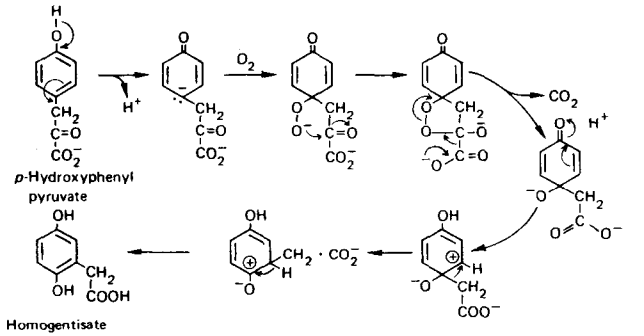



(9)

mulated *cis*-benzene glycol from benzene [Eq. (9)] and also incorporated two atoms of $^{18}O_2$ into this compound. Cell-free extracts were also found to contain a NAD-dependent dehydrogenase that converted *cis*-benzene glycol into catechol. Further, the mutant oxidized toluene to its corresponding *cis*-glycol, namely (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-1,4-diene (Gibson *et al.*, 1970b). Reiner and Hegeman (1971) also have studied the enzymic oxidative decarboxylation of benzoate to give catechol, a reaction frequently encountered in pseudomonads but obscured for many years. They have established a reaction sequence involving the *cis*-dihydrodiol of benzoate as the precursor of catechol [Eq. (9)]. The formation of *cis*-diols by microbial systems, probably by the reduction of peroxides, stands in contrast to hydroxylations catalyzed by rat liver microsomes. These preparations oxidize naphthalene, for example, to give an epoxide that is then hydrolyzed to a *trans*, rather than a *cis*, dihydrodiol (Jerina *et al.*, 1968), whereas *Pseudomonas* forms *cis*-1,2-dihydro-1,2-dihydroxynaphthalene from the hydrocarbon (Jerina *et al.*, 1971). Another compound that appears to be metabolized by reactions in this category is kynurenic acid. Cell-free extracts of *Pseudomonas fluorescens*, supplied with NADH, hydroxylated the benzene nucleus of this quinoline derivative to give 7,8-dihydroxykynurenic acid. Taniuchi and Hayaishi (1963) showed that the 7,8-dihydrodiol was formed as an intermediate in the reaction, but its stereochemistry was not investigated.

When tyrosine is degraded, either by liver or bacteria, *p*-hydroxyphenylpyruvate is converted into homogentisate. This remarkable reaction encompasses three simultaneous chemical events—namely, oxidative decarboxylation of the pyruvate side chain to give carboxymethyl, insertion of a second hydroxyl in a position *para* to that originally present in the substrate, and migration of the carboxymethyl group from this *para* position to an adjacent carbon. The interest of this complex reaction for this survey lies in the fact that, although one additional hydroxyl group results, both atoms of $^{18}O_2$ are incorporated into the substrate; that is, one atom is inserted into

the OH group and the other into the COOH group. Further, no external supply of electrons is required; as illustrated in Eq. (10), these are furnished internally when $CO_2$ is released. Equation (10), is that proposed by Lindblad *et al.* (1970) to account for their results using




(10)

$^{18}O_2$, and it suggests that a cyclic peroxide is formed during the course of the reaction. The decarboxylation of this peroxide would result in a quinonoid structure, the bonds of which may rearrange themselves to give a cationic intermediate as shown. The migration of the side chain is a consequence of a further bond redistribution. Such migrations, following hydroxylation of the benzene nucleus, have been termed "NIH shifts" (Guroff *et al.*, 1967; Daly *et al.*, 1968). For microsomal systems, they have been shown to occur for methoxyl, methyl and chlorine substituents in addition to the example given in Eq. (10). Guroff *et al.* (1967) found that halogen substituents may be displaced from carbon-4 to carbon-3 when phenylalanine is hydroxylated by *Pseudomonas*; thus, it is probable that further examples of the NIH shift will be encountered in future studies of the microbial degradation of chlorinated pesticides.

It appears that the conversion of *p*-hydroxyphenylpyruvate into homogentisate [Eq. (10)] may be a specific example of a new general class of oxygenases for which the oxidative decarboxylation of a 2-keto acid provides the necessary reducing power. Thus, the oxygenation of thymine by *Neurospora crassa* to give 5-hydroxymethyluracil—and hence 5-carboxyuracil—is coupled to the oxidative decarboxylation of 2-ketoglutarate (Holme *et al.*, 1971). A similar system operates in a species of *Pseudomonas* for the hydroxylation of γ-butyrobetaine (Lindstedt *et al.*, 1970). One atom of molecular oxygen is incorporated into the substrate by these oxygenases, and the other atom appears in succinate formed from 2-