Microbial Co-Metabolism and the Degradation of Organic Compounds in Nature

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INTRODUCTION

The phenomenon of co-oxidation was first reported by Leadbetter and Foster (37) when they noted the oxidation of ethane to acetic acid, propane to propionic acid and acetone, and butane to butanoic acid and methyl ethyl ketone during growth of *Pseudomonas methanica* on methane, the only hydrocarbon capable of supporting growth of the organism. The term "co-oxidation" was used to describe the process in which a microorganism oxidized a substance without being able to utilize the energy derived from this oxidation to support growth.

Subsequently, Jensen (34) suggested that the more general term "co-metabolism" be used to describe this process and to expand the concept to include dehalogenation reactions frequently carried out by microbial species. However, co-oxidation and co-metabolism have been used interchangeably so frequently in the literature that these terms are now considered to be synonymous.

Although co-metabolism does imply the concomitant oxidation of a non-growth substrate during growth of a microorganism on a utilizable carbon and energy source, it also describes oxidation of non-utilizable substrates by resting cell suspensions grown at the expense of substances capable of supporting microbial growth. Therefore, usage of co-metabolism refers to any oxidation of substances without utilization of the energy derived from the oxidation to support microbial growth and does not infer presence or absence of growth substrate during the oxidation.

Although this phenomenon appears to occur widely in microbial metabolism, interpretation of experimental results as indicating co-metabolism requires considerable care. For example, certain hydrocarbons may, in some way, increase the endogenous respiration of the test organism without being oxidized. If oxygen is consumed by an organism in the presence of a hydrocarbon, which does not support growth, at a level only slightly higher than endogenous, the question of stimulation of respiration versus co-metabolism must be raised (41). In such cases, proof of disappearance of substrate and accumulation of end products is required to clearly demonstrate a co-metabolic process.

Caution must also be used not to overlook the occurrence of co-metabolic phenomena. For example, Broadbent and Norman (8) offered no explanation for the fact that soil organic matter became a better source of nutrient for the microbial soil population when readily decomposable organic matter was added. It has been demonstrated that microorganisms capable of a co-metabolic degradation of organic pollutants can be enriched for by application of biodegradable analogues of the pollutant to the microbial ecosystem (26). This process, called "analogue enrichment," (D. D. Focht, *personal communication*) appears to account for the enhanced rate of decomposition of organic matter noted by Broadbent and Norman (8).

Burge (9) reported rapid decomposition of the herbicide, dalapon, without an increase in bacterial numbers in soil samples. His interpretation of these results indicated breakdown of the herbicide by a chemical process or de-

composition by fungi, but the possibility of a co-metabolic degradation of dalapon appears to be equally probable. Caseley (11) likewise failed to consider co-metabolism as the process responsible for the breakdown of pentachloronitrobenzene (PCNB) by *Streptomyces aureofaciens*, although the fungicide was not utilized as a sole source of carbon for growth. It was clearly shown that the fungicide was degraded only during the active growth phase of the microorganism, establishing biological participation in the decomposition of PCNB, and the accumulation of the product pentachloroanaline in the medium certainly supports the occurrence of a co-metabolic process.

Chambers and Kabler (12) noted in their study of the relationship between chemical structure and biodegradability of phenols that some cultures had a high oxygen consumption with a substance in manometric tests but failed to utilize the compound as the sole source of carbon and energy for growth in a mineral-salts medium. If manometric experiments repeatedly show oxygen consumption to be well in excess of the endogenous respiratory rate, as in this study, the occurrence of co-metabolism is indicated. Finally, Matsumura and Boush (40) reported the degradation of dieldrin by 12 soil isolates growing in mannitol-yeast extract medium. The lack of growth of these microorganisms at the expense of dieldrin, in the absence of the additional carbon and energy source, mannitol, indicates that co-metabolism was responsible for breakdown of this pesticide by the soil isolates.

Evidence for co-metabolism can also be obtained by means of enzyme induction experiments. It was reported by van Eyk and Bartels (55) that diethoxymethane induced enzymes necessary for growth of *Pseudomonas aeruginosa* on paraffins and stimulated oxygen consumption to a level far in excess of endogenous respiration, but failed to support growth. The ability of diethoxymethane to induce enzymes and the high level of oxygen consumption with this substance indicate that oxygen uptake with this compound was due to co-metabolism and not merely to stimulation of endogenous respiration. Demonstration of substrate disappearance or product accumulation would have provided clear-cut evidence for co-metabolism in this work.

Similarly, Hegeman (22) noted that both *p*-chloromandelate and *p*-bromomandelate induced enzymes involved in mandelate metabolism by *P. putida*. Both of the halogenated compounds were oxidized by enzymes of the mandelate group but neither supported growth

of the organism. These results appear to be interpretable on the basis of co-metabolism.

In spite of the many overlooked examples of co-metabolism, this phenomenon has been observed so frequently that it appears to represent a very important type of microbial metabolism. Table 1 lists those microorganisms which have been clearly shown to possess a co-metabolic type of metabolism. The substances which have been studied in regard to this oxidative mechanism are listed in Table 2 with the products resulting from co-metabolism of each compound. Those substances for which the product of co-metabolism is unknown are not presented in this table.

Undoubtedly, the list of microorganisms exhibiting this property and the substances acted upon by this mechanism are far from complete and will be greatly expanded by future investigation.

BIOCHEMICAL MECHANISM OF CO-METABOLISM

The process of co-metabolism represented to the microbial physiologist a new oxidative mechanism for which there was no adequate explanation. Why could a microorganism oxidize a substance which it could not use as a source of carbon and energy for growth?

Foster (18) stated that the inability to grow at the expense of a substrate was not

TABLE 1. *Microbial species exhibiting the phenomenon of co-metabolism*

Microorganism	Reference
Achromobacter sp.	12, 23, 27, 29
Arthrobacter sp.	27
Aspergillus niger	53
Azotobacter chroococcum	56
Azotobacter vinelandii	56
Bacillus megaterium	35
Bacillus sp.	27, 40
Brevibacterium sp.	24, 25, 27
Flavobacterium sp.	12, 27
Hydrogenomonas sp.	16
Microbacterium sp.	27
Micrococcus cerificans	14
Micrococcus sp.	27
Nocardia erythropolis	51, 54
Nocardia sp.	13, 44
Pseudomonas fluorescens	31, 32, 33
P. methanica	37
P. putida	21, 22
Pseudomonas sp.	12, 19, 20, 27, 44
Streptomyces aureofaciens	11, 53
Trichoderma viride	40
Vibrio sp.	6, 10
Xanthomonas sp.	12, 27

TABLE 2. *Organic substances subject to co-metabolism and accumulated products*

Substrate	Product	Reference
Ethane	Acetic acid	37
Propane	Propionic acid, acetone	37
Butane	Butanoic acid, methyl ethyl ketone	37
m-Chlorobenzoate	4-chlorocatechol, 3-chlorocatechol	27, 33, 56
o-Fluorobenzoate	3-Fluorocatechol, fluoroacteate	15, 33, 54
2-Fluoro-4-nitrobenzoate	2-Fluoroprotocatechuic acid	54
4-Chlorocatechol	2-Hydroxy-4-chloro-muconic semialdehyde	23, 29
3,5-Dichlorocatechol	2-Hydroxy-3,5-dichloro-muconic semialdehyde	23, 29
3-Methylcatechol	2-Hydroxy-3-methyl-muconic semialdehyde	23, 29
o-Xylene	o-Toluic acid	44
p-Xylene	p-Toluic acid, 2,3-dihydroxy-p-toluic acid	44
Pyrrolidone	Glutamic acid	35
Cinerone	Cinerolone	53
n-Butylbenzene	Phenylacetic acid	13
Ethylbenzene	Phenylacetic acid	13
n-Phopylbenzene	Cynnamic acid	13
p-Isopropyltoluene	p-Isopropylbenzoate	13
n-Butyl-cyclohexane	Cyclohexaneacetic acid	13
2,3,6-Trichlorobenzoate	3,5-Dichlorocatechol	25
2,4,5-Trichlorophenoxy-acetic acid	3,5-Dichlorocatechol	24
p,p'-Dichlorodiphenyl methane	p-Chlorophenylacetate	17
1,1-Diphenyl-2,2,2-trichloroethane	2-Phenyl-3,3,3-trichloropropionic acid	17

always the result of an organism's inability to attack the substrate but often resulted from its inability to assimilate the products of oxidation. Although this observation was correct, it merely described the process of co-metabolism and failed to offer an adequate explanation for the incomplete oxidation of the substrate and accumulation of end products.

Hughes (31) suggested that co-metabolism of halogenated aromatics could result from an inability of the organism to cleave the halogen substituent from the benzene ring and carry metabolism to a point where the carbon could be assimilated. This proposal failed to explain the initial attack on the halogenated·aromatic but was later supported by the work of Kennedy and Fewson (36). These investigators showed that cells possessing the benzoate oxidase enzyme could oxidize only benzoic acid and the monofluorobenzoates. This was attributed to the similarities of the van der Waal radii of the hydrogen and fluorine atoms, since

chloro- and bromo-substitutions gave compounds which were inactive as substrates.

This mechanism of co-metabolism does not appear to be universally applicable in view of the many reports of chloro-, bromo-, and iodobenzoates subject to this action. In addition, co-metabolism of alkyl and aryl-alkyl compounds would tend to eliminate the substituent as the sole cause of this phenomenon.

Tranter and Cain (54) had proposed that the failure of many halogenated aromatic compounds to support growth of bacteria which could oxidize them could result from an accumulation of toxic products. Horvath (25) showed that co-metabolism of 2,3,6-trichlorobenzoate did result in accumulation of 3,5-dichlorocatechol and development of a toxic environment to the cells. However, this explanation is again applicable only to halogenated aromatic compounds. Furthermore, it does not account for the accumulation of oxidation products which eventually result in production

of toxic conditions.

An enzymatic explanation of co-metabolism resulting from work on halogenated catechols was offered by Gibson, Koch, Schuld, and Kallio (21). They suggested that the halogenated dihydroxybenzenes inhibited the enzyme system catalyzing the incorporation of oxygen into the aromatic nucleus by chelating the iron at the active center of the enzyme. Although the biochemical mechanism of co-metabolism did appear to involve enzyme catalysis, inhibition of enzyme action by chelation could not account for the co-metabolic attack of such diverse organic molecules as ethane, halogenated benzoates, halogenated catechols, and alkyl-aryl compounds such as ethylbenzene and propylbenzene.

Reports that enzymes required for complete metabolism of growth-supporting substrates could be induced by non-growth-supporting analogues (6, 10, 22, 27, 31, 51, 55) indicated that initial co-metabolic attack of the analogues involved the same enzyme(s) as that used for oxidation of the growth substrate. In addition, the substrate, in many cases, induced formation of enzymes which co-metabolically oxidized the substituted analogues (27, 33, 36, 44, 54). This provided further evidence for an enzyme common to initial attack of substrate by complete metabolism and co-metabolism of substituted analogues.

This evidence indicated the initial mode of co-metabolic attack but did not account for the incomplete oxidation of substrate and, hence, accumulation of end products of co-metabolism. The actual mechanism of co-metabolism of m-chlorobenzoate by Arthrobacter sp. was elucidated by Horvath and Alexander (27) using whole-cell suspensions and cell-free enzyme preparations. Benzoate-grown cells oxidized m-chlorobenzoate without a lag, and preincubation of cells with the halogenated analogue induced the cells to metabolize both benzoate and m-chlorobenzoate. Also, uninduced cells exhibited the same lag period in oxygen uptake when incubated with either compound. It therefore seemed likely that both benzoate and m-chlorobenzoate were metabolized by the same enzyme system. These results were consistent with those described above.

The product which accumulated in this study, 4-chlorocatechol, did not inhibit oxygen uptake on benzoate, the substituted analogue or unsubstituted catechol, thus eliminating toxicity or enzyme inhibition of the cause of co-metabolism. The relatively unspecific nature of the benzoate oxidase enzyme and the

specificity of the ring-cleaving enzyme for an unsubstituted catechol appeared to account for co-metabolism by this Arthrobacter (27).

This explanation was shown to be applicable to co-metabolism of 2,3,6-trichlorobenzoate (2,3,6-TBA) by a Brevibacterium (25). Oxidation of this herbicide was catalyzed by inducible enzymes through the intermediate products 2,3,6-trichloro-4-hydroxy-benzoate and 2,3,5-trichlorophenol to the end product, 3,5-dichlorocatechol, which accumulated in the medium. This catechol did accumulate to concentrations which resulted in production of a toxic environment to the cells, but toxicity of end products was shown to be the result rather than the cause of co-metabolism. Again, specificity of the ring-cleaving enzyme for an unsubstituted catechol accounted for the accumulation of 3,5-dichlorocatechol and the phenomenon of co-metabolism of 2,3,6-TBA by the Brevibacterium.

This mechanism can be proposed to account for the accumulation of alkyl substituted catechols as well, but no evidence to support this view is available at present. It is likely that specificity of enzymes which catalyze reactions subsequent to the initial oxidation is involved, but definitive evidence to verify this is yet to be obtained.

CO-METABOLISM OF ENVIRONMENTAL POLLUTANTS BY PURE CULTURES OF MICROORGANISMS

Evidence indicating the ecological importance of co-metabolism is rapidly accumulating. It has been suggested that co-metabolism may account for the degradation of many pesticides which do not sustain microbial growth (3), and laboratory data are now available to support this view.

Compounds such as diphenyl aliphatics (DDT and related molecules) and polychloroaromatics [2,3,6-TBA and 2,4,5-trichlorophenoxyacetate (2,4,5-T)] have been described as recalcitrant molecules because of repeated failures to isolate organisms capable of utilizing them as sole sources of carbon and energy for growth. However, co-metabolism of these materials has been shown to occur under laboratory conditions and may be an important process in the removal of these pesticides from the environment.

For example, the acaricide chlorobenzilate, a diphenyl aliphatic structurally related to DDT, was subjected to oxidation by the yeast Rhodotorula gracilis in a medium containing

yeast extract and mannitol (42). The initial degradation step was hydrolysis of chlorobenzilate to 4,4'-dichlorobenzilic acid. This substance was further degraded by decarboxylation and dehydrogenation to the end product, 4,4'-dichlorobenzophenone, which was not subject to further attack.

DDT also appears to undergo microbial degradation by a co-metabolic process. Wedemeyer (57, 58) reported oxidation of DDT to p,p'-dichlorodiphenylmethane, a process which must certainly be co-metabolic since no carbon would be available to support growth of the microorganism. This substance was shown to be subject to further co-metabolic attack by a *Hydrogenomonas* (16). The product resulting from this oxidation, p-chlorophenylacetic acid, established the susceptibility of at least one ring of the DDT molecule to microbial cleavage.

It is obvious from these reports that co-metabolism of these pesticides does not result in a complete mineralization of the molecule to inorganic chloride, carbon dioxide, and water, but it does eliminate the toxicity of the pesticide in the environment. In addition, co-metabolism of the pesticides by natural microbial populations present in the environment may result in complete degradation of the substances.

This idea derives support from consideration of herbicide degradation by co-metabolism. The herbicide 2,3,6-TBA was shown to be oxidized by a co-metabolic process to the end product 3,5-dichlorocatechol (25). The herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T) was also reported to undergo co-metabolic oxidation (24). The product resulting from this oxidation was also identified as 3,5-dichlorocatechol.

This chlorocatechol, resulting from co-metabolism of both 2,3,6-TBA and 2,4,5-T, had previously been shown to be an intermediate in the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) by an *Arthrobacter* and to be completely metabolized by this organism (38). In addition, the co-metabolism of 3,5-dichlorocatechol by *Achromobacter* sp. had been clearly demonstrated (23, 29). Thus, it appears that both 2,3,6-TBA and 2,4,5-T might be completely degraded by the action of two or more microbial species by the processes of co-metabolism and metabolism or by a series of co-metabolic reactions.

Examples of co-metabolism of environmentally important compounds are by no means limited to pesticides. Degradation of the surfactant alkyl benzene sulfonate (ABS) also appears to occur via a co-metabolic reaction series (30). The microbial degradation of ABS represents a unique combination of co-metabolism of a portion of the molecule and complete metabolism of the remainder of the molecule. Initial oxidation of the surfactant apparently required an expenditure of energy since co-metabolism of ABS by the *Pseudomonas* did not occur in the absence of an energy source, glucose. Co-metabolism of ABS by this organism yielded isopropanol from side-chain oxidation and catechol from aromatic ring oxidation. Isopropanol, once formed, was capable of supporting growth of the isolate, indicating complete metabolism of this substance. Desulfonation of ABS occurred by the process of co-metabolism and involved a coupled reaction between phenol, an intermediate product of ABS co-metabolism, and a sulfonated alkyl benzene intermediate. Catechol, the end product of aromatic ring co-metabolism, accumulated in stoichiometric amounts and appeared to result from oxidation of phenol via the coupled reaction.

Although the laboratory investigations cited indicate that degradation of environmental pollutants can be accomplished by microorganisms isolated from natural habitats, care must be taken in extrapolating these findings to natural ecosystems. The co-metabolic phenomena discussed have occurred in pure cultures in the absence of adverse conditions caused by competition. Demonstration of a similar process in soil and aquatic ecosystems is required to establish susceptibility of these substances to microbial degradation under field conditions.

CO-METABOLISM OF ENVIRONMENTAL POLLUTANTS BY NATURALLY OCCURRING MICROBIAL POPULATIONS

Evidence for the co-metabolic degradation of environmental pollutants by naturally occurring microbial populations is now available and indicates the importance of this phenomenon in the ecosystem.

Pure culture studies of Horvath and Koft (30) had established the occurrence of co-metabolism in the degradation of ABS, indicating the possibility of this degradative mechanism under mixed culture conditions. Benarde et al. (7) extended these findings by demonstrating the degradation of ABS under simulated natural conditions, thereby establishing the applicability of data obtained from pure culture studies to the situation obtaining in the biosphere. Degradation of ABS was shown to occur

in an aquarium containing lake water and ABS, to the extent of 90% in 40 days. The addition of an energy source (glucose) to this system resulted in a 100% decrease of the surfactant concentration in only 20 days. Although the participation of co-metabolism was not clearly demonstrated in this study, two factors indicate the occurrence of this phenomenon. The enhancement of the rate of degradation of ABS by the addition of a utilizable energy source implicates the existence of a co-metabolic process in this system. Secondly, the inability of the investigators to isolate from this system a microorganism which could utilize ABS as its sole source of carbon and energy for growth indicates that co-metabolism is responsible for the decomposition of ABS in this report (7).

Similar results were obtained by Horvath (26) with the herbicide 2,3,6-TBA. Decomposition of this chlorinated aromatic substance was accomplished by microbial populations present in lake water under laboratory conditions. Approximately 35% of the 2,3,6-TBA supplied was degraded in 18 days, and this degradation was not accompanied by a significant increase in microbial numbers. The technique of "analog enrichment" (D. D. Focht, *personal communication*) was employed and involved addition of a biodegradable simulant of the herbicide, benzoic acid, to the system. This technique resulted in an increase in both the rate of degradation (35% in 4 days) and total amount of the herbicide degraded (80% in 14 days). The increase in microbial numbers obtained under these conditions was attributable only to the concentration of benzoic acid provided. The lack of microbial growth at the expense of 2,3,6-TBA degradation and the increased rate of decomposition resulting from the addition of benzoic acid clearly demonstrate the role of co-metabolism in this investigation and establish the importance of the "analog enrichment" (D. D. Focht, *personal communication*) technique in biodegradability studies. Additional evidence of co-metabolism comes from the fact that the investigator was unable to isolate, from the natural population, an organism which could grow at the expense of 2,3,6-TBA as its sole carbon and energy source.

The investigations cited indicate that natural microbial populations are capable of effecting a co-metabolic degradation of environmental pollutants. A major objection however, is that these investigations employed conditions which deviate from the natural environment in that both studies involved closed systems. A constant flow system would more closely resemble the conditions found in nature, and research in this area is necessary before definite conclusions can be drawn.

This objection appears to have been overcome in the report of Sethunathan and Pathak (50). These investigators noted a rapid inactivation of diazinon within 3 to 5 days of its incubation with water from a rice field that had received several applications of the substance. Degradation of this pesticide in water from an untreated rice field was not significant during the same time period. The data clearly establish that diazinon treatment had induced a microbial population capable of degrading this compound. Of special significance was the finding that the *Arthrobacter* isolated from paddy water of treated fields was capable of degrading diazinon only in the presence of additional carbon and energy sources, specifically ethyl alcohol or glucose. This clearly shows a co-metabolic decomposition of the pesticide by microbial action under natural environmental conditions.

Admittedly, reports concerning the occurrence of microbial co-metabolism under natural conditions are few in number, but they do demonstrate and emphasize the importance of this phenomenon in the environment. Microbial populations are clearly capable of degrading pollutants by the process of co-metabolism. Furthermore, microorganisms capable of this co-metabolic degradation can be enriched for by repeated applications of the substance (50) or by application of biodegradable analogs of the pollutant (26) to the microbial ecosystem.

The method of "analog enrichment" (D. D. Focht, *personal communication*) may present a valuable technique to be used during the application of pesticides to natural ecosystems. Application of both the pesticide and a biodegradable simulant of the pesticide may allow man to have both the benefit of action of the pest control agent and a rapid oxidation of the compound, thus eliminating the environmental hazard which might otherwise accompany its use (26).

A NEW LOOK AT MOLECULAR RECALCITRANCE

In light of the available data indicating the role of co-metabolism in the microbial degradation of environmentally important compounds, a reexamination of the concept of molecular recalcitrance (2) appears to be in order. This concept assumes that there are

many compounds which microorganisms are unable to degrade, either under any circumstances or at sufficiently rapid rates to prevent their accumulation in the environment (3). The term "recalcitrant" has been used to describe organic pollutants that remain unchanged because of the fallibility of microorganisms (3).

It should be noted that investigations concerning the basis for recalcitrance of compounds are relatively recent, and definitive conclusions cannot yet be drawn (3). In addition, only a few non-biodegradable substances have been identified (5), and there is now some question as to the validity of this label of recalcitrance. Are these compounds truely recalcitrant due to microbial fallibility or are the methods employed in biodegradability studies fallible, thus leading to false conclusions regarding the microbial decomposition of the substances?

It has been stated that microbiologists can find evidence for microbial fallibility in their own discipline. For example, bacterial spores and certain fungi and protozoa can remain viable in a dormant state for years, thus indicating an inability of microorganisms to degrade these structures (4). This evidence for molecular recalcitrance appears to stem from human fallibility rather than microbial fallibility. The term recalcitrant, if it is to be used at all, should be applied to individual organic compounds, not to living systems.

However, the failure to demonstrate biodegradation of some pesticides and detergents did appear to be significant and was interpreted as indicating that microorganisms cannot decompose many of the materials provided to them (3). It now appears that this failure may again be attributed to human rather than microbial fallibility. Application of the term recalcitrant to detergents of the ABS type and pesticides such as 2,3,6-TBA, 2,4,5-T, and DDT may be the result of fallible procedures which do not consider the co-metabolic phenomenon, rather than to inactivity on the part of microorganisms.

Demonstration of biodegradability has been based on three approaches (43), none of which takes into account the possibility of a co-metabolic degradation of the substance under investigation. The first approach is a determination of the ability of some commonly stocked prototrophic microorganisms to grow at the expense of the organic material. Because co-metabolism of a substance yields neither carbon nor energy to the microorganisms involved, this approach clearly eliminates consideration of this metabolic process in the decomposition of the compound.

The second method for evaluating degradability is the "die-away" test (43). This procedure has generally involved relatively rapid spectrophotometric methods (39) to follow the disappearance of ultraviolet absorbance caused by cleavage of the aromatic nucleus of detergents and chloro-aromatic pesticides. The process of co-metabolism has been shown to account for a significant alteration of the ABS molecule, the 2,3,6-TBA molecule, and the 2,4,5-T molecule with a catechol-like compound resulting as the end product in each case. Obviously, spectrophotometric methods would be inadequate for the measurement of degradation of each compound since the aromatic nucleus was not cleaved, and thus no significant change in ultraviolet absorbance could be recorded. Thus, this method would not have detected the oxidation of each compound discussed previously. In addition, use of the die-away test under pure culture conditions eliminates the possibility of complete mineralization by co-metabolism, which appears to be accomplished by mixed cultures (26, 50).

Nevertheless, a die-away evaluation procedure can be employed to detect co-metabolic degradation of a substance if gas chromatographic analysis is used. This method allows detection of any alteration of the molecule under consideration as well as a quantitative measurement of degradation of the substance. The use of natural microbial populations such as found in soil, lake water, or sewage oxidation lagoons is to be encouraged in these studies for obvious reasons.

The third approach in demonstrating biodegradability involves the elective culture method (43). Those organisms capable of utilizing the compound of interest should increase in number during the enrichment period due to the competitive advantage they enjoy in this system. Isolation, by this procedure, of microorganisms capable of utilizing the substance for growth has generally been considered to be a necessary step in establishing that the microbial population is involved in the decomposition process (1). For example, 2,4,5-T has been regarded as a recalcitrant molecule because an isolate capable of growing at the expense of this substance has not been isolated by the elective culture method (1). This procedure again completely ignores co-metabolism as a factor in the decomposition of organic materials, and in fact rules out co-metabolism as a mechanism by

virtue of the requirement for growth at the expense of the substance. The existence of co-metabolism represents a severe drawback to the use of growth as a criterion of biodegradability of substrates. Compounds which had been previously designated as recalcitrant have now been clearly shown to be subject to co-metabolic degradation.

Thus, the inability to demonstrate microbial transformations of certain compounds may result from an emphasis on techniques designed to select for microorganisms that can utilize such compounds as the sole source of carbon and energy rather than from an inability of the microorganisms to actively oxidize these compounds. Results obtained with co-metabolism indicate that the concept of molecular recalcitrance (2) may no longer be valid.

CO-METABOLISM AS A BIOCHEMICAL TECHNIQUE

The process of co-metabolism presents to the microbial physiologist more than merely a new metabolic phenomenon to be investigated. It has been employed as a technique for the biochemical study of microbial aromatic metabolism, and in this regard, may prove to be as valuable a tool as simultaneous adaptation experiments have been (52).

Gibson et al. (21) used co-metabolism as a technique to isolate and identify products resulting from oxidation of halogenated benzenes and p-chlorotoluene when these substances were incubated with Pseudomonas putida. This technique was necessary because both benzene and toluene were oxidized so rapidly by this organism that reaction products failed to accumulate (19, 20). Use of co-metabolism aided study of the mechanism of oxygen fixation into the aromatic nucleus by allowing for isolation of early intermediates in the degradation of these compounds.

Focht and Alexander (16) also employed co-metabolism as a technique in their investigation of the microbial degradation of DDT metabolites. Their findings indicated that p,p'-dichlorodiphenylmethane, a known metabolite in DDT metabolism (57, 58), was oxidized to p-chlorophenylacetic acid by the Hydrogenomonas organism used, thus establishing cleavage of one of the two rings of this substrate.

The microbial oxidation of 2,3,6-TBA was also elucidated by means of co-metabolic techniques (25). The accumulation of 3,5-dichlorocatechol, in stoichiometric amounts, assured complete recovery of the end product and al-lowed for demonstration of the pathway employed by the Achromobacter. Because co-metabolism involves very few oxidative steps, it was possible to determine the sequence of oxygenation, decarboxylation, and dehalogenation reactions involved in 2,3,6-TBA degradation.

Co-metabolism appears to be common in the microbial oxidation of halogenated aromatic substances; thus, it should be possible to obtain accumulation of a variety of halogen-substituted metabolites (28). It has also been observed that bacteria capable of growing on halogenated compounds co-metabolize the corresponding unsubstituted products (38). Thus, accumulation of non-halogenated metabolites should also be possible by exploitation of co-metabolism.

For example, Ribbons and Senior (47) employed this technique for the quantitative estimation of 2,3-dihydroxy-p-toluate by spectrophotometric measurement. This compound occurs as a metabolite in certain aromatic hydrocarbon fermentations, and use of co-metabolism allowed a rapid, sensitive, and specific method of assay for the substance. This technique may thus offer a simple and rapid method for the preparation of biochemicals useful for tracing metabolic pathways (28).

Refinements in co-metabolic procedures may also offer an attractive means of accumulating products important in the fermentation industry. Raymond, Jamison, and Hudson (45) reported the use of anion-exchange resins to significantly increase the yields of acidic products in co-metabolic systems. When the resin was incorporated in shake flasks or agar plates, p-toluic acid, 2,3-dihydroxy-p-toluic acid, and α,α -cis,cis-dimethylmuconic acid resulting from the oxidation of p-xylene by Nocardia sp. accumulated on the resin. Final product concentration was shown to increase with increasing resin concentration, and mineral balances were not affected if the resin was properly conditioned before use.

Co-metabolic accumulation of at least one product important in the pharmaceutical industry has been shown to be economically feasible. Co-metabolism was used in the aromatization of steroids into equilin by Nocardia rubra (49). Resting cell suspensions of this organism converted 19-hydroxy-androsta-4,7-diene-3,17-dione into equilin with yields of 40% equilin for a substrate concentration of 1 g/liter.

Co-metabolism appears to be an ideal technique for aromatization, and specific hydroxylation of many compounds produced by the

fermentation industry and future research should provide many examples of presently used processes which might be carried out more efficiently or more easily by the technique of co-metabolism.

The importance of co-metabolism is not limited to the accumulation of biochemical products. Co-metabolism has also been used for the detection and demonstration of specific enzyme action. Demonstration of the action of catechol-1,6-dioxygenase, a new metacleaving enzyme possessed by an *Achromobacter*, was accomplished by use of the technique of co-metabolism (23, 29). The accumulation of end products allowed for the isolation and identification of metabolites and the determination of specific bonds of substituted catechols which were cleaved by the co-metabolic process. The products identified as 3,5-dichloro-2-hydroxymuconic semialdehyde, 3-methyl-2-hydroxymuconic semialdehyde, and 4-chloro-2-hydroxymuconic semialdehyde which resulted from co-metabolism of 3,5-dichlorocatechol, 3-methylcatechol, and 4-chlorocatechol, respectively, could be formed only by cleavage of the bond between carbon atoms 1 and 6 of the aromatic ring. The importance of co-metabolism as a technique for the study of microbial enzyme specificity and action is clearly indicated by this report (23, 29).

Ribbons and Senior (46, 48) also utilized co-metabolism to determine the site of cleavage of 2,3-dihydroxy-benzoate. These investigators used an analogue, 2,3-dihydroxy-*p*-toluate, which had been isolated as a co-metabolic product from *p*-xylene oxidation (44) as a substrate for 2,3-dihydroxybenzoate oxygenase. Whole cells co-metabolized this analogue and accumulated 2,6-dioxoheptenoic acid. These results were consistent only with ring cleavage of 2,3-dihydroxy-*p*-toluate in the 3,4-position and indicated that the site of cleavage of 2,3-dihydroxybenzoate was also in the 3,4-position.

Applications of co-metabolism as a technique for biochemical and metabolic studies appear to be limited only by the imagination of investigators. Future studies should establish many more uses for this new and potentially valuable technique.

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