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Biodegradation of the Phthalates and Their Esters by Bacteria

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Recent studies on the biodegradation of phthalate esters in natural ecosystems, sewage, and laboratory cultures are reveiwed. There is ample evidence to demonstrate that bacteria are major elements in the biodegradative processes and that in most situations complete oxidation of the aromatic ring occurs; much less is known about the catabolism of the alcoholic moiety, e.g., 2-ethylhexanol. Evidence is presented to support catabolic pathways in pseudomonads and micrococci that are initiated by successive hydrolyses of the diesters to give the phthalate anion. Thereafter a dioxygenase catalyzes the formation of 4,5-dihydro-4,5-dihydroxyphthalate, which is oxidized by an NAD-dependent dehydrogenase to give 4,5-dihydroxyphthalate. Protocatechuate, formed by decarboxylation of 4,5-dihydroxyphthalate, is the substrate for ring cleavage enzymes. Whereas flurorescent pseudomonads use the β-ketoadipate pathway, the nonfluorescent strains and micrococci examined use a *meta*-cleavage (4,5-) route. All the intermediates proposed have been accumulated by enzymes purified from *Pseudomonas fluorescens*. Isophthalate and terephthalate (anions) are readily used as carbon sources by aerobic bacteria, and preliminary evidence is consistent with catabolic routes for these isomers converging at the ring-cleavage substrate protocatechuate. Some possible effects and interactions of synthetic organic chemicals with the natural microflora, and the influence of other vectors, is discussed in relation to the maintenance of the carbon cycle and environmental pollution.

Phthalic acid esters (PAEs) have been synthesized on a massive scale for the last two to three decades, mainly for the formulation of plastics. In 1972 production was in excess of 10^9 lb/yr (*1*). A conference, sponsored by the National Institute of Environmental Health Sciences four years ago, focused attention on the accumulation of phthalic acid esters in the environment, and how this might be potentially disadvantageous for the normal development of many life forms, including man. The papers presented were collectively published (*1*) and two excellent commentary reviews of the meeting appeared in *Science* (*2*) and *Chemical and Engineering News* (*3*). A statement made by Marx (*2*) attracted our attention: "A few scientists apparently believe that the phthalates are relatively biodegradable . . . but most consider that the evidence for biodegradation is still inconclusive." Examination of the evidence for the biodegradation of di-2-ethylhexyl phthalate and butyl benzyl phthalate by activated sludge, reported by Graham (*4*), however, led us to concur with the minority opinion, namely, that these esters are biodegradable. Our position was influenced by the knowledge that *o*-phthalate (anion) is readily used as sole source of carbon and energy by fluorescent (*5*) and nonfluorescent (*6*) pseudomonads, as were the anions of the two isomers, isophthalate and terephthalate (Sherratt and Ribbons, unpublished observations). The isolation and study of the bac-

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teria that grew at the expense of *o*-phthalate led to the formulation of catabolic pathways that had common intermediates, 4,5-dihydroxyphthalate and protocatechuate, but which diverged when 3,4- or 4,5-dioxygenases catalyzed the opening of the benzene ring (Fig. 1). Discovery of the 4,5-dioxygenative cleavage of protocatechuate was largely due to the studies of Dagley and his colleagues with a nonfluorescent pseudomonad (*7-9*) now known to be *Pseudomonas testosteroni* (*10*), that grows with *p*-cresol, and a similar species that was isolated with *o*-phthalate as sole carbon source (*6,9*). Since we knew that *o*-phthalate and its two isomers were readily metabolized by bacteria which are easily isolated from soils and water, we expected that phthalate esters would also be readily metabolized, since esterases frequently possess wide substrate specificities (*11*). Indeed, observations made twenty years ago during studies of biodeterioration of plastics showed that several plasticizers (including phthalate esters) were subject to microbial degradation (*12-14*). This topic has been reviewed by Autian (*15*) and Mathur (*16*); the latter author also observed phthalate ester utilization by *Serratia marcescens*.

During the last three years, a number of reports on the biodegradation of phthalate esters have appeared. These have conclusively shown that phthalate esters are easily biodegradable in the laboratory with pure cultures of bacteria and in several environments with their natural communities of microflora. Most of the studies reported do not allow the conclusion to be drawn

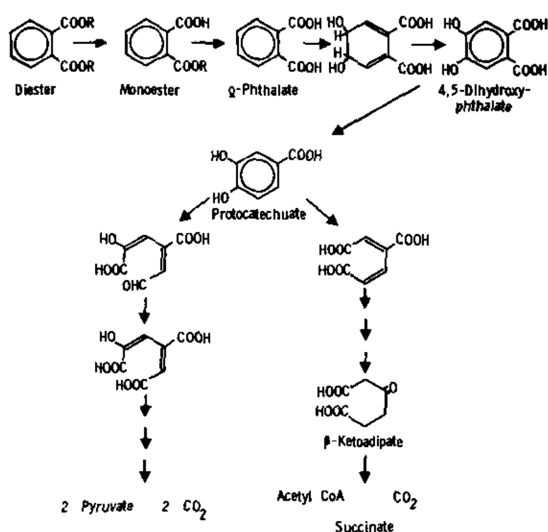

FIGURE 1. Catabolic pathways for *o*-phthalate and its esters.

that the entire molecule is mineralized (or assimilated in the various experimental situations. This point was clearly realized by Engelhardt, Wallnofer, and Hutzinger (*17*), who demonstrated that either the esterifying alcohol or the aromatic moieties of phthalate esters could support the growth of different bacteria or fungi. Several coryneforms, brevibacteria, and pseudomonads isolated with *o*-phthalate (anion), grew with dibutyl phthalate. Evidence was presented to show that monobutyl phthalate, phthalate and protocatechuate were intermediates (Fig. 1). They also made the interesting observation that pure cultures from dibutyl phthalate (DBP) enrichments formed mono-*n*-butylphthalate almost quantitatively as the only metabolite, suggesting that the butanol hydrolyzed from one of the ester groups served as carbon and energy source for growth. That a single hydrolysis may be a biologically important event, is supported by results of studies on the metabolism of phthalate esters by rats. The major metabolites observed by Albro and his collaborators (*18,19*) after administration of di-2-ethylhexyl phthalate (DEHP) were oxidized derivatives of the monoester; free phthalic acid accounted for less than 3% of the metabolites in the urine. The accumulation of mononesters of the phthalates has also been observed in hydrosoils. Johnson and Lulves (*20*) showed that two of the esters, DEHP and DBP, most frequently used, were readily degraded in hydrosoils, but that anaerobic conditions retarded these degradative processes. Of particular interest in the light of the ease of hydrolysis to give monoesters was the observation that after aerobic incubations of [^{14}C-carbonyl]-labeled diesters, monoesters, and anions in these hydrosoils, mono-*n*-butyl phthalate and phthalate anion were completely degraded within 7 days, whereas only 50% of the ^{14}C was recovered as carbon dioxide from the diester during the same period. Since after 1 day exposure of the hydrosoil to the diester about 50% had been hydrolyzed to the monoester, it is possible that the diester may exert a partial inhibition of its own biodegradation under some conditions.

A study of the kinetics of the biodegradation of five commonly used phthalate esters was made by Saeger and Tucker (*21,22*) which convincingly showed that they readily underwent biodegradation. The data indicated that phthalate esters with short (or partially oxidized) alcohol chains, e.g., DBP were more easily degraded than the long chain esters, e.g. DEHP, a finding that was also observed in activated sludge digestions (*22*) and hydrosoils (*20*).

The purpose of this article is to summarize the present state of knowledge about the biochemistry of the transformations of the phthalates brought about by pure cultures of bacteria. Some of the data produced from this laboratory have been reported previously (*23,24*).

Isolation and Identification of the Bacteria

Over 100 enrichment cultures were made with minimal media (*25*) supplemented with the phthalate carbon source (0.1% for the anions and about 1 ml/100 ml of medium for the esters). The media were inoculated with several sources of natural habitat including soils, plant debris, running and still fresh water, raw sewage and marine and brackish waters. Most of these primary sources gave successful enrichments. Media containing the esters were much slower to develop; however pure cultures of the bacteria isolated from them grew easily with the esters. Marine waters did not yield bacteria able to degrade any of the substrates provided, whereas brackish waters did. This probably reflects the inability of marine bacteria to survive or proliferate in the absence of high salt concentrations, since our colleague, Dr. Barrie Taylor of the Marine Institute of this University has obtained numerous phthalate utilizers from enrichments using synthetic sea water basal media, for which they show a dependence.

Gram-negative rods frequently appeared as the predominant bacterial populations in the enrichments with the phthalate anions. However, Gram-positive cocci were the preponderant type seen when phthalate esters were supplied as the carbon source. A few of the cultures obtained as morphologically pure strains were selected for further studies on the basis of their stability and growth rate. Among the Gram-negative strains, both fluorescent and nonfluorescent species were found; their speciation was based on the biochemical and nutritional criteria provided by Stanier et al. (*10*). All of the phthalate ester utilizers selected, were strictly aerobic Gram-positive catalase-positive cocci, and appear to be species of *Micrococcus* (*26*); this classification is based on their inability to produce acid from glucose anaerobically, which distinguishes them from *Staphylococci* (27).

Catabolism of *o*-Phthalate and its Esters: Whole Cell Experiments

The respiratory activities of cells grown with *o*-phthalate (anion or diester) or glucose were measured to indicate the likely inducible catabolic routes used for oxidation of the phthalates. The results for representative strains are shown in Table 1. In most cases tested, both 4,5-dihydroxyphthalate and protocatechuate were oxidized at least as fast as *o*-phthalate when grown with *o*-phthalate anion; 4-hydroxy-*o*-phthalate, a possible intermediate, does not stimulate respiration above endogenous rates, nor does it support growth of the bacteria, whereas 4,5-dihydroxyphthalate and protocatechuate do. None of the substrates stimulate the respiration of glucose-grown cells.

When *Micrococcus* sp.strain 12B is grown with dimethyl phthalate in the presence of yeast extract, the medium turns purple due to the accumulation of metabolites and their possible reaction with Fe^{3+} ions. Extraction of acidified culture filtrates has allowed the tentative identification of two compounds of *o*-phthalate catabolism by this strain, protocatechuate and 3-carboxy-oxalocrotonate, both of which give purple colors with Fe^{3+} ions. The latter would be an expected oxidation product of protocatechuate after 4,5-dioxygenative cleavage (*28*, Fig. 1). Phthalate esterase activity was detected in cells of the Micrococcus grown with phthalate esters or with succinate. Activity was measured by titration of proton release with phthalate esters as substrates, and therefore, phthalate esterase activity is a constitutive property of this micrococcus. However, oxidation of phthalate anion by this strain is accomplished only after growth of the species with *o*-phthalate or some of its esters. One remarkable feature of the respiratory patterns of strain 12B grown with dimethyl phthalate, is the failure of cells to oxidize protocatechuate. Extracts of these cells, however, possess a very active protocatechuate 4,5-oxygenase, which is also inducible.

Enzymic Activities in Extracts of Cells

All of the enzymic activities necessary to account for the reaction sequences proposed in Figure 1 have been demonstrated in extracts of various cells. With extracts of *o*-phthalate-grown *P. fluorescens* strain PHK, *o*-phthalate (in the presence of NADH) is oxidized to β-ketoadipate. Approximately two moles of oxygen are consumed and two moles of carbon dioxide are released for each mole of β-ketoadipate formed from *o*-phthalate. Both 4,5-dihydroxyphthalate and protocatechuate are rapidly oxidized to β-ketoadipate by the same extracts, but only one mole of oxygen is consumed in each case.

Table 1. Oxidation of potential intermediates of o-phthalate, isophthalate, and terephthalate catabolism by washed suspensions of bacteria.

Substrate supplied	O_2 consumed, nmole/min-mg dry cells					
	P. fluorescens, grown on o-phthalate	P. testosteroni EN 5a grown on			P. sp EN_3, grown on isophthalate	Marine bacterium OP, grown on o-phthalate
		o-phthalate	Isophthalate	Terephthalate		
None	29	10	50	7	10	20
o-Phthalate	68	194	50	7	9	90
Isophthalate	27	7	252	7	7	N.D.[a]
Terephthalate	27	7	50	79	60	N.D.
4,5-Dihydroxyphthalate	60	168	N.D.	N.D.	N.D.	96
4-Hydroxyphthalate	28	12	N.D.	N.D.	N.D.	N.D.
4-Hydroxyisophthalate	N.D.	14	130	7	36	N.D.
Protocatechuate	64	237	309	79	18	225
Gentisate	14	40	180	29	7	N.D.

[a]N.D. = not determined.

Whereas 2 moles of CO_2 are released when 4,5-dihydroxyphthalate is oxidized, the oxidation of protocatechuate releases one mole of CO_2 (Table 2). Temporal separation of the proposed decarboxylation of 4,5-dihydroxyphthalate to give protocatechuate was observed during simultaneous measurements of O_2 and CO_2 concentrations in reaction mixtures, with specific electrodes (Fig. 2). Release of carbon dioxide, when 4,5-dihydroxyphthalate is the substrate, precedes oxygen consumption; the converse is apparent when protocatechuate is provided as substrate (not shown).

With extracts of *P. testosteroni* EN5A, pyruvate was found as one end product of protocatechuate and 4,5-dihydroxyphthalate oxidation, and the characteristic yellow absorption spectrum of the product of the 4,5-dioxygenative cleavage was observed (λ_{max} 410 nm at pH values > 7). A similar intermediate accumulated when extracts of *Micrococcus* sp. strain 12B were incubated with protocatechuate, an observation which was in accord with the suggestion that 3-carboxyoxalocrotonate had accumulated in culture filtrates, mentioned before.

Constitutive esterase activities in the whole cell experiments using *Micrococcus* sp. strain 12B (dimthyl phthalate grown) were demonstrated in extracts of cells but not in the supernatants of culture media. With extracts of cells, hydrolysis of dimethyl, diethyl, and dibutyl phthalates occured readily; diethyl phthalate was most rapidly hydrolyzed of the three esters provided.

Table 2. Stoichiometry of gas exchange during oxidation of o-phthalate by extracts and purified enzymes from *P. fluorescens* PHK.

Enzyme	Substrate	O_2 consumed, moles/mole substrate	CO_2 Produced, mole/mole substrate
Crude extract	o-Phthalate[a]	2	2
	4,5-Dihydroxyphthalate[a]	1	2
	Protocatechuate[a]	1	1
Highly purified o-Phthalate 4,5-dioxygenase	o-Phthalate[b]	1	0
Partially purified o-phthalate 4,5-dioxygenase	o-Phthalate[c]	1	0
Crude component C of o-phthalate 4,5-dioxygenase	4,5-Dihydroxyphthalate[a]	1	2
	Protocatechuate[a]	1	1
4,5-Dihydroxyphthalate decarboxylase	4,5-Dihydroxyphthalate[d]	0	1
Protocatechuate 3,4-dioxygenase	Protocatechuate[e]	1	0

[a]β-Ketoadipate accumulates.
[b]Dihydrodiol accumulates.
[c]4,5-Dihydroxyphthalate accumulates.
[d]Protocatechuate accumulates.
[e]β-Carboxy-*cis*-*cis*-muconate accumulates.

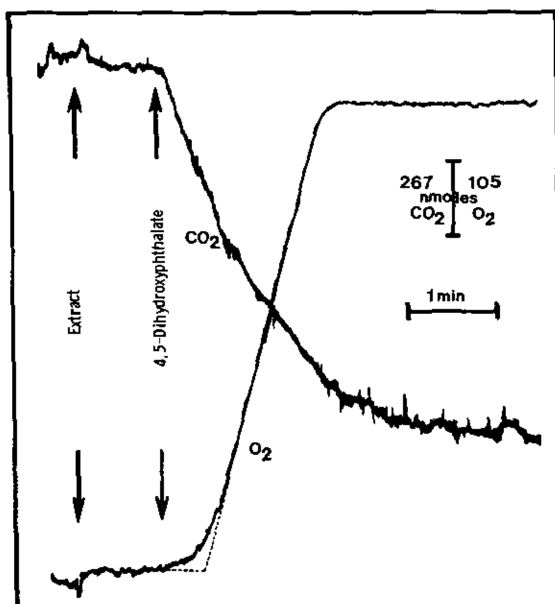

FIGURE 2. Oxidation and decarboxylation of 4,5-dihydroxyphthalate by cell-free extracts of *P. fluorescens* PHK.

Enzymology of the *o*-Phthalate Pathway in *P. fluorescens* PHK

We have purified some of the enzymes induced during growth of *P. fluorescens* PHK in the presence of *o*-phthalate. Two of the enzymes of the *o*-phthalate catabolic pathway, 4,5-dihydroxypthalate decarboxylase and protocatechuate 3,4-dioxygenase (Fig. 1) have been obtained fortuitously in pure form as by-products of a chromatographic procedure used for the purification of one component of the initial enzyme system of the catabolic sequence, namely *o*-phthalate 4,5-dioxygenase. Affinity chromatography was an important final procedure developed for the isolation of homogeneous preparations of the decarboxylase and protocatechuate 3,4-dioxygenase (*24,29*). Separation of the decarboxylase and 3,4-dioxygenase was first observed during chromatography of an unresolved mixture on Sepharose 4B with 4,5-dihydroxyphthalate as ligand. Similar chromatography on columns of Sepharose 4B (with protocatechuate as ligand) allow the preparation of both enzymes in pure form, as indicated by disc-gel electrophoresis after denaturation with sodium dodecyl sulfate (*29*).

The initial enzyme system of the catabolic sequence, *o*-phthalate 4,5-dioxygenase, catalyzes the NADH- and O_2-dependent oxidation of phthalate. It has been resolved into two protein components (designated A and C) by $(NH_4)_2SO_4$ fractionation, both of which are necessary for activity (*24,30*). Each fraction has been substantially purified by chromatographic separations on DEAE-cellulose, Sephadex, and hydroxylapatite. Fraction A is red-brown with absorption maxima at 465 and 320 nm, and fraction C is yellow with absorption maxima at 400 and 460 nm. Polyacrylamide electrophoresis of the fractions indicated that neither of these protein components were yet homogeneous.

The availability of the proteins of the *o*-phthalate 4,5-dioxygenase complex, and the decarboxylase and protocatechuate 3,4-dioxygenase has allowed us to show that a dihydrodiol, presumably 4,5-dihydro-4,5-dihydroxyphthalate (Fig. 1), is probably a metabolite between *o*-phthalate and 4,5-dihydroxyphthalate. When the two purified proteins (A and C) of the *o*-phthalate 4,5-dioxygenase complex are incubated with phthalate and NADH aerobically, one mole of O_2 and of NADH are consumed for each mole of phthalate supplied, and a compound more polar than phthalate accumulates. This compound is not 4-hydroxyphthalate, since the two are well separated by thin-layer chromatography, and their reaction to Gibb's reagent is different. Thin-layer chromatography also excludes 4,5-dihydroxyphthalate and protocatechuate as the accumulated intermediate; additionally this compound does not serve as a substrate for homogeneous preparations of 4,5-dihydroxyphthalate decarboxylase, nor those of protocatechuate 3,4-dioxygenase (*30*). The intermediate does give a positive reaction with triacetylosmate (*31*), and can be converted into a compound with the same mobility as 4-hydroxyphthalate, by heating in acid. Also, β-ketoadipate is formed when crude extracts of *Ps. fluorescens* are incubated with the intermediate accumulated from *o*-phthalate. With less pure preparations of the *o*-phthalate 4,5-dioxygenase system, 4,5-dihydroxyphthalate has been shown to accumulate. The consumption of NADH is less than equimolar with oxygen under these circumstances. This allows the inference that these preparations contain a dihydrodiol dehydrogenase which can use NAD as electron acceptor (Fig. 1), which has been confirmed in subsequent experiments (P. Keyser, unpublished).

Catabolism of Isophthalate and Terephthalate

Much less is known about the pathways used by bacteria for the catabolism of isophthalate and terephthalate, and we believe this is the first report of their biological oxidation. They have received much less publicity as potential environmental pollutants—possibly because they are used in lesser quantities than the orthoisomer. Terephthalate is, however, manufactured on a very large scale as a monomer for the textile industry; it is often removed from industrial effluents as a calcium salt, although the acid itself is not very soluble in water.

Isolation of isophthalate and terephthalate (anions) utilizing bacteria presented little difficulty. All of the bacteria obtained were Gram-negative rods and strictly aerobic. The ability to utilize isophthalate terepthalate was also a property of one strain isolated with *o*-phthalate as carbon source. Protocatechuate was well oxidized by suspensions of cells grown with isophthalate and terephthalate (but not after growth on glucose), whereas other potential ring cleavage substrates (*32*), namely, catechol(s), gentisate(s), 2,3-dihydroxybenzoate(s) were not oxidized.

One strain, isolated with *o*-phthalate as carbon source and identified as *P. testosteroni*, could grow also with either isophthalate or terephthalate. Gentisate markedly stimulated the respiration of this strain, whether or not the cells had been grown at the expense of one of the phthalates. Gentisate 1,2-dioxygenase is known to be a constitutive enzyme in this species (*33*). The only other potential intermediate of those tested, which stimulated respiration, was 4-hydroxyisophthalate, and this was observed only with isophthalate grown cells. Extracts of isophthalate and terephthalate grown cells always possessed high levels of protocatechuate oxygenase activities. Enzyme systems for the oxidation of isophthalate and terephthalate (and 4-hydroxyisophthalate) have not yet been obtained.

These results allow the tentative formulation of catabolic pathways for isophthalate and terephthalate which converge at protocatechuate as the ring cleavage substrate (Fig. 3), like those for *o*-phthalate (Fig. 1); the position of 4-hydroxyisophthalate as a metabolite is quite uncertain; this compound has been shown to be an intermediate in the catabolism of 2,4-xylenol by *Pseudomonas* sp. (*34*). Isophthalate (and terephthalate may be metabolized by a benzoate-type dioxygenase (*35*) to give a hydrodiol intermediate, which is decarboxylated concomitantly with rearomatization to give protocatechuate. Much remains to be learned about these catabolic processes.

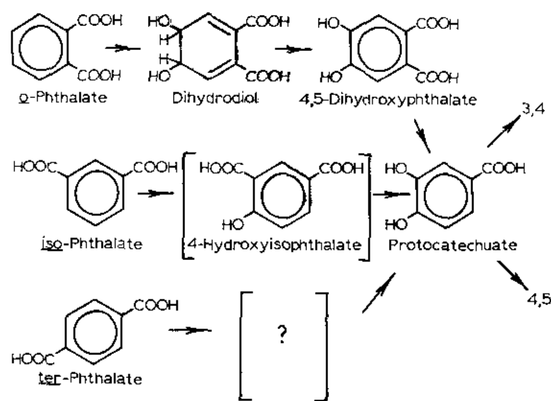

FIGURE 3. Catabolic pathways proposed for the three isomeric phthalates.

Concluding Note

That the phthalates and their esters are easily biodegradable is supported by several recent reports of their disappearence in a variety of natural ecosystems and sewage and the delineation of the catabolic pathways used by pure cultures of bacteria capable of growth with them as sole source of carbon and energy. Photochemical transformations of the phthalates may also occur, but we are not aware of detailed reports of them. Nor is it clear how stable the esters are to chemical hydrolysis, possibly catalysed by clay minerals or other agents. The most important mechanism for the mineralization of organic compounds is by the catabolic activities of microorganisms. The oxidation of organic compounds produced mainly by plants, but increasingly by industrial syntheses represents an essential part of the carbon cycle. The importance of the very diverse catabolic processes used to accomplish this, so that the carbon cycle may be maintained has been well documented by Dagley in a number of recent essays (*36, 37*).

The detection of phthalic acid esters in soils, waters, and animal tissues is not too surprising, in view of the sensitivities of present day analytical methods, and the ease with which they are leached and volatilized from some formulations (*1, 4, 38*). Whether the levels reported are affecting the balance and development of

natural communities of organisms or not, is quite uncertain. The experiments of Metcalf with his elegant laboratory model ecosystems demonstrated that the phthalate esters are quickly accumulated in plant and animal tissues, as are many other lipophilic compounds of recent industrial origin (*39*).

In order to demonstrate that phthalate esters are mutagenic or teratogenic, fairly large doses were administered to animals (*1*, *15*, *16*); the possible subtle toxic effects of the esters on man after long-term exposure to low doses are unknown (*15*). These reports clearly indicated that further research on the metabolism of the phthalate esters is necessary in plants, animals and microorganisms. Some attention ought also to be directed towards the effects of synthetic organic compounds on the natural metabolic activities of the microflora. Additionally, knowledge of the effects of synthetic chemicals on the poise of consortia of microbial populations is necessary, as these are the major agents for the return of carbon in the carbon cycle. In this respect the selection of a number of Gram-positive, catalase-positive cocci from enrichments with phthalate esters as carbon source suggests that these compounds may be partially counter-selective for Gram-negative rods normally obtained with phthalate anion enrichments.

Mixed populations of microbes, rather than pure cultures of bacteria selectively isolated in the laboratory, are likely to be more effective for the complete biodegradation of some chemicals in natural habitats, as has been well-documented by Alexander and his colleagues (*40*), in particular for the degradation of DDT (*41*). Similar consortia of microbes would appear to be responsible for the anerobic digestion of carbon compounds in the rumen (*42*, *43*), and other situations where CH_4 is the ultimate end-product of carbon metabolism. This, like the former example, utilizes different microbes to achieve different portions of the overall transformation. Indeed the outcome of events would be entirely different in the anaerobic systems, were methanogenic bacteria absent, as has been amply demonstrated and reconstructed in the laboratory by Wolin and Bryant (*43*). Furthermore, there is abundant evidence that the course of metabolic processes mediated by the microflora is radically altered by seemingly small and innocuous changes in ionic composition. For example, the presence of sulfate ions tends to depress methanogenesis, not by inhibition of the process, but by allowing other bacteria (sulfate-reducers) to compete for the available reducing power (H_2) produced by fermentation. Similar competitive processes may also occur in aerobic environments during the biodegradation of synthetic chemicals.

Nature has had billions of years to develop mechanisms for the oxidation of compounds which have been biosynthesized. And it is probably true to say that all compounds synthesized by living organisms, are also oxidized by the micro-flora. On the other hand the rapid development of the chemical industry, and in particular that associated with the petroleum industry (*44*) has placed severe demands on, and also umprecedented benefits to, different forms of life. For microbes to become equipped for the recognition, uptake and transformation of the synthetic compounds, many novel to nature, may require time periods orders of magnitude over the one to three decades that have elapsed since their appearance. Evolution of enzymes for new catabolic processes is a slow process, and catabolic pathway evolution would be expected to be very much slower. It is therefore a remarkable fact that many industrial chemicals, which would be considered rare in nature (and even antiseptic) are easily oxidized by bacteria and fungi. The directed selection and evolution of mutant strains for the oxidation or transformation of compounds, which are not utilized by the parent strains has been studied for a number of years (*45*,*46*). The discovery of several systems for gene transfer in the laboratory for the transmission of parts or all of catabolic pathways, inter-species and inter-generic, suggests that this mechanism for acquisition of function may predominate in mixed populations that occur naturally. Clearly, much remains to be learned about the effects, interactions, and fates of synthetic chemicals in natural environments; rational approaches for understanding these processes rests on a more complete knowledge of the catabolic pathways and enzyme mechanisms used by microorganisms, and also the effects that intruding synthetic chemicals have on the normal metabolic activities as they occur in nature.

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