PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Co-Metabolism [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1982 **297**, 481-496
doi: 10.1098/rstb.1982.0056

Phil. Trans. R. Soc. Lond. B **297**, 481–496 (1982)
Printed in Great Britain

Co-metabolism

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There have been numerous instances reported when potentially recalcitrant compounds have been modified by microorganisms or completely mineralized by mixed communities of organisms; an example is pesticide biodegradation. Both situations rely upon the ability of microorganisms to transform compounds that they cannot utilize as sole sources of carbon and energy. This phenomenon of co-oxidation or co-metabolism has been fraught with confusion for many years as a result of the ambiguous use of terms and definitions. A redefinition of co-metabolism is proposed in an attempt to alleviate the problem:

> Co-metabolism – the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound.

The term 'non-growth substrate' describes compounds that are unable to support cell replication as opposed to an increase in biomass. This definition was devised primarily as a result of non-growth substrate metabolism studies with methane-utilizing bacteria. These studies are described in the text. The possible impact of endogenous polymer reserves on co-metabolic events is discussed. A number of examples where non-growth substrate metabolism is of environmental importance are presented, in particular the potential role of methane-oxidizing bacteria in the removal of CO from the environment. The evolutionary significance, if any, of fortuitous metabolism or co-metabolism is discussed, as are potential applications of these phenomena.

Introduction

There are many organic compounds such as chlorinated pesticides that are biologically modified in the environment, but the individual organisms that can use such compounds as carbon or energy sources have been difficult to isolate (Horvath 1972; Alexander 1981). In many instances it appears that several distinct organisms may be responsible in which one organism modifies the substrate such that the second and subsequent organisms can now use the product as a substrate and effect further modifications. In such cases no organism in pure culture would use the substrate as sole carbon and energy source; it was necessary to add an alternative carbon source to provide energy for growth (see Slater & Somerville 1979).

The process whereby a substrate is modified but not utilized by an organism growing on another substrate has been called co-metabolism and in this paper we intend to concentrate on the biochemical, physiological and ecological basis of co-metabolism by pure cultures of microorganisms. Other papers will cover the metabolism of substrates by mixed populations of microbes. This phenomenon has attracted interest in recent years owing to increasing concern over pollution of the environment by widespread use of pesticides and other chemicals, many of which are modified by microbes in co-metabolic processes.

Historical perspectives

Definitive studies on co-metabolism were initiated over 20 years ago when Leadbetter & Foster (1958) observed that the methane-utilizing bacterium *Pseudomonas methanica* would oxidize ethane but was not capable of using it as a sole carbon and energy source. This was the first in a series of papers from Foster's group on the phenomenon of non-growth substrate oxidation by hydrocarbon-utilizers. Leadbetter & Foster (1959, 1960) subsequently observed that when *Ps. methanica* was grown on methane it would concomitantly oxidize different hydrocarbon co-substrates to homologous oxidation products. When ethane was present they found ethanol, acetaldehyde and acetic acid as products; with propane as the co-substrate, propan-1-ol, propionic acid and acetone were detected. Neither the co-substrates nor any of their oxidized products would serve as growth substrates for the methanotroph, which is restricted in its choice of growth substrates to C_1 compounds only. From these early studies Foster concluded that the organism did not grow on the co-substrates because of its inability to assimilate the oxidized products into central metabolic pathways.

Table 1. Examples of co-metabolic activities of pure cultures of microorganisms

organism	growth substrate	co-metabolic substrate	product(s)
Methylomonas methanica	methane	ethane	ethanol, acetaldehyde, acetic acid
Nocardia salmonicolor	hexadecane	toluene	2,3-dihydroxybenzoic acid, α-methyl muconic acid
	hexadecane	*p*-xylene	2,3-dihydroxytoluic acid, *p*-toluic acid
	hexadecane	ethylbenzene	phenylacetic acid
Achromobacter	benzoate	*m*-chlorobenzoate	4-chlorocatechol, 3-chlorocatechol
Corynebacterium	hexadecane	naphthalene	salicyclic acid
	glucose	anthracene	2-hydroxy-3-naphthoic acid
Mycobacterium	propane	cyclohexane	cyclohexanone
Pseudomonas	glucose	limonene	perillic acid, perillyl alcohol
Nocardia salmonicolor	hexadecane	*p*-cymene	cumic acid

This production of oxidized compounds from co-substrates in the presence of the growth substrate was called 'co-oxidation' by Foster (1962). The definition was later expanded by Jensen (1963) to include other reactions, for example dehalogenations, for which he proposed the term 'co-metabolism' and dropped the obligate requirement for the presence of the growth substrate.

Towards a definition of co-metabolism

A review by Horvath (1972) lists many examples of co-oxidative and co-metabolic studies in which the conditions for observing co-metabolism varied quite considerably between different researchers and more often than not a stimulation of respiration was observed rather than true co-metabolism. This highlights a problem pervading this whole area of research, i.e. the ambiguous use of either term in the literature leading to confusion as to which phenomenon is actually being investigated. This confusion has prompted Hulbert & Krawiec (1977) to criticize the whole concept of co-metabolism and co-oxidation on the grounds that the transformations observed (i.e. the transformations of non-growth substrates by microbes) do not constitute novel metabolic events and are indeed manifestations of existing mechanisms of anabolism and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

catabolism. Furthermore, these authors assert that very few observations of co-metabolism have been studied carefully enough to establish that the four features of co-metabolism (which they assembled from Horvath's review) are fulfilled; they merely reflected the bias of the experimenter who happened to observe the unexpected biotransformation of a certain compound.

The four features of co-metabolism were:

(1) the co-metabolite does not support growth of the organism;

(2) products are stoichiometrically accumulated from the co-metabolite;

(3) transformation of co-metabolite is associated with increased oxygen consumption;

(4) transformation of co-metabolite involves adventitious utilization of existing enzyme systems.

Although many of Hulbert and Krawiec's criticisms are valid, we feel, as does Alexander (1981), that maintaining a separate term to describe the phenomenon is justified on the grounds that the environmental consequences of co-metabolism are clearly quite important (see below) and it helps in understanding the wealth of literature devoted to this area of metabolism.

In an attempt to rationalize the observations by researchers in this area and to take account of the above criticisms we have proposed the following definition (Stirling & Dalton 1979):

> Co-metabolism – The transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound.

It is also suggested that the term 'non-growth substrate' be used to describe compounds that do not support cellular division (as opposed to increase in biomass), because it is possible that such compounds could be incorporated into cellular components although they were not essential for growth. The definition of co-metabolism remains true to the Foster definition of co-oxidation but includes other types of reactions in addition to oxidations, and extends the range of co-substrates to include non-growth substrates as well as growth substrates.

We suggest that the transformation of non-growth substrates in the absence of another substrate be referred to simply as fortuitous metabolism, oxidation, dehalogenation, etc., and not be classed as a novel metabolic event.

The biochemical basis of co-metabolism in methane-oxidizing bacteria

The justification for these new definitions has stemmed largely from studies on the methane-oxidizing bacteria. Although there are clearly examples in the literature that could serve equally well to illustrate the various points we wish to make, the choice of these organisms is due in part to the interest that these organisms have engendered from Foster's original work and is a reflection of our own research efforts in this area.

Studies in our laboratory had shown that cell-free extracts of methane-oxidizing bacteria catalyse the NAD(P)H-driven insertion of oxygen into a wide variety of compounds, which included *n*-alkanes, haloalkanes, alkenes, ethers and aromatic alicyclic and heterocyclic compounds (Colby *et al.* 1977; Stirling *et al.* 1979; Stirling & Dalton 1980) (table 2). The enzyme responsible for these conversions was the methane monooxygenase (MMO) (figure 1). Other monooxygenases and dioxygenases have been isolated that also show a broad substrate specificity, although none with such an extensive catholicity as MMO.

Since organisms possessing an enzyme with wide substrate specificity might be important

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

both environmentally, in the biotransformation of many organic pollutants, and industrially, in effecting specific chemical oxidations, we investigated the ability of whole cells of *Methylococcus capsulatus* (Bath) to effect these enzymic transformations (Stirling & Dalton 1979). Of the 31 compounds shown to be oxidized by the enzyme only 5 were oxidized by resting cell suspensions, although 7 more were oxidized in the presence of 4 mM formaldehyde (table 3).

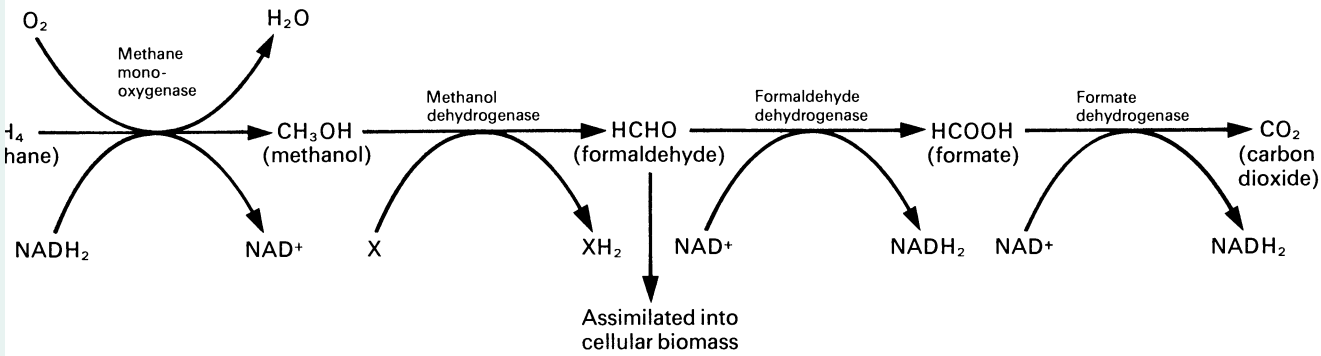

FIGURE 1. Microbial oxidation of methane.

TABLE 2. SUBSTRATES AND PRODUCTS OF METHANE MONOOXYGENASE

substrate	product(s)	substrate	product(s)
methane	methanol, formaldehyde	ethene	epoxyethane
ethane	ethanol, acetaldehyde	propene	epoxypropane
chloromethane	formaldehyde	benzene	phenol, hydroquinone
methanol	formaldehyde	toluene	benzyl alcohol, *p*-cresol
dimethyl ether	methanol, formaldehyde	pyridine	pyridine-*N*-oxide
carbon monoxide	carbon dioxide	ammonia	hydroxylamine, nitrite

None of the compounds that were shown to be oxidized would support growth and replication of the organism incubated at the growth temperature for 10 days. These results suggest that some substrates were not oxidized at all unless there was an exogenous supply of reducing power available. Since the catalytic oxidation of substrate by the MMO in cell extracts required the cofactor NADH it has been assumed that such a reductant is required *in vivo* also. This was satisfied by the oxidation of formaldehyde to carbon dioxide, 1 mol of which will produce 2 mol NADH (Stirling & Dalton 1978). Recent findings by Hou *et al.* (1980) indicate that exogenously supplied methane metabolites would stimulate epoxidation of propylene by methane oxidizers, whereas the oxidation of *n*-alkanes was not stimulated, presumably because the further oxidation of the *n*-alkane by non-specific alcohol and aldehyde dehydrogenases would generate sufficient reducing power for the initial oxygenation.

In those substrates that were oxidized in the absence of formaldehyde, the exogenous reducing power either had no effect on the oxidation rate or it was stimulatory. These particular oxidations did not involve the recruitment of new enzymes and are good examples of fortuitous oxidation by the methane monooxygenase. Furthermore, chloromethane, bromomethane and dimethylether are all oxidized to formaldehyde (Dalton, unpublished; Stirling & Dalton 1980) and therefore could fuel their own oxidation by subsequent oxidation of formaldehyde. In theory, any substrate that could be metabolized to formaldehyde should be a growth substrate

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

for a methanotroph and yet the three compounds cited above are not growth substrates. The resolution of this paradox must await further experimentation, although such possibilities as the production of toxic metabolites, differences between oxidation products *in vivo* and *in vitro*, and the non-involvement of methanol as an intermediate, the oxidation of which has been estimated to provide more than 65 % of electron transport to oxygen in methylotrophs (Anthony 1981), could explain the lack of growth on these compounds.

TABLE 3. OXIDATION OF VARIOUS SUBSTRATES BY WHOLE CELLS OF *METHYLOCOCCUS CAPSULATUS* (BATH)

(From Stirling & Dalton (1979).)

	oxidation rate (nmol mg^{-1} d.m. min^{-1})		
substrate	no formaldehyde	+4 mM formaldehyde	product
chloromethane	170	170	
bromomethane	88	88	
dimethyl ether	7	125	
diethyl ether	0	16	
carbon monoxide	0	520	carbon dioxide
ethane	0	25	acetaldehyde
propane	0	4	propionaldehyde
ethene	4	22	epoxyethane
propene	6	22	epoxypropane
1-butene	0	23	1,2-epoxybutane
cis-2-butene	0	27	*cis*-2,3-epoxybutane, *cis*-buten-1-ol
trans-2-butene	0	52	*trans*-2,3-epoxybutane, *trans*-2-buten-1-ol

TABLE 4. OXIDATION PRODUCTS OF HALOALKANES AND DIMETHYL ETHER BY MMO

(From Stirling & Dalton (1980) and unpublished observations.)

$CH_3Cl \longrightarrow HCHO$
$CH_2Cl_2 \longrightarrow CO$
$CHCl_3 \longrightarrow CO_2$
$CH_3Br \longrightarrow HCHO$
$CH_3{-}O{-}CH_3 \longrightarrow CH_3OH + HCHO$

Substrates that were oxidized only in the presence of a methane metabolite (Hou *et al.* 1980) or methane itself (Higgins *et al.* 1979) are examples of co-metabolism as defined here.

Whole cell oxidations of a much wider range of substrates have been observed in the methane-utilizer, *Methylosinus trichosporium*, by the Kent group (Higgins *et al.* 1979). Using higher cell densities and longer incubation times than we used they have reported oxidations in the presence and absence of methane. Furthermore, cultures grown under carbon-excess conditions generally contained up to 25 % of cell dry mass as poly-β-hydroxybutyrate (PHB), whereas carbon-limited cells contained less than 0.5 % (Best & Higgins 1981). Since a loss of PHB has been correlated with a loss of the ability of methane oxidizers to oxidize non-growth substrates (Thomson *et al.* 1976), then cells grown under carbon-limiting conditions might not be expected to fortuitously oxidize non-growth substrates owing to the absence of readily generated $NADH_2$ for the MMO.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The absence or presence of endogenous reserves may therefore provide a physiological explanation for fortuitous metabolism and co-metabolism.

Cells in which endogenous reserves may be readily mobilized to provide cofactors (e.g. $NADH_2$ and ATP) for enzymes such as MMO would be able, fortuitously, to transform non-growth substrates in the absence of an exogenous supply of energy. Cells that do not form these polymers (either because of an intrinsic lack of the anabolic and catabolic enzymes for polymer metabolism or because of growth conditions) could only transform non-growth substrates fortuitously if a product of the transformation could be used to generate the necessary cofactors required for the initial transformation. Otherwise an exogenous source of cofactor regeneration would be required and would therefore be manifest as co-metabolism.

In this context it is interesting to note that D. Leak in our laboratory has observed that type II methane-oxidizers (which readily synthesize PHB under oxygen-limiting conditions) are capable of active non-growth substrate biotransformation in the absence of exogenous donors. Type I organisms, on the other hand (which synthesize only limited quantities of PHB or none at all in shaking flask cultures), usually required exogenous donors for biotransformations. This fundamental difference between the two types of methane oxidizer may explain why *M. trichosporium* (type II) was able to fortuitously oxidize more substrates than *M. capsulatus* (type I) (Higgins *et al.* 1979; Stirling & Dalton 1979). It is therefore possible that a non-growth substrate could be transformed either by fortuitous or co-metabolic means, depending on the growth conditions. If the organism is grown under conditions in which there are no endogenous reserves to generate necessary cofactors, the non-growth substrate could only be transformed if an exogenous source of cofactor were available. If the cell were rich in these endogenous reserves, no exogenous substrate would be necessary and the non-growth substrate would be transformed fortuitously. In the methane oxidizers it appears that their ability to transform non-growth substrates is due to the extremely non-specific nature of the MMO. There are, of course, many other microbial enzymes that are normally involved in growth substrate metabolism but are relatively non-specific. The result of this is that they will catalyse the conversion of a non-growth substrate to a product that may or may not be a substrate for another enzyme in that organism. If the product is not a recognizable substrate for other enzymes it accumulates; if it is a substrate for a different enzyme then it may be further transformed but there will come a stage when eventually the compound is no longer a substrate for any enzyme and it again accumulates. Thus we may have examples in which a compound is only slightly modified by an organism (e.g. the oxidation of propene to epoxypropane by methane-oxidizers) or it is substantially modified (e.g. the degradation of chlorobenzilate to 4,4′-dichlorobenzophenone by *Rhodotorula gracilis* (Miyazaki *et al.* 1970)). If the organism responsible for the transformation is to grow it must, of course, be supplied with its growth substrate. Although we have considered only enzymes from a co-metabolic point of view that are actively involved in the catabolism of the growth substrate, this is not always so. For example, a species of *Nocardia* was isolated on *n*-hexadecane but was capable of co-metabolizing *p*-xylene to 2,5-dimethyl-*cis*,*cis*-muconate (Jamison *et al.* 1969). The metabolism of *p*-xylene to the co-metabolic product involved a catabolic pathway that was not involved in hexadecane metabolism and was presumably induced by *p*-xylene. A further anomalous situation has been observed in the microbial degradation of the surfactant alkyl benzene sulphonate (ABS) by a pure culture of a *Pseudomonas* species (Horvath & Koft 1972). The organism could not grow on the ABS and therefore required glucose to effect its co-metabolism. However, the energy-

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

dependent metabolism of ABS yielded propan-2-ol from oxidation of the side chain, and catechol from the oxidation of the aromatic ring. The only product to accumulate, however, was catechol because the propan-2-ol was completely metabolized, being a growth substrate for the organism.

The significance of co-metabolism in Nature

Many hydrocarbons and their derivatives are continually released into the environment. These organic molecules rarely accumulate because they are either chemically modified in soils and water or they serve as growth or co-metabolic substrates for microorganisms. Some, of course, persist for long periods and are clearly of ecological concern: these include many synthetic organic polymers that are not modified by chemical or biological activities in the environment and have been referred to as 'recalcitrant' molecules (Alexander 1965). The biological basis for molecular recalcitrance probabaly resides in two factors: firstly the lack of suitable enzymes to effect even a minor modification to the substrate and secondly the inability of the substrate to enter the microbial cell (Alexander 1981), although other possibilities do exist (Slater & Somerville 1979).

It is probably impossible to quantify how much biodegradation of organic molecules proceeds by normal growth metabolism and how much is due to co-metabolism. What is clear is that many compounds will not support the growth of individual organisms but are degraded by them. For example, only a few reports on the isolation of pure cultures that can readily degrade unsubstituted cycloparaffins have appeared in the literature (Imelik 1948; Stirling *et al.* 1977) and yet such compounds are readily degraded by mixed microbial populations in soil (Beam & Perry 1973, 1974). A propane-utilizer, *Mycobacterium vaccae*, was observed to be co-metabolizing cyclohexane to cyclohexanone in the presence of propane, which was then utilized by a cyclohexanone-degrading organism as a source of carbon and energy. *M. vaccae* has subsequently been shown to be responsible for the initial functionalization of a whole range of cycloparaffins (see Perry 1979) to their respective ketones, which would then serve as growth substrates for other organisms. Presumably the non-specific propane monooxygenase, in conjunction with an equally non-specific secondary alcohol dehydrogenase, would produce the ketone, which would not be metabolized further and was therefore excreted by the organism.

Of course, these experiments were performed on growing cultures, which, as we have already seen with the methane-oxidizers, is not necessary if one is to see the transformation of non-growth substrates. Since the organisms responsible for these bioconversions do not need to be increasing in numbers to effect the transformation of a non-growth substrate, we can ask the question, 'What is the significance of fortuitous metabolism and co-metabolism in Nature?'

Most environmental situations impose fairly severe restrictions on microbial growth such that growth rates in Nature are much lower than those attainable in laboratory cultures (Brock 1971), and organisms are frequently either growing extremely slowly or not at all because of the low concentrations of available nutrients. Under such circumstances fortuitous metabolic activities may become significant, particularly if the carbon growth substrate is limiting or absent.

In the methane-oxidizing organisms methane oxidation by whole cells is inhibited by carbon monoxide in what is presumed to be a competitive process (Ferenci 1974; Patel *et al.* 1976).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Now that sufficient evidence has accumulated to suggest that both CO and CH_4 are oxidized by the MMO enzyme (Ferenci *et al.* 1975; Tonge *et al.* 1977; Colby *et al.* 1977), it is presumed that both substrates would compete for the same active site on the enzyme. Therefore under methane-limiting conditions the oxidation of CO to CO_2 may well become significant, particularly since the K_m for CO by whole cells was one fifth of that for CH_4 (Ferenci *et al.* 1975).

Since it has been estimated that microbes are responsible for the removal of between 10 % (Heichel 1973) and 50 % (Liebl & Seiler 1976) of the global CO generated each year, the obvious candidates for this role might well be the aerobic CO-utilizing carboxydobacteria (Zavarzin & Nozhevnikova 1977). However, recent evaluations of the role of authentic carboxydobacteria have concluded that these organisms cannot make any significant contribution to CO removal from the atmosphere at the soil surface (Conrad *et al.* 1981), and it has been suggested that co-metabolic oxidation of CO is the major microbial removal mechanism (Bartholomew & Alexander 1979). The reported K_m values for CO by the methane utilizer *Methylomonas methanica* was one-eighth that of the carboxydobacteria (Ferenci *et al.* 1975; Conrad *et al.* 1981) and their co-oxidation rates are nearly twice as high (cf. Stirling & Dalton 1979; Conrad *et al.* 1981). Furthermore, the numbers of methane-oxidizing bacteria in soils and lake sediments can be in the region of 10^6 to 10^8 per gram (Adamse *et al.* 1972; Whittenbury *et al.* 1976) and would therefore be extremely popular candidates for CO removal from such environments.

TABLE 5. KINETIC PARAMETERS FOR CARBON MONOXIDE OXIDATION

(Data from Ferenci *et al.* (1975), Conrad *et al.* (1981) and Stirling & Dalton (1979).)

	K_m / μM	V_{max} / nmol min^{-1} mg^{-1}
Ps. carboxydovorans	21	226†
Ps. methanica	2.7	85
M. capsulatus Bath	—	520

† Assuming that protein content is 50 % of dry mass.

Because of their ubiquitous nature and ability to transform a variety of compounds either by fortuitous means or by co-metabolic means, the methanotrophs may also be important in the biotransformation of pollutants such as ammonia and halogenated alkanes because these will also act as co-metabolic substrates for these organisms. Indeed correlation between the disappearance of ammonia and the incidence of methane-oxidizers in laboratory 'model' systems has been observed, although unfortunately no measurements were made of other autotrophic or heterotrophic ammonia oxidizers (Whittenbury *et al.* 1976) and so the correlation cannot be absolute. The observations that ammonia and methane are both oxidized by ammonia-oxidizing and methane-oxidizing bacteria and their extracts (Suzuki *et al.* 1976; Hutton & Zobell 1953; Dalton 1977; O'Neill & Wilkinson 1977; Drozd *et al.* 1978), although at different K_m and V_{max} values, suggest that both groups of organisms could be competing for the same substrates in the environment. Under these circumstances a number of factors would be important in determining which organism would predominate. These include the overall K_s and V_{max} for the substrate by the individual microbes, the population size, and the availability of growth nutrients (for a complete discussion of these factors see Harder & Dijkhuizen, this

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

symposium). Direct measurements of these variables are often difficult in natural environments, so one can only gauge the microbial contribution from studies on laboratory cultures, which give a potential rate for co-metabolism, and then attempt to correlate these figures with what is observed in Nature.

Evolutionary significance of co-metabolism

Although plausible explanations have been put forward to explain why organisms do not grow on transformable non-growth substrates (Horvath 1972), the reason and significance why the non-growth substrate should be transformed at all is a subject of some conjecture. Obviously the ability of an enzyme to modify a variety of substrates other than its physiological catabolic substrate is of major importance in the manifestation of co-metabolism. For example, the broad substrate specificity of MMO confers upon methane-oxidizers the ability to oxidize many different non-growth substrates to non-assimilable products. Because the enzyme requires NADH to effect these transformations the metabolic significance of these reactions must remain obscure since the requirement for energy clearly represents a drain on cells that are already limited in their supply of NADH (Anthony 1978). On the other hand, it has been argued that the MMO is responsible for the initiation of useful metabolism (Higgins *et al.* 1980). For example, the ω-hydroxylation of alkanes, followed by further oxidation to fatty acids and thence β-oxidation to yield reducing equivalents and eventually acetate, could supplement metabolism or facilitate survival under severe methane limitation. Indeed it has been demonstrated that *Methanomonas methanooxidans* will incorporate acetate into lipids and amino acids (Wadzinski & Ribbons 1975) so that the ability to oxidize higher *n*-alkanes may have some survival value to the organism. Unfortunately these organisms cannot grow on alkanes other than methane, and because they lack isocitrate lyase and malate synthase (Trotsenko 1976) they cannot use acetate as a carbon and energy source. If, as has been argued, there has been a selection pressure for the evolutionary development of a broad-specificity MMO to allow the organism to scavenge other carbon sources, then one might have expected to turn up at least one methane oxidizer with the ability to grow on higher *n*-alkanes or any of the co-metabolic substrates discussed earlier. To date, the only methane utilizer that grows on heterotrophic sources (none of which are modified by MMO) is a facultative strain in which the MMO is apparently plasmid-encoded and readily lost when grown on substrates other than methane (Hanson 1980; O'Connor 1981). Clearly one can make a case for the retention of an MMO that can hydroxylate higher *n*-alkanes also; however, the intrinsic ability of the enzyme to oxidize many compounds that are not even structurally related to alkanes and which are not transformed into metabolically useful products must require some other explanation.

Jensen (1976) has argued that primitive organisms possessed enzymes that had a broad substrate specificity that enabled them to react with a wide range of related substrates. Subsequent elaboration of additional enzymes would then permit a higher degree of specialization and improved metabolic efficiency. Thus substrate ambiguity in enzymes offered primitive cells the opportunity to develop metabolic pathways for substrate utilization with minimal gene content. Obviously a large proportion of substrates metabolized by the primitive enzymes gave rise to products that were not further utilized by any of the other enzymes in the cell, and selective pressures forced the organism to maximize its growth efficiency on one or a few particular substrates.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

There are many elegant laboratory studies to show that enzymes do evolve (see, for example, Hegeman & Rosenberg 1970; Clarke 1974; Jensen 1976) in which a pre-existing enzyme of broad substrate specificity is recruited to perform a novel role, often by a single mutational change. Accompanying this change in the reactivity of an enzyme to its new catalytic function there may be a loss of normal regulation, thereby allowing the evolved enzyme to function metabolically with a new substrate.

What we may be observing with many catabolic enzymes like MMO is that their broad substrate specificity is giving the organism the opportunity to react to changes in availability of carbon substrates in the environment and is manifested either as fortuitous metabolism or co-metabolism.

TABLE 6. CHANGES IN ENZYME SPECIFICITIES AS A RESULT OF POINT MUTATIONS IN AN EVOLVED β-GALACTOSIDASE (*ebg*) OPERON (AFTER HALL 1981)

		V_{max}/K_m† for various substrates					
class	number of point mutations in *ebg*A gene	*o*-NPG	*p*-NPG	lactose	lactulose	galactosyl arabinose	lactobionate
0	0	2730	1530	4	1	0.81	0
I	1 (region I)	4013	2810	160	2	12.7	0
II	1 (region II)	23300	21800	40	73	14	0
IV	2 (region I and region II)	55923	28655	1805	55	244	6.7
V	3	21500	14800	850	33	70	123

† V_{max} is in reciprocal milligrams; K_m is in millimolar substrate concentration.

The substrate specificity of some enzymes in microbes is clearly changed when subjected to the appropriate environmental stress, and enzymes have evolved to their new function often to the detriment of their previous function (Senior *et al.* 1976; Hall 1981). Some enzymes, however, that show substrate ambiguity have not managed to overcome the co-metabolic consequences of their active site chemistry. For example, the ribulose 1,5-bisphosphate carboxylase (RuBPCase) found in all autotrophs, both prokaryotic and eukaryotic, has both carboxylation and oxygenation functions. The prime anabolic function of the enzyme is to fix carbon dioxide into phosphoglyceric acid in the presence of ribulose 1,5-bisphosphate. The oxygenase activity, which still requires the presence of carbon dioxide as activator, yields phosphoglycollate, which in many organisms is excreted as glycollate. This oxygenase activity has not been eliminated over a long period of biochemical evolution and is a wasteful process for these cells. Some organisms have reconciled this inevitable consequence by evolving mechanisms to harness the glycollate thus produced. For example, in higher plants and algae the serine–glycine pathway plays an important role in the assimilation of this glycollate. A similar pathway may operate in the chemolithotroph *Thiobacillus neapolitanus* (Beudeker *et al.* 1981) although incorporation of glycollate into malate was probably more important. Interestingly *M. capsulatus* Bath, which also has an RuBPCase, has the rudiments of a serine cycle, which may be involved in glycollate assimilation (Taylor *et al.* 1981). The RuBPCase from anaerobic photosynthetic bacteria also has an oxygenase function, but because oxygen is absent

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

from their ecological niche there has been no environmental pressure on them to evolve glycollate-scavenging mechanisms and so they are absent in these cells.

A similar situation is found in organisms that can fix dinitrogen. Nitrogenase, the enzyme responsible for the reduction of dinitrogen to ammonia, produces dihydrogen as an intrinsic part of its mechanism. Dihydrogen production by nitrogenase is an ATP-dependent process that represents a loss of energy to the cell. These organisms have elaborated a dihydrogen-recycling system, probably via a respiratory cytochrome chain, to produce ATP and aid in dioxygen protection of the nitrogenase (Smith *et al.* 1976; Bothe *et al.* 1977; Emerich *et al.* 1979).

TABLE 7. DUAL ACTIVITY OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE

$$\text{D-ribulose-1,5-bisphosphate} + CO_2 + H_2O \xrightarrow[\text{carboxylase activity}]{Mg^{2+}} \text{2,3-phospho-D-glycerate}$$

$$\text{D-ribulose-1,5-bisphosphate} + O_2 \xrightarrow[\text{oxygenase activity}]{Mg^{2+},\ CO_2} \text{3-phospho-D-glycerate} + \text{2-phosphoglycollate}$$

In some cases the co-metabolic substrate may well be toxic to the organism whereas the transformed product is not. Under these circumstances the organism that possesses the co-metabolic ability will have some selective advantage over those that do not. On the other hand, co-metabolic activities often result in the formation of toxic products from relatively non-toxic substrates (e.g. the conversion of phenolic compounds to quinones, the production of methylated mercury from elemental mercury, the formation of reactive epoxides from alkenes, and the oxidation of arylhydrazines to the toxic diazene derivatives) and often represent an energy drain on the cell. The formation of lethal halogenated catechols and acyl halides, which are conceivably produced from halogenated substrates by *Pseudomonas*, arise as a result of the co-metabolic activities of the *meta*-cleavage enzymes in these organisms and has, in fact, been used as a method for obtaining mutants defective in this pathway (Wigmore & Ribbons 1981). Obviously this happenstance co-metabolic synthesis of lethal compounds is detrimental to the cell and may contribute to the biological recalcitrance of halogenated compounds in Nature.

It seems, therefore, that substrate ambiguity of an enzyme, which appears manifestly as co-metabolism, can be detrimental to the cell unless other mechanisms have been developed to allow the co-metabolic products to be utilized. An organism faced with two substrates that could be transformed by a single enzyme in the cell would enter a competitive situation in which the K_s and V_{max} for each substrate would play an important part in determining how much of each was transformed. It does not always follow that the natural catabolic substrate is preferentially oxidized. For example, the K_s values for methane and carbon monoxide by methane-oxidizing cells were 15 and 2.7 μM and the V_{max} values were 100 and 85 nmol mg^{-1} min^{-1}, respectively (Ferenci *et al.* 1975).

Therefore we contend that the broad substrate specificity of certain enzymes may be a consequence of the rather non-specific nature of the active site(s) of the enzyme and that the advantage this confers upon an individual cell is to allow it the potential of being able to adapt to a new environmental situation. This presumes, of course, that the new situation is not of an ephemeral nature.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Possible applications of co-metabolism

Horvath (1972) has already indicated that co-metabolism may be used as a biochemical technique for establishing the mode of action of substrate-ambiguous enzymes. He suggested that applications of co-metabolism for biochemical and metabolic studies were limited only by the investigators' imagination. In this respect Horvath has been quite correct. Since 1972 there have been countless reports on the use of this phenomenon in the investigation of enzyme mechanisms, enzyme evolution, metabolic pathway elucidation, strain construction, pesticide and toxic chemical breakdown and, quite importantly in this biotechnological age, the production of industrially important compounds.

It is in this latter respect that a considerable amount of interest has been aroused in recent years. It was Foster (1962) who recognized that the phenomenon of co-metabolism could have potential technological use, and Quayle (1980) who suggested that microbes with co-metabolic capabilities be given the apt term 'chemical cutting tools'. These chemical cutting tools have, of course, been used for many years by the pharmaceutical industry for the production of modified steroids (Marsheck 1971). Microbes have been isolated that are capable of modifying the steroid but are not capable of assimilating it. The reason that microbes should transform such complex molecules but not grow on them may be the presence of steroid-transforming enzymes that act as detoxifying agents to protect the cells against the deleterious effects of the steroid. Judging from the number of modifications to the steroid nucleus that are effected by different microbes, it is possible that these transformations are the result of enzymes involved in similar reactions in the cell and can therefore be considered as co-metabolic (Marsheck 1971). For example, the fungi, which have been successfully exploited in this respect, can introduce hydroxyl groups into many compounds in addition to steroids. These include substrates such as benzene, chlorobenzene, biphenyl and naphthalene, as well as D-α-pinene by a number of mechanisms, including hydration at a double bond, oxygenation at an allylic position to a double bond or direct oxygenation at a double bond (Cain 1980).

The biotransformation of a number of organic compounds of interest to the chemical industry is already the subject of many papers and patent applications (see Higgins *et al.* 1980), not least of which are those catalysed by the methane-oxidizing organisms. Many of the oxygenation reactions catalysed by the MMO are either difficult or expensive to effect by traditional chemical means such that the co-metabolic activities of these organisms might be commercially exploitable. We have investigated the ability of these organisms to insert an atom of oxygen into several of the substrates given in table 2. The oxidation rate of propene to epoxypropane depends very much on the strain of organism used (figure 2) and could dictate which organism should be used in a commercial process. In the presence of the growth substrate methane, the rate of epoxidation is inhibited by about 50% (Hou *et al.* 1979) as would be expected in a co-metabolic process in which two substrates are competing for the same enzyme. An obvious solution to this problem would be to grow the organism in the presence of a substrate other than methane under conditions that permitted active epoxidation of propene. Methanol is a prime candidate for this role and has been used as a growth substrate for *Methylosinus trichosporium* in batch culture while still permitting active propene oxidation (Best & Higgins 1981). Unfortunately methanol is also a substrate for the MMO (Colby *et al.* 1977) and so it is necessary to ensure that the MMO is not oxidizing this to the detriment of propene.

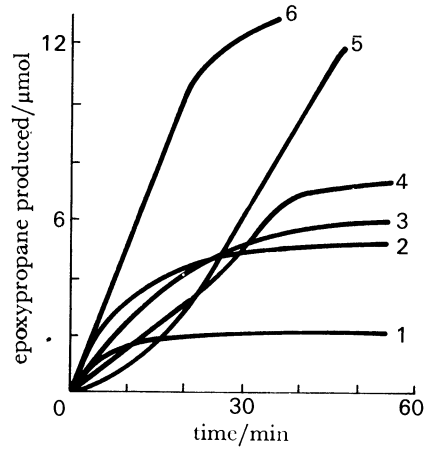

FIGURE 2. Whole cell oxidation of propene to epoxypropane by six different strains of methane-oxidizing bacteria.

There are still many obstacles to be overcome if these organisms, or similar ones, are to be used industrially. However, the possibilities that exist for the industrial exploitation of co-metabolism are, in Horvath's words, 'limited only by the investigators' imagination'.

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Discussion

J. R. Quayle, F.R.S. (*Department of Microbiology, University of Sheffield, U.K.*). I have two questions. The first is: is there any evidence for decreased growth yields in a culture of an organism when a non-growth substrate is added to the growth medium and is co-metabolized by the organism without producing utilizable energy?

The second question arises from the first. If co-metabolism could be deleterious to an organism in terms of reducing its growth yield when presented with a cosubstrate, is there any evidence that the enzyme in question, e.g. methane oxygenase, could be protected from the cosubstrate by morphological means as with RuBP carboxylase in a C_4 plant cell?

H. Dalton. We have maintained cultures of *Methylococcus capsulatus* (Bath) in a methanol-limited chemostat and have observed a decrease in the cell yield in the steady state when the non-growth substrate propylene was fed to the cells. Under these conditions the propylene was quantitatively co-metabolized to epoxypropane. The epoxidation of propylene was catalysed by the methane monooxygenase, which is still present in methanol-grown cells, and therefore consumes energy in the form of reduced pyridine nucleotide. We would presume, therefore, that the extra requirement for reducing power by the MMO is responsible for the decrease in cell yield in the presence of the co-metabolite.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

In reply to the second question, I should say that we do not have any specific evidence for compartmentalization of the enzyme when grown in the presence of non-growth substrates although Scott *et al.* (*J. gen. Microbiol.* (1981) **125**, 63–72) have reported a change in the location of the enzyme in a different methane-oxidizer, *Methylosinus trichosporium* OB3b, between the soluble and membrane fractions of the cell in response to oxygen, but this, of course, is not a non-growth substrate. The Kent group had previously shown the enzyme to be associated with the membranes of the cell, whereas we had only ever found it in the soluble fraction. Presumably the differences in these observations may be due to the oxygen régime during growth.

I. J. Higgins (*Biotechnology Unit, Cranfield Institute of Technology, U.K.*). In relation to the possible consequences of generation of toxic compounds by methanotrophic co-metabolism, Professor Quayle raised the question of whether there is any evidence based on chemostat experiments, which would be expected to show decreased yields. We have not done such experiments but have been more concerned with the possibility that co-metabolism of some methane analogues, e.g. higher alkanes, may lead to increases in yield under C_1 limitation conditions. We have proposed that such metabolism might be called 'supplementary metabolism' in the event that the co-metabolized substrate yields some utilizable carbon and energy. We have evidence from chemostat experiments that such growth yield increases do indeed occur, but they are relatively small. We feel that this aspect is more important from the ecological point of view since there are substantial concentrations in the environment of potential supplementary metabolites, especially alkanes, while there are few naturally occurring substances that would lead to toxic products as a result of methanotrophic co-metabolism. Of course, in recent times substantial quantities of xenobiotics have been released into the environment and it is possible that co-metabolism of some of these may yield substances with increased toxicity to methanotrophs and other microorganisms.

Professor Quayle also raised the question of whether there is any evidence for physiological adaptations to handling the effects of the lack of specificity to methane monooxygenase, such as mechanisms for metabolizing co-metabolic products or isolation of the enzyme in some way, *in vivo*. Our recent studies with *Methylosinus trichosporium* suggest that the location of the enzyme *in vivo* changes in response to growth conditions. Low oxygen tension leads to enzyme that is strongly membrane-bound, with some characteristics differing from enzyme found in soluble fractions of disrupted organisms grown under other conditions. In particular, the rates of oxidation of methane and other substrates are higher in organisms grown under oxygen limitation and the inhibitor profiles of the MMOs are different. However, organisms with both enzyme arrangements show extensive co-metabolic activities, although there are variations in the nature and amounts of products generated.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES