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Strategies of Mixed Substrate Utilization in Microorganisms [and Discussion]

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Strategies of mixed substrate utilization in microorganisms

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In natural and man-made environments microorganisms often grow in the presence of a diversity of functionally similar substrates. The pattern of utilization of these mixed substrates is generally dependent upon their concentration. When substrates are present in high (not growth-limiting) concentrations, sequential utilization and diauxic growth is often observed and the substrate that supports the highest growth rate is utilized preferentially from the mixture. When the substrate concentrations are growth-limiting, simultaneous utilization of the various compounds present in the mixture appears to be the general response. Recent studies on mixed substrate utilization in both batch and continuous cultures have thrown light on the strategies of the control mechanisms that, in microbes, govern the utilization of the various substrates. But perhaps more importantly these studies have indicated the possible significance of mixed substrate utilization in microbial competition in nutrient-limited natural ecosystems.

Introduction

In Nature a multiplicity of different microorganisms is involved in the cycling of the various elements. These organisms not only differ in their morphology and adaptation to various physical environmental variables such as pH, p_{O_2} and temperature, but also display an almost continuous spectrum of physiological types. Studies on the specific role of one type of organism in a certain environment have often involved separation of that organism from the more or less complex community existing in that environment, and the classical batch-type enrichment technique has been most widely used for this purpose (see Veldkamp 1970). However, major drawbacks of this method are that reproducible enrichments are usually confined to organisms of pronounced metabolic specificity and that substrate concentrations must be used that are far in excess of those found in most natural environments. Thus, selection is based on differences in maximum specific growth rate (at substrate saturation) of organisms and it does not immediately follow that organisms isolated in this way play any significant role in the nutrient-limited environment from which they have been isolated.

More recently, continuous enrichment methods have been employed in an attempt to overcome this inadequacy and several studies (see Harder *et al.* 1977; Kuenen & Harder 1982) have indicated that the organisms (e.g. pure cultures or stable communities) isolated in this way do indeed differ from those obtained with the classical enrichment techniques. In the operation of these continuous-flow enrichments nutrient-limited growth is usually established by a single constituent of the culture medium. While this procedure acknowledges that most naturally occurring ecosystems are nutrient-limited, it does not recognize that in many natural and certain man-made environments the growth of microorganisms takes place in the presence of low concentrations of a diversity of mixed substrates. The use of the term 'mixed substrate' in this context refers to the presence of a multiplicity of sources of nutrients that serve a similar physiological function (e.g. carbon source, energy source, nitrogen source). A few examples are

shown in table 1 to illustrate the point. These are obviously the simplest mixtures; in many natural and man-made environments they are no doubt much more complex. In recent years there has been an increasing interest in mixed substrate utilization by microbes. These studies were initiated in recognition of the suggested importance of this process in Nature and in waste water purification systems and also by the fact that culture media for commercial fermentations usually contain complex mixtures of nutrients, whose composition may have a

TABLE 1. SOME EXAMPLES OF FUNCTIONALLY SIMILAR MIXED SUBSTRATES IN CHEMOTROPHIC MICROORGANISMS

substrates	metabolic function	type of organism
glucose + acetate	mixed org. carbon and energy sources	heterotroph
succinate + H_2/CO_2	mixed org. and inorg. carbon and energy sources	fac. chemolithotroph
$H_2 + CO$	mixed inorg. energy sources	(fac.) chemolithotroph
CO_2 + acetate	mixed carbon sources	(fac.) autotroph
$O_2 + NO_3^-$	mixed electron acceptors	denitrifier
$NH_4^+ + NO_3^-$	mixed nitrogen sources	
$PO_4^{3-} + PO_3^{3-}$	mixed phosphorus sources	

significant effect on the yield of a product. Thus, a wealth of new information has been generated, particularly with respect to the control of mixed carbon and energy source metabolism in certain groups of organisms. But perhaps more importantly, very recent studies have indicated that there are in Nature microbes that appear to be specially adapted to growth under mixed nutrient-limited conditions. These organisms have been obtained by continuous-flow enrichments by using mixed substrate limitation, and it seems not unreasonable to postulate that such organisms may play an important role in nutrient cycling in those natural ecosystems in which the turnover rates of various nutrients is low. These recent findings amply illustrate that there are compelling reasons for studies of mixed substrate utilization by microorganisms, particularly under nutrient-limiting conditions.

It is the purpose of the present contribution to discuss mixed substrate utilization by microorganisms at both high and low concentrations of substrate, and to highlight the strategies of the control mechanisms governing the utilization of the various substrates. The possible significance of mixed substrate utilization in microbial competition in nutrient-limited natural environments is also considered. Because of constraints of space we shall limit ourselves to a discussion of selected examples. For more detailed information on other aspects the reader is referred to Harder & Dijkhuizen (1976) and Bull & Brown (1979).

MIXED SUBSTRATE UTILIZATION IN PURE CULTURES

Substrate-sufficient conditions

When bacteria are grown in batch culture on a mixture of two carbon and energy sources, the most pronounced response is that of diauxic growth (Monod 1942), which is caused by the fact that the substrates are used sequentially. Although growth in this manner was first observed on a mixture of two sugars, it is certainly not restricted to these compounds. For instance, *Pseudomonas oxalaticus* grows diauxically in media containing a mixture of two organic acids, acetate and oxalate (figure 1). In the first phase of growth only acetate is utilized. After the

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exhaustion of acetate, in a lag phase of growth, synthesis of the oxalate-metabolizing enzymes occurred, which allowed a second, slower growth phase on oxalate (Dijkhuizen *et al.* 1980). Thus, in this organism, despite its name, acetate is the preferred substrate over oxalate.

Diauxic growth is not the sole response observed when mixed substrate utilization is studied. A number of substrates do not interfere with utilization of each other (the combination may even stimulate growth), in which case the two compounds are metabolized simultaneously.

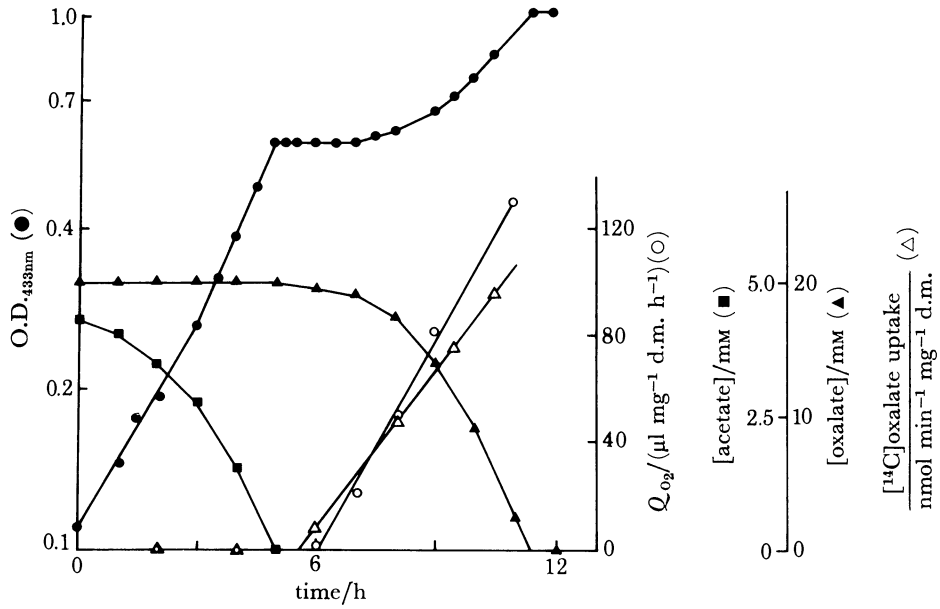

FIGURE 1. Diauxic growth of *Pseudomonas oxalaticus* OX1 on a mixture of acetate (5 mM) and oxalate (20 mM). The inoculum was pregrown on acetate. ■, Acetate concentration; ▲, oxalate concentration; ●, optical density at 433 nm; ○, Q_{O_2} for oxalate; △, rate of [^{14}C]oxalate transport in whole cells. (Data of Dijkhuizen *et al.* (1980).)

Another possibility is that adaptation to the second substrate is not prevented but that its utilization is inhibited. Although this results in a sequential utilization of the two substrates it does not lead to diauxic growth. Examples are growth of *Escherichia coli* on mixtures of glucose and galactose (Standing *et al.* 1972) and glucose and fructose (Clark & Holmes 1976). In general, different bacteria do not give the same response to a combination of two substrates. A combination that gives diauxic growth in one organism may not in another. Furthermore, a substance that is the preferred substrate for one organism may be a secondary substrate for another. Glucose, for instance, often is the preferred substrate for enteric bacteria, whereas organic acids are preferred by *Pseudomonas* (Clarke & Ornston 1975) and *Arthrobacter* species (Krulwich & Ensign 1969). There is generally a relation between the ability of a certain compound to act as a preferred substrate and the growth rate that it will sustain. In most cases, although not invariably, the presence of a substrate permitting a higher growth rate prevents the utilization of a second, 'poorer', substrate in batch culture (see Harder & Dijkhuizen 1976). The metabolic events underlying mixed substrate utilization have been studied in a number of cases and this has contributed significantly to our understanding of cellular control mechanisms and their evolution (see Baumberg 1981). There are at least three different levels at which the utilization of a certain substrate from a mixture can be controlled, namely (*a*) regulation of its

uptake from the medium into the cell, (*b*) regulation of the synthesis of pertinent enzymes, and (*c*) regulation of the activities of these enzymes.

(*a*) *Regulation of substrate uptake*

The addition of glucose to cultures of *E. coli* growing on a wide variety of substrates generally results in the rapid inhibition of their utilization (catabolite inhibition). In many cases this is caused by the interference of glucose with specific uptake systems involved in the utilization of these substrates. An example is growth of *E. coli* on a mixture of glucose and fructose. It was found that glucose inhibited fructose utilization, which caused sequential utilization of the sugars. In this organism glucose and fructose both are translocated via the phosphoenolpyruvate: sugar phosphotransferase (PT) system, and it has been reported that glucose interferes both with the activity and the synthesis of the fructose-specific part of this PT system (Clark & Holms 1976). Studies with mutants (Kornberg & Jones-Mortimer 1977) showed that glucose itself does not inhibit uptake of fructose and various other so-called PT sugars, but that it has to be phosphorylated to become effective. Inhibition of the synthesis of the fructose-specific part of the PT system by glucose is most probably mediated by cyclic AMP (Kornberg & Jones-Mortimer 1977). It is thought that this is due to an interaction between glucose and some component of the PT system that causes inhibition of adenyl cyclase (this enzyme catalyses the production of cyclic AMP from ATP). Saier & Roseman (1976) showed that in *E. coli* and *Salmonella typhimurium*, PT-mediated inhibition also interferes with the uptake of several non-PT substrates, such as melibiose, maltose, lactose and glycerol. This inhibition by glucose and other PT sugars again requires interaction of the sugar with a membrane-bound sugar-specific (enzyme II) component of the PT complex. Although the detailed mechanisms are unknown, the general effect of these phenomena in enteric bacteria is that the presence of glucose blocks the uptake of many substrates. Further induction of their specific catabolic enzyme systems is thereby also prevented; this phenomenon has been given the appropriate name inducer exclusion.

As mentioned above, organic acids are generally 'better' substrates for representatives of the genus *Pseudomonas* in the sense that they allow a higher rate of growth than sugars. In *Pseudomonas aeruginosa*, for example, this preference results in diauxic growth in batch culture on a mixture of citrate and glucose, with citrate used first (Hamilton & Dawes 1959). A similar behaviour was reported for *P. fluorescens* during growth on mixtures of citrate or malate plus glucose (Lynch & Franklin 1978). A detailed and elegant study of the regulation of glucose metabolism in *P. aeruginosa* was made by Dawes and coworkers (Dawes *et al.* 1976). This organism possesses a dual pathway system for glucose catabolism. One pathway that is expressed under glucose-limiting conditions has a high-affinity glucose transport system (K_m = 8 μM), and intracellular glucose is converted via glucose 6-phosphate and 6-phosphogluconate. The other pathway, which is present when the extracellular glucose concentration is high, involves the oxidation of glucose by periplasmic glucose dehydrogenase (K_m = 1 mM) and gluconate dehydrogenase to gluconic and 2-oxogluconic acids, which are taken up by the cells by specific transport systems. The inducible (high-affinity) glucose transport system is strongly inhibited by citrate, gluconate and a number of other organic acids. This explains the observed preferential utilization of organic acids from mixtures of these acids and glucose in batch cultures of this organism. The finding that gluconate inhibits glucose uptake has interesting implications for the metabolism of glucose when it is present as the sole carbon and energy source, in that it leads to

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extracellular accumulation of gluconate and 2-oxogluconate. The significance of this is not entirely clear, although two explanations have been suggested (Dawes *et al.* 1976; Bull & Brown 1979). Interference with glucose uptake by various compounds in a way similar to that observed in *P. aeruginosa* has also been reported for *Arthrobacter crystallopoietes* (by succinate), *Thiobacillus intermedius* and *T. novellus* (by thiosulphate) (Krulwich & Ensign 1969; Romano *et al.* 1975; Matin *et al.* 1980).

(*b*) *Regulation of enzyme synthesis*

In catabolic pathways there are usually two kinds of control, responsive to the presence or absence of substrate and to levels of metabolites or energy within the cell, that regulate the synthesis of the various enzymes. This may be illustrated by a well known example of diauxic growth, namely that of *E. coli* on glucose and lactose, with glucose used first. The regulation of the synthesis of the lactose enzymes, whose genes are linked together in an operon on the chromosome, has been studied in detail (see Beckwith & Zipser 1970). Upon the addition of lactose as the sole source of carbon and energy to uninduced cells, part of it is translocated into the cell, by a specific permease, and converted into the actual inducer, allolactose, by β-galactosidase. Initially, low levels of these enzymes are present in the cell. Inducer production, however, inactivates the repressor molecule, which is bound to the operator site, and permits binding of the RNA polymerase to the promotor thus facilitating transcription of the *lac* operon. This results in the synthesis of high levels of the lactose enzymes. However, during growth on glucose plus lactose, transcription of the *lac* operon is blocked, indicating that the presence of the inducer in the medium is not a sufficient condition to initiate enzyme synthesis. In addition to inducer exclusion (see above), further control mechanisms exist. Glucose is not directly responsible for this negative effect, but its metabolism leads to a lowered intracellular concentration of cyclic AMP. This compound, in a complex with a specific protein called catabolite-gene activator protein (CAP), must bind to the promotor of the *lac* operon before RNA polymerase can bind and initiate transcription. This inhibitory effect of glucose on the synthesis of enzymes coded for by the *lac* operon, and on several other glucose-sensitive systems, has been termed (carbon) catabolite repression (Magasanik 1961). The signal metabolite for this in enteric bacteria is cyclic AMP. Its physiological effect is that it prevents any unnecessary synthesis of catabolic enzymes in cells that already have available a carbon and energy source that allows fast growth. Glucose not only represses synthesis of the lactose enzymes, but, when added to a culture growing on lactose alone, also strongly inhibits any existing enzyme activities (catabolite inhibition). These combined regulatory mechanisms cause diauxic growth on a mixture of glucose and lactose. Only after the exhaustion of glucose does the culture behave as if it were freshly transferred to lactose and exhibits a considerable lag phase before all cells resume growth. Catabolite repression in *E. coli* is not restricted to glucose, nor is *lac* the only sensitive operon. Other examples are the arabinose (Englesberg 1971) and the histidine (Magasanik 1976) operons. In pseudomonads, succinate is among the substrates producing a most pronounced catabolite repression (Clarke & Ornston 1975). It represses the synthesis of inducible enzymes required for the catabolism of many compounds, e.g. acetamide, histidine and mandelate.

A more complex situation arises when one of the organic compounds in the mixture contains both carbon and nitrogen atoms (e.g. amines, amides and amino acids). Often such compounds can be used both as a carbon and energy source and as a nitrogen source. Synthesis of enzymes

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involved in the utilization of these substrates may therefore be dependent not only on the energy status of the cell but also on the nitrogen status. A classical example is the regulation of histidine catabolism in *Klebsiella aerogenes* (Magasanik 1976). The histidine-degrading enzymes are induced by urocanate, the product of the first enzyme, histidase. Neidhardt & Magasanik (1957) showed that cells of *K. aerogenes*, when incubated in mineral medium with glucose, ammonium sulphate and histidine, do not use histidine until ammonium has become depleted. The synthesis of histidase and the other enzymes of the *hut* operon is strongly repressed under these conditions, whereas succinate is a weaker source of catabolite repression. This repression can be partly relieved by the addition of cyclic AMP (Prival & Magasanik 1971), which shows that the *hut* system is subject to regulation by CAP and cyclic AMP (see above). In the absence of ammonia *K. aerogenes* manages to escape from catabolite repression exerted by glucose and does produce the *hut* enzymes. By using a mutant defective in adenylcyclase, Prival & Magasanik (1971) were able to show that the synthesis of histidase and utilization of histidine is possible in the absence of cyclic AMP. Evidence was obtained that expression of the *hut* operon is activated during ammonia-limitation by glutamine synthetase (Magasanik *et al.* 1974; Tyler 1978). If available in excess, ammonia is usually assimilated via glutamate dehydrogenase (GDH). When the ammonia concentration is low (less than 1 mM), GDH cannot function biosynthetically because it has a low affinity for ammonia (Brown 1976). Under these conditions the GDH level in *K. aerogenes* is low, and ammonia is assimilated via glutamine synthetase (GS). A relation has been found between the levels of GS in the cell and synthesis of the *hut* enzymes (Magasanik 1976) in that the activities of GS and histidase were high in nitrogen-poor media (i.e. with histidine as the nitrogen source) and low in nitrogen-excess media (i.e. with ammonia). Furthermore, mutants unable to synthesize high levels of GS did not synthesize the *hut* enzymes when grown under ammonia-limiting conditions, whereas mutants constitutive for the synthesis of GS possessed high levels of histidase, even when grown in glucose-minimal medium with excess ammonia. It has been reported that the same system regulates the proline and arginine catabolizing enzymes (Magasanik 1976). Recently, some doubt has arisen whether the activator protein is actually GS itself. It has been suggested that the products of three genes, *gln*L, *gln*G (which are closely linked to *gln*A, the structural gene for GS) and *gln*F (unlinked to *gln*A) are involved in regulating various 'nitrogen-controlled' genes in *E. coli* and *S. typhimurium* (McFarland *et al.* 1981). Whatever the precise identity of the activator protein(s) may be, the strategy is clearly to control the synthesis of enzymes involved in the degradation of various amino acids (and possibly other nitrogenous compounds) in relation to the nitrogen requirements of the cell. Thus it appears that with organic compounds containing both carbon and nitrogen atoms, metabolic control functions in such a way that enzymes for their metabolism are only formed when these compounds are required either as a source of carbon and energy or as a source of nitrogen.

In enteric bacteria this type of control of the utilization of nitrogen-containing compounds appears to be fairly general. Little is known about this in other microorganisms, although there is evidence that in *P. aeruginosa* (Leslie & Neidhardt 1967) and in *Arthrobacter* P1 (Dijkhuizen *et al.* 1981) a similar mechanism might operate. The latter organism is able to utilize methylamine as a source of carbon, energy and nitrogen (Levering *et al.* 1981). In media containing glucose, methylamine and ammonia, growth was diauxic, with glucose used first. The enzymes of methylamine metabolism, a specific amine carrier and amine oxidase, were only synthesized when the glucose concentration had dropped to a low level. In the absence of ammonia in the

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medium, simultaneous utilization of both glucose and methylamine was observed. These results indicate that relief of catabolite repression, exerted by glucose, on the metabolism of nitrogen-containing secondary substrates, may be a common response under nitrogen-limiting conditions.

(*c*) *Regulation of enzyme activity*

Control of the activity of existing enzymes, which may be considered as a 'quick action' control mechanism, plays an important role in regulating the flow of metabolites to suit the needs of the cells (see Baumberg 1981). Unfortunately, only a few cases of the involvement of this mechanism in the regulation of mixed substrate utilization have been reported. One example is, as discussed above, the strong inhibition of several substrate uptake systems in the presence of a second, preferred, substrate. In many bacteria the activity of glutamine synthetase is regulated via a reversible, enzyme-catalysed, adenylylation and deadenylylation (Tronick *et al.* 1973), dependent upon the nitrogen status of the cell. In the presence of excess ammonia the enzyme is largely in the inactive, adenylylated form. Ammonia deficiency activates the enzyme by deadenylylation and also results in an increase in the level of GS (see Tyler 1978). As discussed above, this increase in levels of active GS is generally accompanied by the activation of transcription of several operons coding for enzymes involved in the catabolism of amino acids.

Many other examples of the regulation of existing enzyme activities by modification or modulation (allosteric enzymes) are known (see Baumberg 1981). For instance in *Alcaligenes eutrophus* H16 (and in many other hydrogen bacteria) hydrogen, the inorganic energy source, suppresses the utilization of several organic substrates (Blackkolb & Schlegel 1968*a*). Thus, incubation of fructose-grown cells in fructose media placed under a hydrogen–oxygen atmosphere caused inhibition of the utilization of this sugar. No inhibition by hydrogen of gluconate utilization was observed, which indicated that hydrogen caused inhibition of the reactions leading to this compound. Blackkolb & Schlegel (1968*b*) subsequently showed that glucose 6-phosphate dehydrogenase in *A. eutrophus* is strongly inhibited by ATP and NADH, both of which are generated during hydrogen oxidation (Bowien *et al.* 1974). Although the strategy of this type of control is not entirely clear, it may be speculated that its major function is to prevent accumulation of metabolites to 'traumatic' levels that would embarrass the metabolic machinery of the cell.

Substrate-limiting conditions

Most of our knowledge of regulatory mechanisms involved in the control of mixed substrate utilization has been obtained by studying the behaviour of microorganisms at high substrate concentrations in batch cultures. As discussed above, this often results in diauxic growth with preferential utilization of the substrate supporting the highest growth rate. However, in Nature most substrates are available at low, sub-saturating concentrations and thus the question must be raised whether the regulatory mechanisms observed at high substrate concentrations also play an important role under substrate-limiting conditions. For this purpose continuous culture is a useful tool, since it enables studies of microbial metabolism under steady-state conditions at low concentrations of the growth-limiting substrate (Herbert *et al.* 1956). A number of studies have been reported of the behaviour of microorganisms when exposed simultaneously to two growth-limiting substrates in continuous culture. An example may serve to illustrate the general response observed. In the previous section diauxic growth of *P. oxalaticus* on a mixture of acetate plus oxalate in batch culture was described. Growth on this

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mixture has also been studied in a continuous culture limited by carbon and energy source (Dijkhuizen, unpublished). As shown in figure 2, at dilution rates below 0.15 h^{-1}, *P. oxalaticus* utilized both organic acids simultaneously and completely. The maximum specific growth rate on oxalate alone is approximately 0.20 h^{-1} and on acetate 0.35 h^{-1}. At dilution rates above 0.15 h^{-1} an increasing proportion of the oxalate in the feed remained unutilized by the culture. This was paralleled by a significant drop in the specific activity of oxalyl CoA reductase, which

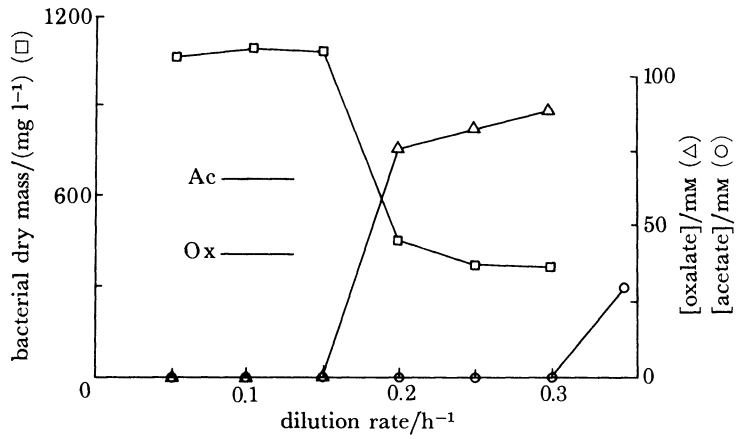

FIGURE 2. Growth of *Pseudomonas oxalaticus* OX1 on a mixture of acetate (S_r = 30 mM) and oxalate (S_r = 100 mM) in a continuous culture limited by carbon and energy. □, Bacterial dry mass on the mixture; △, ○ residual oxalate and acetate concentration, respectively. The dry masses observed during growth of the organism on 30 mM acetate (Ac) or 100 mM oxalate (Ox), separately, are also given. (Data of Dijkhuizen, unpublished.)

is a key enzyme of carbon assimilation during growth on oxalate. In contrast, no residual acetate was detected in the culture supernatant, up to a dilution rate of 0.30 h^{-1}, and the activity of isocitrate lyase, a key enzyme of carbon assimilation during growth on acetate, remained high. The inability to use oxalate at the higher dilution rates was also reflected in the culture dry mass, which decreased to a level (360 mg l^{-1} at D = 0.30 h^{-1}) even below that usually observed (630 mg l^{-1}) on 30 mM (S_r) of acetate alone. This is probably caused by the toxic effect of high oxalate concentrations on the organism (Dijkhuizen & Harder 1975). These results indicate that *P. oxalaticus*, in a continuous culture limited by carbon and energy source, can use oxalate and acetate simultaneously provided that the growth rate is low. Growth at higher dilution rates results in a situation comparable with that observed in batch culture, namely that of impaired utilization of oxalate. Similar observations have been reported by Mateles *et al.* (1967), during growth of *E. coli* on a mixture of glucose and fructose, and Silver & Mateles (1969) during growth of *E. coli* B6 on glucose plus lactose in carbon-limited chemostats. In both cases the two sugars were used simultaneously at low dilution rates whereas fructose and lactose utilization, respectively, was progressively impaired at the higher dilution rates.

In *P. aeruginosa* aliphatic amidase is induced during growth in the presence of its substrate, acetamide. Synthesis of the enzyme is severely repressed by succinate, and in batch culture, during growth on a mixture of succinate and acetamide, this results in diauxic growth with succinate used first (Boddy *et al.* 1967). The underlying regulatory mechanisms were studied in detail by Clarke *et al.* (1968) during growth of *P. aeruginosa* in a carbon-limited continuous culture on a mixture of succinate and acetamide (figure 3). It was found that amidase activity

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increased as the dilution rate was raised and reached a maximum at $D = 0.30$ to $0.35\ h^{-1}$. The observed maximum in amidase activity was thought to result from a balance between induction and catabolite repression. This explanation was supported by work with an amidase-constitutive mutant, which revealed that amidase activity in the mutant increased steadily with decreasing D (figure 3), reflecting the decreasing effect of catabolite repression (Clarke *et al.* 1968).

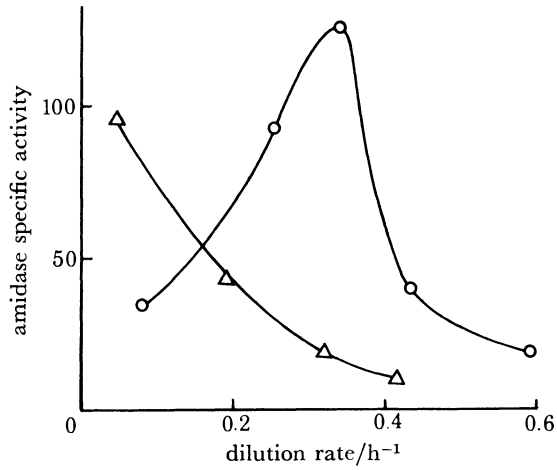

FIGURE 3. Relation between amidase activity and dilution rate during growth of *Pseudomonas aeruginosa* wild-type (○) and a mutant (C11, fully constitutive for amidase) (△) in continuous culture on a mixture of succinate (S_r = 10 mM) and acetamide (S_r = 20 mM). (Data of Clarke *et al.* (1968).)

Except for a few cases it is generally not known in which way and to what extent the utilization of a second substrate influences the metabolism of a certain compound when they are used simultaneously. There is some evidence (see below) to indicate that under certain conditions the metabolism of each of the substrates may proceed completely independently as if the second substrate were absent. There are, however, also instances in which the metabolism of a certain compound is significantly influenced by the presence of another, even when both are utilized to completion. The following examples may serve to illustrate this. In a study of *Beneckea natriegens*, Linton *et al.* (1981) observed that the presence of formate exerted significant effects on the molar growth yield on glucose. This organism cannot grow in mineral media with formate as the sole source of carbon and energy. In a mixture with glucose, however, formate can function as an additional energy source and consequently addition of this compound (30 mM) to the medium reservoir of a glucose-limited chemostat ($D = 0.37\ h^{-1}$) caused the molar growth yield, based on glucose alone, to increase from 84 to approximately 93 g dry mass per mole. A further increase in the formate concentration (to 88 mM) did not result in a corresponding increase in yield but caused accumulation of unused formate (19.4 mM) in the culture.

A more complex situation arises when the utilization of formate from a mixture is studied in an organism that can actually grow on this substrate. This is so for instance in *P. oxalaticus*, an organism that is able to use the energy derived from formate oxidation to fix carbon dioxide via the Calvin cycle. During growth on formate this is the only carbon assimilation pathway and high levels of ribulose 1, 5-bisphosphate carboxylase (RuBPCase) and phosphoribulokinase (PRK) are present in the organism. These enzymes are not detectable during growth on other

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substrates, such as oxalate and acetate, when carbon assimilation occurs via heterotrophic routes (the glycerate pathway and the glyoxylate cycle, respectively). The absence of autotrophic enzymes under these conditions indicated the existence of regulatory mechanisms governing their synthesis. These were studied by growing *P. oxalaticus* on mixtures of formate and various other substrates. In batch culture the general response was one of severe repression of RuBPCase synthesis by 'fast-growth' substrates. On a mixture of formate with acetate, for instance, both

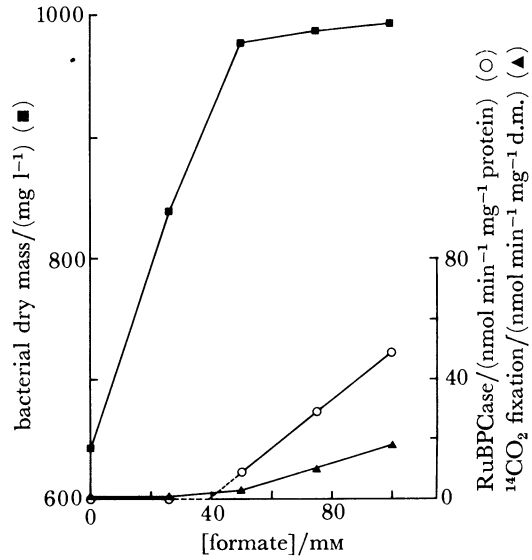

FIGURE 4. Effect of increasing concentrations of formate (0–100 mM) in the reservoir of an acetate-limited (S_r = 30 mM) continuous culture of *Pseudomonas oxalaticus* OX1 at D = 0.10 h^{-1} on a number of steady-state culture parameters. ■, Bacterial dry mass; ○, RuBPCase activity; ▲, $^{14}CO_2$ fixation rate by whole cells. (Data of Dijkhuizen & Harder (1979*a*).)

substrates were used simultaneously, but formate only served as an energy source (Dijkhuizen *et al.* 1978). A subsequent continuous culture analysis of the behaviour of *P. oxalaticus* during growth on mixtures of formate with acetate or oxalate revealed several interesting features of the regulation of the synthesis of enzymes involved in formate metabolism (Dijkhuizen & Harder 1979*a*, *b*). Addition of acetate (0–30 mM) to the medium reservoir of a formate-limited continuous culture (D = 0.10 h^{-1}) led to the simultaneous and complete utilization of both substrates at steady state. As expected, the synthesis of RuBPCase became progressively repressed, although it was established that (at this dilution rate) its remaining activity was sufficient to allow both autotrophic and heterotrophic carbon assimilation pathways to function as if the other substrate were absent. Addition of formate (0–100 mM) to the medium reservoir of an acetate-limited continuous culture (D = 0.10 h^{-1}) also resulted in simultaneous and complete utilization of the two substrates at steady state. Although when there was less than 40 mM formate in the feed, RuBPCase was not detectable in the culture (figure 4), the presence of this amount of formate increased the bacterial dry mass in the culture by about 50 %. Since acetate was the only available carbon source, acetate carbon must have been redistributed over the dissimilatory and assimilatory pathways, i.e. more acetate was assimilated and less was dissimilated under these conditions. Thus, formate at concentrations of below 40 mM in the mixture only served as an ancillary energy source for the assimilation of acetate carbon.

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Above this concentration, RuBPCase synthesis was derepressed and increased linearly with increasing formate concentration in the feed. Once RuBPCase appeared, the increasing contribution of the energetically very expensive autotrophic CO_2 fixation to the biosynthesis of cell material was reflected in a remarkable drop in the amount of dry mass synthesized per millimole of formate added (figure 4). A calculation of the specific activity of RuBPCase necessary to synthesize the same amount of cell material from CO_2 during growth on the mixture

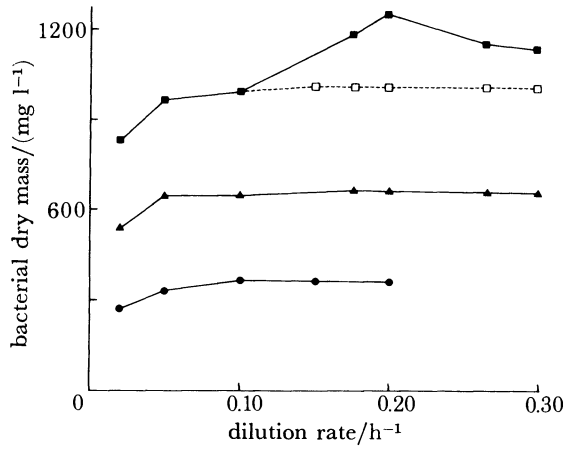

FIGURE 5. Relation between bacterial dry mass and dilution rate of *Pseudomonas oxalaticus* OX1 during growth on: ●, formate ($S_r = 100$ mM); ▲, acetate ($S_r = 30$ mM); ■, a mixture of formate and acetate. □, Sum of the bacterial dry masses obtained during growth on formate and acetate separately. (Data of Dijkhuizen & Harder (1979*a*).)

as during growth on formate alone showed that this is only achieved with 100 mM of formate in the feed. At lower concentrations part of the energy derived from formate oxidation is used to enhance assimilation of acetate carbon. At 50 mM formate this resulted in a 19 % higher yield on the mixture than expected when acetate and formate were metabolized independently. These results indicate that in *P. oxalaticus* the synthesis of RuBPCase is controlled by (de)repression and is responsive to the ratio of acetate to formate in the feed. In a further series of experiments the organism was grown at different dilution rates in a chemostat supplied with a medium containing a constant mixture of acetate (30 mM) and formate (100 mM) (figure 5). In this experiment the activity of RuBPCase reached a maximum at $D = 0.05\ h^{-1}$ and decreased at both lower and higher dilution rates. It was calculated that at dilution rates below $0.10\ h^{-1}$ the specific activity of RuBPCase that was required to synthesize the same amount of cell material from CO_2 as during growth with formate alone was sufficiently high to allow the cells to assimilate CO_2 via the Calvin cycle as if acetate were not present. The growth yield data indicate that this is actually so, since the dry mass of organisms on the mixture is exactly the same as the sum of the dry masses produced during growth on the single substrates. However at dilution rates above $0.10\ h^{-1}$ the RuBPCase activities are too low to accomplish this. This results in the utilization of part of the energy derived from formate oxidation to assimilate acetate carbon. At $D \geqslant$ of $0.10\ h^{-1}$, the energy-saving effect of this is reflected in a significant increase in the bacterial dry mass produced compared with values expected when the metabolism of acetate and formate was unimpaired (figure 5). At $D = 0.20\ h^{-1}$ this increase is approximately 23 %. That redistribution of acetate carbon in favour of carbon assimilation had

actually occurred is also strongly indicated by the fact that, at $D \geqslant 0.10\ h^{-1}$, the specific activity of isocitrate lyase during growth on the mixture is higher than on acetate alone. The maximum specific growth rate of *P. oxalaticus* on formate alone is close to $0.20\ h^{-1}$. However, during growth on the mixture, formate was also utilized at higher dilution rates, albeit not to completion. The residual formate concentration in the culture increased from 0 at $D = 0.20\ h^{-1}$ to 15.9 mM at $D = 0.30\ h^{-1}$. These continuous culture experiments confirm the general view derived from experiments in batch culture that synthesis of RuBPCase is very sensitive to repression by acetate. However, they also illustrate that under substrate-limiting conditions repression may be totally absent or only partial, depending on the ratio of acetate to formate in the medium and on the growth rate. A molecular interpretation of these phenomena has been presented elsewhere (Dijkhuizen & Harder 1979*a*).

Gottschal & Kuenen (1980*a*) studied growth of the facultative chemolithotroph *Thiobacillus* A2 under dual limitation by acetate and thiosulphate in a continuous culture at $D = 0.05\ h^{-1}$ and also observed simultaneous and complete utilization of the two substrates at all ratios in the medium reservoir. Synthesis of RuBPCase in this organism is very sensitive to repression by acetate, and no RuBPCase activity was detectable at ratios of thiosulphate to acetate in the medium below 2:1 (20 and 10 mM, respectively). Only at higher ratios of thiosulphate to acetate did synthesis of this enzyme occur and its specific activity increased in proportion to the relative thiosulphate concentration in the inflowing medium. The cell yields of *Thiobacillus* A2 were, at maximum, 30 % higher than expected from the sum of the yields observed on the single substrates. This again reflects an energy-saving effect exerted by repression of CO_2 fixation via the Calvin cycle and indicates that (part of) the energy produced from thiosulphate oxidation is used for the assimilation of acetate carbon. This was subsequently shown to be so by [^{14}C]-acetate assimilation experiments and by gas analysis.

Similar results have been obtained during growth of *Paracoccus denitrificans* in continuous culture with mannitol plus methanol as the limiting substrates (Van Verseveld *et al.* 1979). Mixed substrate utilization by *Thiobacillus* A2 has also been studied in continuous cultures limited by glucose and thiosulphate (Smith *et al.* 1980) and by glucose and formate (Wood & Kelly 1981), and by *T. novellus* during growth on a mixture of glucose plus thiosulphate (Leefeldt & Matin 1980). In cultures of the latter two organisms the two substrates were consumed simultaneously and it was found that the presence of glucose severely repressed RuBPCase synthesis. However, during growth on these mixtures the amounts of bacterial dry mass produced were exactly the sum of those obtained on the single substrates. Apparently, in the presence of glucose, metabolic energy produced from thiosulphate or formate does not result in an enhanced growth yield, although in *Thiobacillus* A2 the percentage of glucose that was assimilated increased compared with that observed during growth on glucose alone. An interesting case of mixotrophic growth of a marine pseudomonad on manganese plus succinate in continuous culture has also recently been encountered (P. E. Kepkay & K. H. Nealson, personal communication).

Mixed electron acceptor utilization by microbes has also been studied. Again an example serves to illustrate this. *Hyphomicrobium* X is able to grow in mineral media containing dimethylamine, both under aerobic and anaerobic (with nitrate) conditions (Meiberg *et al.* 1980). During growth of the organism in a dimethylamine-limited continuous culture in the presence of nitrate at $D = 0.10\ h^{-1}$, synthesis of nitrate and nitrite reductases was observed at dissolved oxygen tensions (d.o.ts) below 15 mmHg (*ca.* 2000 Pa). This was paralleled by the start of

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nitrate consumption. The activities of these two enzymes increased linearly with decreasing d.o.ts. In a further series of experiments, Meiberg *et al.* (1980) showed that denitrification in the presence of oxygen was dependent on the growth rate of the organism. At relatively high growth rates (0.15 h^{-1}), synthesis of nitrate and nitrite reductases only became apparent at d.o.ts below 6 mmHg (*ca.* 800 Pa). However, at low growth rates (0.01 h^{-1}), synthesis of these two enzymes was already significant at relatively high d.o.ts (up to 50 mmHg) (*ca.* 6600 Pa). These results indicate that *Hyphomicrobium* X is able to carry out denitrification under 'partly aerobic' conditions, particularly at low growth rates.

Utilization of several organic carbon compounds in addition to a special carbon source (i.e. carbon dioxide or a reduced one-carbon compound) may also be an important form of mixed substrate utilization in certain groups of specialized organisms. This has been observed in a number of chemolithoautotrophs, photoautotrophs and obligate methylotrophs (Smith & Hoare 1977). During growth in batch culture, these organisms show a limited ability to regulate enzyme synthesis, and the addition of organic substrates generally does not influence growth rate or growth yield (Matin 1978), although they may contribute significantly to cell carbon content. Increased growth yields only became apparent during growth of obligately chemolithotrophic thiobacilli in chemostat cultures limited by thiosulphate or carbon dioxide (Kuenen & Veldkamp 1973). This may not be a general phenomenon because Meiberg (1979) failed to observe a similar effect in restricted facultatively methylotrophic hyphomicrobia.

General aspects of the regulation of the synthesis of catabolic enzymes

The evidence summarized above poses the question of whether it is possible to draw a generalized picture of the strategies of the control mechanisms involved in the synthesis of a variety of catabolic enzymes in microorganisms and their survival value. Obviously, metabolic control operates in such a way that it enables organisms to grow at a high rate (μ_{max}) under substrate-sufficient conditions, whereas under nutrient limitation they allow concurrent utilization of multiple substrates in organisms of the appropriate genotype. Under the former conditions, mechanisms such as catabolite repression and inducer exclusion prevent the synthesis of enzymes that are not immediately required for fast growth, whereas under conditions of nutrient limitation all the available nutrients can be taken up from the environment as a result of induction or relief of catabolite repression (derepression), or both. Much of our knowledge of the regulation of enzyme synthesis in relation to the extracellular concentration of nutrients has come from continuous culture studies. These studies have shown that the specific activity of a large number of microbial enzymes follows one of five general patterns (table 2) (Dean 1972; Matin 1979). The response most frequently observed is that the activity increases with decreasing dilution rate either throughout the range of dilution rates tested for (repressible) constitutive enzymes (table 2, B), or through a substantial part of it with (repressible) inducible enzymes (table 2, C). This type of response embraces almost all the catabolic enzymes involved in the early metabolism of substrates that have been examined, although there are one or two exceptions (see Matin 1979). An increase in enzyme activity with increasing dilution rate (table 2, A) has been less frequently observed; this response appears to be common for enzymes involved in biosynthetic reactions and those involved in or connected with the respiratory chain. Two other types of response, namely no change in enzyme activity with dilution rate (table 2, E) and a minimal activity at an intermediate dilution rate (table 2, D), are exceptional and will not be considered here.

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Since the most frequent response to decreasing nutrient concentrations in the environment is increased enzyme synthesis, the underlying molecular control mechanism(s) and the potential benefit of this response to the organism must be considered. *A priori* it seems paradoxical that slow-growing organisms should possess higher levels of catabolic enzymes, and perhaps even wasteful to synthesize enzymes that are not immediately required for growth (i.e. constitutive enzymes). However, the potential beneficial effect of this type of response to the organism is not difficult to infer. The K_m of many catabolic enzymes is in the millimolar range and increased

TABLE 2. GENERALIZED EFFECTS OF DILUTION RATE ON BACTERIAL ENZYME SYNTHESIS IN CONTINUOUS CULTURE

A. Specific activity increases as the dilution rate is increased
B. Specific activity increases as the dilution rate is decreased
C. Specific activity passes through a maximum at intermediate dilution rates
D. Specific activity passes through a minimum at intermediate dilution rates
E. No change in activity at different dilution rates

enzyme levels would clearly enhance the competitiveness of bacteria that have to metabolize substrates that are present in micromolar concentrations. This latter situation is not unusual in cultures limited by carbon or nitrogen at low dilution rate, or indeed in Nature. It must be noted that an increase in the level of catabolic enzymes in response to low nutrient availability is not the only way in which an organism can respond. There are also instances recorded in the literature in which the response is to derepress the synthesis of enzymes with an improved affinity (see Brown 1976; Neijssel *et al.* 1975; Dawes *et al.* 1976).

Repression of the synthesis of various catabolic enzymes at high growth rates (table 2, B, C) in all probability constitutes a type of control whose strategy is to prevent the synthesis of enzymes that have no immediate function. Apparently, under these substrate-sufficient conditions the biosynthetic machinery of the cell is geared to fast reproduction, and the overall efficiency of the energetically expensive process of protein synthesis dictates that the synthesis of redundant proteins is shut down. This was the basis of the suggestion (Pardee 1961) that growth rate is probably the guiding principle of bacterial metabolism. But would similar arguments not apply to growth under nutrient limitation at low growth rates? At first sight it would certainly seem wasteful for an organism to produce, under conditions of nutrient constraints, more of certain catabolic enzymes or indeed produce enzymes that have no immediate function. A closer inspection of the machinery of the cell, however, reveals that this is not necessarily true. In order to appreciate this we must consider briefly the effect of growth rate on the macromolecular composition of cells, in particular those components (RNA and protein) that are involved in the protein-synthesizing machinery.

It has been known since the late 1950s (see Herbert 1961) and much evidence has accumulated since (see Nierlich 1978) that the RNA content of a microorganism is an almost linear function of its growth rate. Further studies have shown that most (80–85 %) of this RNA is ribosomal (rRNA) and from this it has been inferred that in growing cells the concentration of ribosomes increases approximately in proportion to the growth rate (Maaløe & Kjeldgaard 1966). The reason for this is that in cells growing at moderate or fast rates, the available catalytic units of the protein-synthesizing machinery are nearly maximally utilized. Except for cells growing very slowly, which may have an excess of ribosomes (Koch 1971), the cellular ribosome

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content is apparently adjusted to the growth rate in such a way that the number of catalytic protein-forming units is just sufficient to sustain the rate of protein synthesis required by the growth rate. At high growth rates it is not unusual for microorganisms to contain up to 25 % RNA (as a percentage of the dry mass) whereas at low growth rates this may be closer to 10 % (Herbert 1961; Esener 1981). The abundance of transfer RNA with respect to total cellular RNA in growing cells is relatively constant and is on average 10–15 %, although it may increase to approximately 20 % in very slowly growing cells (see Nierlich 1978). Also the contribution of messenger RNA is relatively constant and is on average 4 %. These data can be used to predict, for a hypothetical organism, how much of the total protein will be associated with

Table 3. Effect of growth rate on the approximate macromolecular composition of microbial cells

(The data given are for a hypothetical organism and are an average of published experimental data. The figures are expressed as a percentage of dry mass unless otherwise stated.)

macromolecular component	slow-growing cells ($0.05\ \mu_{max}$)	fast-growing cells (μ_{max})
total protein	71	67
total RNA	10	25
ribosomal RNA	8	20
ribosomal protein	5	15
ribosomal protein (percentage of total protein)	7	22

the ribosomes at low and high growth rates. The result of such an exercise (table 3) indicates that at high growth rates more than 20 % of the total protein of a microorganism may be associated with ribosomes (and probably an additional small percentage with the other enzymes involved in protein synthesis), whereas at low growth rates only approximately 7 % of the total protein is involved in protein synthesis. These data, which hold for all microbes that have been examined so far, indicate that the transcription pattern changes drastically with growth rate and may explain why organisms have to control carefully which proteins shall be synthesized at high growth rates because so much of it is required for the protein-synthesizing machinery itself. At the same time these considerations imply that it is not particularly wasteful for an organism to synthesize more of certain catabolic enzymes whose synthesis is derepressed or even produce a variety of catabolic enzymes that are not immediately functional when the organism grows slowly. Indeed, the fact that the total protein content of a slow-growing microbe is at least as high or even higher than that of a fast-growing cell (Herbert 1961; Esener 1981) even requires that more of other proteins must be synthesized when the amount of protein associated with the protein-forming machinery decreases. It has become clear in recent years (see Nierlich 1978) that 5′-diphosphoguanosine 3′-diphosphate (ppGpp) plays an important role as regulator for the expression of genes coding for rRNA and ribosomal proteins and possibly (all) other proteins of the protein-synthesizing machinery. An inverse relation between intracellular ppGpp concentration and growth rate has been observed, suggesting that ppGpp is a negative effector of ribosome synthesis. Although several other factors are involved in the regulation of the synthesis of rRNA and ribosomal protein (e.g. DNA gyrase, post-translational feedback regulation of ribosomal protein synthesis; see Oostra 1981), this preferential inhibitory effect of the synthesis of rRNA and ribosomal protein by ppGpp is possibly of overriding importance and helps the organism to control the priorities of protein synthesis at the different rates at

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which it can grow. The potential importance of this coarse type of control is also indicated by the observation that the rate of transcription of several operons coding for catabolic enzymes (such as *lac*) is stimulated by ppGpp (see Nierlich 1978). Thus, the very fact that so much protein is involved in the protein-synthesizing machinery of microbial cells and the accurate control over the 'size of the protein-synthesizing factory' enable an organism to become optimally adjusted to a low-nutrient environment by allowing the synthesis of various catabolic enzymes. It may even be speculated that this feature of the protein-synthesizing machinery has been so successful in the course of evolution that this explains why ribosomes contain so much protein.

It is clear from the above discussion that there is an overriding coarse control of protein synthesis operating in microorganisms that enables versatile microbes to utilize simultaneously various nutrients under conditions of nutrient limitation. This of course only applies to organisms of the appropriate genotype. Organisms that are highly specialized with respect to the nutrients that they can use for growth of course do not have this option. These, however, may respond to nutrient limitation by synthesizing large amounts of enzymes involved in the early metabolism of their substrates.

Significance of mixed substrate utilization in Nature

The enormous diversity of microorganisms encountered in Nature illustrates the equally enormous diversity of ecological niches that harbour these organisms. Adaptation of microorganisms to a particular environment occurs both at the genotypic and phenotypic level and has led on the one hand to organisms that have become highly specialized and on the other hand to organisms that are very versatile in that they have a high potential for phenotypic changes. While the significance of mixed substrate utilization by those representatives of the former group of organisms that are metabolically highly specialized (e.g. obligate chemolithotrophs, obligate methylotrophs) may be limited, it is to be expected on the basis of the evidence presented in the previous sections that mixed substrate utilization by more versatile organisms may be of great importance, particularly in environments that contain low concentrations of a variety of substrates and are characterized by relatively low turnover rates (see below). In these environments the ability to utilize various substrates simultaneously may even confer a competitive advantage on such organisms, which could mean that they are of major importance in the cycling of nutrients in such environments.

It has become clear from studies on mixed substrate utilization under substrate sufficient conditions (see above) that metabolic control mechanisms in microbes generally prevent the utilization of more than one substrate at the same time. This means that the classical batch-type enrichment cultures, even when mixtures of substrates are employed, tend to select for specialists and discourages the development of the more versatile microbes that may have an advantage under conditions where mixed substrates are present in low concentrations. Also single nutrient-limited continuous enrichments favour selection of specialists that are optimally adapted to those conditions (Kuenen *et al.* 1977). However, it is possible that there are organisms in Nature that are optimally adjusted to growth under low mixed nutrient conditions for which the appropriate selection method has not been used. Such organisms may play a significant role in at least some environments. Recent experiments employing mixed substrate-limited enrichment procedures have indicated that these considerations are essentially correct and have pointed the way for further experimentation. A few examples may serve to illustrate the point.

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Kuenen and his colleagues (see Gottschal & Kuenen 1981) investigated the physiological and ecological significance of facultative chemolithotrophy and mixotrophy in chemolithotrophic bacteria. The question that was studied was how the facultatively chemolithotrophic and chemolithoheterotrophic thiobacilli would be able to compete successfully with their specialized obligately chemolithotrophic counterparts and with specialized obligately heterotrophic bacteria in view of the fact that their maximum specific growth rates are often much lower than those of the specialists. Although it had been postulated (Rittenberg 1969) that the ecological advantage of facultative chemolithotrophs could lie in their ability to grow on mixtures of

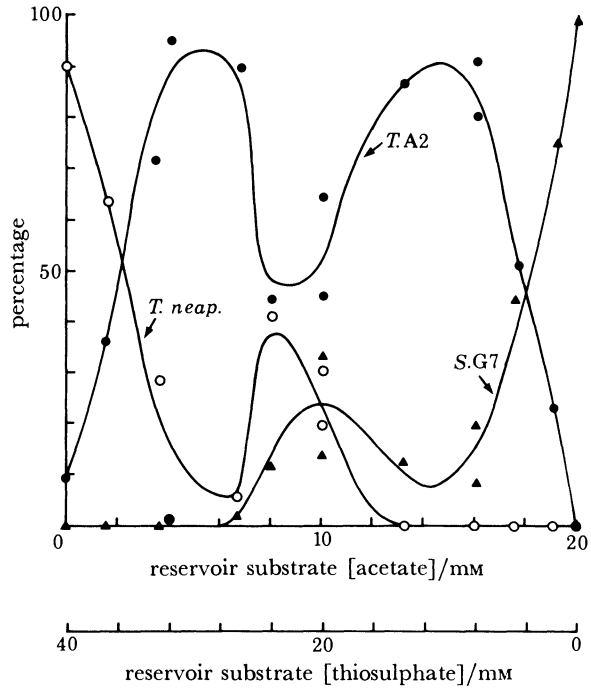

FIGURE 6. Competition between *Thiobacillus* A2 (●), *T. neapolitanus* (○) and *Spirillum* G7 (▲) for thiosulphate (S_r = 0 to 40 mM) and acetate (S_r = 0 to 20 mM) as growth-limiting substrates in the chemostat at D = 0.075 h^{-1}. (Data of Gottschal *et al.* (1979).)

inorganic and organic compounds, these organisms almost never became dominant in batch-type enrichment cultures containing mixtures of an organic substrate and an inorganic reduced sulphur compound. Consequently the organisms of this type that have been isolated are few. However, when Rittenberg's suggestion was tested in competition experiments under dual (thiosulphate and acetate)-limited conditions, it appeared that the more versatile facultatively chemolithotrophic *Thiobacillus* A2 had a competitive advantage over the more specialized organisms (Gottschal *et al.* 1979). In a thiosulphate-limited chemostat, the specialized *T. neapolitanus* outcompeted the versatile *Thiobacillus* A2 at D = 0.025 h^{-1}, clearly demonstrating the advantage of the specialist under single nutrient limitation. However, when acetate or glycollate was added to the inflowing medium of a mixed continuous culture run at a dilution rate of 0.07 h^{-1}, the percentage of *Thiobacillus* A2 in the culture increased with increasing concentration of organic substrate. The final result of the competition was dependent upon the ratio of thiosulphate to the organic compound in the inflowing medium; when this ratio exceeded a certain value, the specialist was completely eliminated from the culture. Similar

results have been obtained with mixtures of thiosulphate and glucose (Smith & Kelly 1979). In a subsequent series of experiments, Gottschal *et al.* (1979) studied competition between the versatile thiobacillus and a heterotrophic specialist, *Spirillum* G7. With acetate as the sole growth-limiting substrate *Thiobacillus* A2 was completely outcompeted by the specialist at dilution rates of 0.07 and 0.15 h^{-1}. However, increasing concentrations of thiosulphate in the inflowing medium eventually led to the elimination of the heterotrophic specialist. This apparent advantage of mixotrophic growth was also expressed in competition experiments between *Thiobacillus* A2 and the two specialists in a three-membered mixed culture (figure 6). Another example of competition between a specialist and a more versatile organism for mixed substrates has been reported by Laanbroek *et al.* (1979).

The above experiments clearly indicate a possible ecological advantage of versatile organisms. When the conditions employed in the dual substrate-limited chemostat experiments are translated to relative turnover rates of two substrates in Nature, then it must be expected that in environments where the turnover rates of two substrates are similar a more versatile organism would have an advantage, whereas under conditions of extreme turnover ratios specialist organisms would be favoured. The general validity of this principle is indicated both by the results of many batch-type and single nutrient-limited enrichments and by the dual nutrient-limited enrichments reported recently by Gottschal & Kuenen (1980*b*). These authors performed enrichments for facultatively chemolithotrophic thiobacilli by using continuous cultures with varying mixtures of acetate and thiosulphate and showed that such organisms can be reproducibly enriched from fresh water environments. These results therefore show that among the thiobacilli mixotrophic growth can have a significant ecological advantage and also indicate that similar principles may apply to other types of versatile organisms. We have recently obtained evidence that this may also be true for methylotrophs (Marijke Smits & W. Harder, unpublished). These experiments also provided the first proof, to our knowledge, for the prediction (Yoon *et al.* 1977) that growth yield is an important property of an organism and may be decisive in the outcome of the competition for two (or more) substrates.

Concluding remarks

In many microorganisms the utilization of mixed substrates is carefully controlled when these substrates are present in sufficiently high concentrations. In general it has been found that the substrate that supports the highest growth rate is utilized preferentially from such mixtures and much has become known about the underlying regulatory mechanisms that govern the principle (Pardee 1961) that growth rate is of prime importance in microbial metabolism. However, this may only be true for the organisms that have been studied so far. Most of these have been obtained by batch-type enrichment procedures in which this very property of microorganisms is selected for and thus it is not really surprising to find that these organisms behave as they should.

In most natural environments we may expect that low concentrations of multiple substrates prevail and although it has been the general observation that microbes, selected for their capability to grow fast under substrate-sufficient conditions, will utilize at least two substrates simultaneously when these are present in growth-limiting concentrations, this does not necessarily exclude the possibility that there are in Nature organisms that are specially adapted to life under mixed nutrient limitation. Indeed, whereas organisms that have specialized to grow

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at a high substrate concentration have an advantage in environments in which the turnover rate of that substrate is high, versatile organisms that have specialized in the utilization of low concentrations of a mixture of substrates at the same time may be expected to flourish in those environments where two (or more) substrates are turned over at comparable rates. The results of recent experiments discussed above illustrates that these considerations hold for certain chemolithotrophs and methylotrophs and indicate that they may be of more general validity. Although it is to be expected that organisms of the versatile type are more abundant in natural ecosystems, it is not unlikely that we have so far encountered only very few of those organisms because the selection methods that have been used select *against* them. Consequently, we may have discovered only the tip of an iceberg and will witness a rapid increase in the number of specially adapted organisms as dual-nutrient (or even multinutrient) limited selection procedures become more popular and learn more about the significance of these organisms in nutrient cycling in Nature. It must be borne in mind, however, that other properties of organisms such as reactivity are also important, particularly in environments in which substrates become available in rapidly fluctuating concentrations (Koch 1971).

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Discussion

J. R. Postgate, F.R.S. (*Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K.*). When a chemostat population has been persuaded, by manipulation of growth rate and nutritional status, to utilize two substrates that are incompatible in batch culture, it is important to be sure

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that the *whole* population is derepressed with respect to both substrates. The alternative situation is that half of the population, say, is derepressed with respect to one substrate and the remaining half is derepressed for the other. Chemostat cultures rarely give a clear answer, but the question is important if one wishes to draw physiological conclusions, and supplementary experiments at the single cell level are usually needed.

W. HARDER. It is very true that experiments at the single cell level are required to establish that *all* organisms in a chemostat culture growing on two substrates utilize both compounds at the same time. Such experiments have been done with *P. oxalaticus* and *Thiobacillus* A2 and these clearly demonstrated that all organisms respond in the same way. Therefore, at least in these two organisms, the possibility that one part of the population utilizes one substrate and the other part another substrate can be dismissed. It may be of interest that these experiments involved the use of the so-called slide-culture technique developed by Professor Postgate.

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