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PHYSIOLOGICAL RESPONSES TO NUTRIENT LIMITATION

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INTRODUCTION

The properties of any microbial cell are ultimately determined by the characteristics of its genome. This carries all the information necessary for an organism to become a structural and functional unit and also gives it the potential to respond to changes in its (micro)environment. This latter property is uniquely associated with the life-style of microbes. These organisms only have a limited capacity to control their environment and they almost invariably respond to environmental change by changing themselves. In principle this can occur in two ways, namely, by changes in their genetic constitution or by phenotypic

adaptation. In this review we do not consider the former of these processes (for reviews see 68, 88, 109, 110), but rather focus on the phenotypic responses of microorganisms of a given genotype to environmental change.

The wide variety of phenotypic responses encountered within a certain genotype clearly shows that none of the microbial species studied so far expresses its entire genome under any set of environmental conditions (61). Instead, microbes generally express only that part of their genome that enables them to become structurally and functionally adjusted to a certain set of conditions. This ability has led to the appreciation that most microbes potentially possess an enormous phenotypic variability that confers great versatility. This variability may involve merely one or more quantitative changes in some cellular component or, more drastically, radical qualitative changes in cell structure or function. Thus, an organism of a given genotype is very much a product of its environment, so much so that "it is virtually meaningless to speak of the chemical composition of a micro-organism without at the same time specifying the environmental conditions that produced it" (50). Essentially the same applies to the functional properties of many organisms (117).

Among the environmental parameters that commonly influence the properties of microbial cells in nature, the concentration of essential nutrients is of particular importance. Natural ecosystems frequently are virtually depleted of one or more of these nutrients (129) as a consequence of the (potentially vigorous) metabolic activities of indigenous microbial populations. Hence, microbial growth in natural environments is nearly always nutrient limited so that "nutrient insufficiency is the most common environmental extreme to which micro-organisms are routinely exposed" (118). Consequently, if one wants to understand the behavior of microbial populations in nature (or indeed in many man-made environments), it is necessary to study in which way microorganisms accommodate to nutrient-limiting conditions. Experimentally, such conditions can be imposed upon microbial populations in a flow-controlled continuous culture, and a considerable body of literature has accumulated on the behavior of organisms in such culture systems. This enables one, at least in outline, to discern a number of the physiological problems posed by nutrient limitation and some of the strategies that have evolved in the microbial world that enables organisms to cope with these ubiquitous conditions.

This review discusses published data relating to structural and functional changes in microbes consequent upon exposure to low-nutrient (chemostat) environments. In recent years, a number of reviews on this aspect of microbial (eco)physiology have been published (17, 44, 66, 75, 79, 117, 118), and because of this and of constraints of space, we have not attempted to aim for completeness. Instead, we discuss a number of nutrient-limited conditions that we consider of importance and try to rationalize the responses observed in

terms of microbial (eco)physiology. We restrict our survey of cellular effects brought about by nutrient limitation mainly to the so-called primary potential growth-limiting nutrients, of which the cells contain a sizeable amount, namely carbon, nitrogen, phosphorous, sulfur, potassium, and magnesium, and we limit the discussion to "steady-state" responses.

GENERAL STRATEGIES

Before embarking on a more detailed discussion of the physiological effects of nutrient limitation by major nutrients, a few general remarks are in order. If, as outlined above, one accepts that nutrient-limited growth conditions have been and still are important in natural environments, then it must be expected that these will have exerted a strong selective pressure in the course of evolution for organisms to evolve mechanisms to accommodate to such restrictive conditions. The principal objective of these mechanisms clearly must be to enable an organism to grow as fast as possible at a given, low, environmental concentration of the limiting nutrient (129). For this to be possible, the organism should be able to take up and metabolize that nutrient at the highest possible rate under conditions where its concentration outside the cell is exceedingly low, and to produce cell material with a yield factor (with respect to the limiting nutrient) that is maximal under the prevailing conditions. Conceivably, these mechanisms may include three responses: (a) One is the ability to increase the rate of transport of a nutrient when its concentration becomes growth limiting. At the molecular level, this may be brought about by synthesizing more of the existing uptake system (increase its V_{max}), which would enable an increase in the transport rate at a subsaturating (low) substrate concentration (see 117). Alternatively, organisms could have acquired the ability to synthesize a different "high-affinity" uptake system for the growth-limiting nutrient. And also, mechanisms may have evolved by which the kinetic properties of an existing uptake system can be changed, for instance, through changes in the binding affinity of the substrate, changes in the stoichiometry of the transport process, or modulation of carrier activity. (b) Another is the ability to increase the rate of initial metabolism of the nutrient that has accumulated inside the cell when its intracellular concentration is low. The significance of this response may be understood from the following considerations. The driving force for accumulation of most solutes is composed of components of the proton motive force and of the solute gradient. When this driving force is maintained at a certain value by primary transport systems, the level of accumulation of a solute can be predicted from the translocation mechanism (64). For instance, for a neutral substrate that is accumulated in symport with one proton, the steady-state level of accumulation at a certain value of the proton motive force may be 1000-fold. Thus, if the extracellular concentration of this substrate is in the nanomolar

range, its concentration inside will never exceed micromolar levels. Clearly, if the cell were able to metabolize nutrients that accumulated to concentrations of this order, it would be at a competitive advantage. To acquire this potential, organisms may synthesize more of the existing enzyme(s) involved in the initial metabolism of the substrate (see *a*). Alternatively, a different "high-affinity" enzyme system may be formed, which would have essentially the same effect. (*c*) The third response is the ability to rearrange the chemical composition of cellular structures by redirecting fluxes of metabolites containing the limiting nutrient. The significance of this response would be that it allows more cell material to be produced from a given quantity of the growth-limiting nutrient. In the following, we mainly consider the adaptive responses to nutrient limitation encountered among microorganisms in light of the processes *a-c* listed above.

CARBON-SUBSTRATE LIMITATION

Carbon-substrate limitation has been employed most commonly in studies of microbial response to nutrient constraint in chemoorganotrophs. It has also been used in the study of metabolic regulation in chemolithotrophic bacteria (8, 69) by applying limitation by carbon dioxide under conditions of excess energy source, but studies of this kind have been relatively rare. Not surprisingly, a condition of carbon-substrate limitation in chemoorganotrophs is characterized by a high carbon conversion efficiency in which, in most organisms, diversion of substrate carbon into extracellular products is minimized (120). Under such conditions, organisms tend to derepress the synthesis of their catabolic enzyme machinery, whereas the synthesis of anabolic functions remains adjusted to levels in keeping with the growth rate (23, 45, 46, 50, 77, 92). One consequence of this behavior is that although the rates of catabolism and anabolism may be adequately tuned to a particular condition of nutrient constraint, organisms will frequently catabolize at a high rate any excess substrate suddenly added to such a culture (91).

Regulation of Substrate-Uptake Systems

Substrates can be translocated across the cytoplasmic membrane of bacteria either passively, without the involvement of membrane proteins, or facilitated by specific carrier proteins (64). Carrier-mediated transport systems are probably involved in the transport of most substrates that have to be accumulated at a high rate or against a significant concentration gradient, and therefore it is not surprising to find that cytoplasmic membranes usually contain many of such carrier proteins, each having affinity for only one solute or a group of structurally related solutes. Carriers usually have a high affinity for their substrates. K_m values generally range between 10^{-6} and 10^{-5} M, but affinity constants as low

as 10^{-8} M have also been reported. In gram-negative bacteria, some transport systems require a soluble protein (so-called binding protein) that is present in the periplasmic space and specifically binds the substrates of the transport system.

Facilitated transport systems for a wide variety of potential carbon substrates have been demonstrated in bacteria and fungi. These include amino acids, carboxylic acids, and sugars (for reviews see 31, 58, 63, 64). The number of different carriers present in the cytoplasmic membrane differs from organism to organism and in one organism may further depend upon environmental conditions. A surprisingly large number of carriers is present constitutively in cytoplasmic membranes of some organisms, but inducible carriers have also been found. These have, for instance, been demonstrated for lactose in *Escherichia coli* (57, 106), citrate and malate in *Bacillus subtilis* (64), and oxalate in *Pseudomonas oxalaticus* (30). In a number of instances, the occurrence of multiple uptake systems for a single substrate has been reported (135). This is the case in *E. coli* where at least five systems have been implicated in galactose transport (134), whereas in *Salmonella typhimurium*, two proline transport systems were shown to be present (99). The general situation appears to be that high-affinity (low K_m) systems have a low capacity (low V_{max}), whereas low-affinity systems display a high capacity (135). Initially, there was some doubt as to whether the multiple K_m values seen for the uptake of a given substrate were in fact due to several independent carrier systems. However, the fact that mutants have been isolated that are affected in separate genes each inactivating a single system without at the same time impairing other systems, is evidence that favors the presence of independent systems with different K_m values. Such evidence has been obtained in the case of leucine transport in *E. coli* (4, 42), neutral amino acid transport in a marine pseudomonad (36), gluconate (35) and glutamate transport (107) in *E. coli*, and proline transport in *S. typhimurium* (85).

Except for a few cases, it is not known in which way environmental nutritional constraints affect the synthesis and activity of microbial transport systems and there is clearly a need for much work in this area. Nevertheless, the little knowledge currently available (59, 62, 63, 65, 102, 135) does suggest various ways in which microbes may respond to limitation by a particular nutrient. These include increasing the concentration of a given transport system, preferential synthesis of a high-affinity uptake system, and changing the kinetic properties of an existing carrier system into the direction of higher binding affinity.

There is evidence in the literature to indicate that, at least in some organisms, the level of activity to which transport systems become expressed is adjusted to the rate of uptake of the growth-limiting substrate required by the growth rate of the organism. For instance, this has been reported for the phosphoenolpyru-

ate-dependent sugar phosphotransferase system involved in glucose uptake in *E. coli* (51) and other organisms (19), the uptake rate of the amino acids alanine, glutamate, and arginine in *Streptomyces hydrogenans* (3), and of sugars in *Thiobacillus* A2 (136). Such a control system could not only ensure that the rate of transport of substrates is sufficiently high at higher growth rates, but conceivably might also, by derepression of the synthesis of carriers at very low growth rates, scavenge substrate molecules when their concentration is diminishingly low. This type of control probably does not function under conditions where the carbon substrate is not growth limiting (see below).

Preferential synthesis of a high-affinity uptake system under conditions of carbon-substrate limitation has also been reported. An informative study of the regulation of glucose uptake by *Pseudomonas aeruginosa* may serve as an example (25, 132). This organism possesses two pathways of glucose metabolism, as shown in Figure 1. During nitrogen-limited growth of this organism,

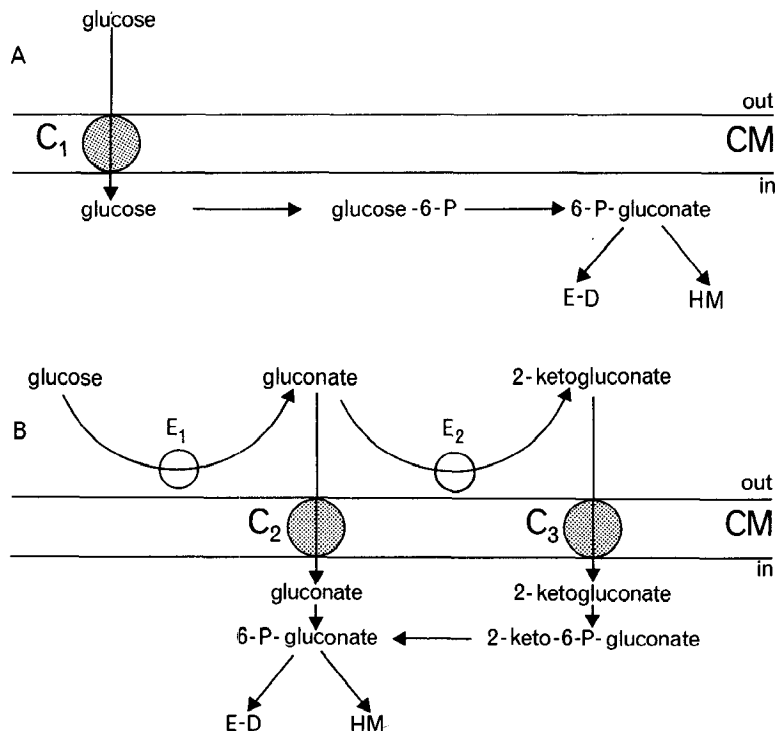


Figure 1 Pathways of glucose metabolism in *Pseudomonas aeruginosa*. CM, Cytoplasmic membrane; C₁, glucose uptake system; E₁, glucose dehydrogenase; E₂, gluconate dehydrogenase; C₂, gluconate uptake system; C₃, 2-keto-gluconate uptake system; E-D, Entner-Doudoroff pathway; HM, hexose-monophosphate pathway.

when excess glucose was present in the culture, the activity of the high-affinity glucose uptake system C_1 (K_m for glucose 8×10^{-6} M; Figure 1A) was low, whereas the activities of the periplasmic dehydrogenases (E_1 and E_2 ; Figure 1B), the gluconate and 2-ketogluconate uptake systems (C_2 and C_3) and the intracellular enzymes metabolizing these compounds were high. Thus, under these conditions, glucose was mainly metabolized via periplasmic glucose dehydrogenase (Figure 1B), whose K_m for glucose is approximately 1 mM. However, during glucose-limited growth, the glucose uptake system C_1 was present in high activity, whereas the activities of enzymes and uptake systems of the low-affinity system were very much reduced. Thus, this organism responded to glucose limitation by elaborating a high-affinity system for its uptake.

Konings & Robillard (65) recently provided evidence for regulation of the *E. coli* transport proteins for lactose and proline by the redox state of these carriers. The reduced form of the carriers had a low K_m , whereas the K_m increased when they became oxidized. A mechanism was proposed in which the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$, or one of its components, alters the ligand affinities of the carriers in that an increase in $\Delta\tilde{\mu}_{H^+}$ caused a reduction in the K_m . In contrast, the affinity of the phosphoenolpyruvate-dependent transport systems was high when the $\Delta\tilde{\mu}_{H^+}$ was low and their activity was inhibited when the secondary transport systems became fully activated. Although it has been suggested that in this way a balanced uptake of energy source and essential solutes can be achieved (67), the physiological significance of these mechanisms under conditions of nutrient constraints remains to be established.

Regulation of Intracellular Enzymes

A wealth of information is available in the literature with respect to the synthesis of microbial catabolic enzymes in response to decreasing concentrations of carbon substrate in their environment. These data concern both enzymes involved in the initial (or early) metabolism of compounds for which carrier systems are supposedly absent, such as glycerol and methanol, and those accumulated by facilitated diffusion against a concentration gradient. Much of our knowledge of the regulation of the synthesis of these enzymes in relation to the extracellular concentration of nutrients has come from continuous culture studies. These studies, reviewed by Dean (27) and Matin (79), have shown that the specific activity of a large number of microbial enzymes follows one of five general patterns (Table 1) (44). The response most frequently observed is that the activity increases with decreasing dilution rate, either throughout the range of dilution rates tested (repressible constitutive enzymes), (Table 1E), or through a substantial part of it (repressible inducible enzymes), (Table 1C). This type of response embraces almost all the catabolic enzymes involved in the early metabolism of substrates that have been examined,

Table 1 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

*Reproduced from Harder & Dijkhuizen, (44), with permission of the Royal Society (London)

although there are one or two exceptions (79). An increase in enzyme activity with increasing dilution rate (Table 1A) 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 1E) and a minimal activity at an intermediate dilution rate, (Table 1D), are exceptional. The molecular details of the observed responses have been discussed elsewhere (44, 79) and are not considered here.

Since the most frequent response to decreasing nutrient concentrations in the environment for catabolic enzymes is increased enzyme synthesis, the potential beneficial effect of this type of response to an organism must be considered. The K_m of many enzymes involved in the initial metabolism of a carbon substrate is in the 0.1–1 mM range. By synthesizing increased levels of these enzymes, organisms clearly enhance the effectiveness of substrate utilization at concentrations in the micromolar range or below (117), a condition not uncommon in carbon-limited cultures of bacteria at low dilution rates, or indeed in many natural environments. That higher levels of a catabolic enzyme are indeed of selective advantage to organisms when exposed to low concentrations of carbon sources is well documented in the literature (for reviews see 45, 68 109). Significantly, evidence (52, 80) also suggests that environments, characterized by a constant low concentration of a specific carbon substrate, tend to select for organisms that have become constitutive with respect to the synthesis of enzymes involved in the early metabolism of that substrate.

An example of derepression of a catabolic enzyme concerns the synthesis of alcohol oxidase during growth of the yeast *Hansenula polymorpha* in a methanol-limited chemostat (125). In this organism, methanol metabolism is initiated by alcohol oxidase, which catalyzes the oxygen-dependent formation of formaldehyde and hydrogen peroxide. The enzyme is contained within peroxisomes formed during growth on methanol (127). Organisms harvested from methanol-limited cultures at low dilution rate (0.03 h^{-1}) had a maximum rate of oxygen uptake in the presence of excess methanol [$Q(0_2)^{\text{max}}$], approximately 20 times the culture $Q(0_2)$, whereas at higher dilution rates (0.16 h^{-1}) the $Q(0_2)^{\text{max}}$ was only $1.6 Q(0_2)$ (124). The basis for this vast oxidative “over-

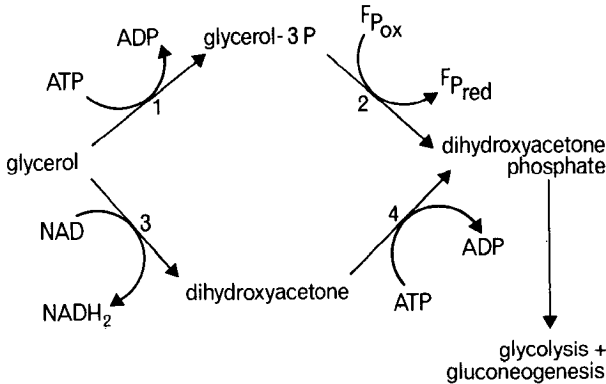


Figure 2 Pathways of glycerol metabolism in *Klebsiella aerogenes* [after Neijssel et al (90)]. 1, Glycerol kinase; 2, glycerol-3-P dehydrogenase; 3, glycerol dehydrogenase; 4, dihydroxyacetone kinase.

capacity” at low growth rates (e.g. low substrate concentration) is the poor affinity of alcohol oxidase for methanol (K_m 1.3 mM), which under these conditions requires that the cells devote approximately 20% of their cellular protein to the synthesis of this enzyme. Not surprisingly, when grown at low dilution rates, the cells contain large numbers of peroxisomes, whose matrix is of a highly regular structure due to the presence of crystalline alcohol oxidase protein (128).

Organisms may also respond to nutrient limitation by synthesizing a different “substrate-capturing” enzyme, which has a lower affinity constant for its substrate. As demonstrated elegantly by Hartley et al (48), this also increases the organism’s affinity for the growth-limiting substrate and thereby confers a competitive advantage. An example of this response was encountered in studies of glycerol metabolism in *Klebsiella aerogenes*. Early studies with batch cultures revealed that this organism possessed two pathways of glycerol metabolism (Figure 2), one involving glycerol kinase, which was expressed when the organism was growing aerobically, and the other involving glycerol dehydrogenase, which was used for anaerobic growth (74). These findings were rationalized as follows. Glycerol 3-phosphate dehydrogenase is a flavoprotein and hence cannot function under anaerobic conditions in the absence of an external electron acceptor. On the other hand, glycerol dehydrogenase was rapidly inactivated in cells placed under aerobic conditions so that two different pathways are required in this organism to enable glycerol metabolism under aerobic and anaerobic conditions. This explanation proved to be incomplete, because subsequent chemostat studies revealed that the route employed is also dependent upon the glycerol concentration in the culture (90). Chemostat-grown glycerol-limited cultures of *K. aerogenes* contained high levels of glycerol kinase but little glycerol dehydrogenase when grown aerobically,

whereas when grown anaerobically, the cultures contained little kinase but very high levels of the dehydrogenase, results that supported the findings of Lin et al (74). However, when glycerol-excess (sulfate or ammonia limited) cultures were studied, there was little kinase activity when grown aerobically; instead, dehydrogenase was present, which, when assayed in the presence of Mn^{2+} (53), showed sufficiently high activities to explain the growth rate observed. Thus, under aerobic conditions, the route of glycerol metabolism employed by *K. aerogenes* depends upon the concentration of glycerol available, and this may be explained in terms of affinities of glycerol kinase (K_m $1-2 \times 10^{-6}$ M) and glycerol dehydrogenase (K_m $2-4 \times 10^{-2}$ M) for glycerol. The high K_m for glycerol of this latter enzyme made it necessary for the organism to synthesize vast amounts of it under anaerobic glycerol-limited conditions.

An interesting observation involving modulation of enzyme activity that resulted in a drastic change in the fermentative metabolism of the carbon source in response to carbon-substrate limitation was reported in *Lactobacillus casei* (29). In glucose-sufficient cultures, the organism fermented the substrate via the homofermentative lactic acid pathway with an ATP yield of 2 mol/mol of glucose. However, when the organism was grown under glucose-limited conditions at dilution rates below 0.25 h^{-1} , fermentation of the substrate involved the phosphoroclastic split and the main fermentation products were acetate, formate, and ethanol. In this case, the ATP yield was close to 3 mol/mol of glucose consumed. These observations were explained on the basis of the kinetic properties of lactate dehydrogenase. This enzyme showed a strong dependency on fructose-1,6-bisphosphate for activity, and the intracellular concentration of this compound was considered to be too low under conditions of glucose limitation at low dilution rates to allow any significant activity.

The above data indicate that limitation by the carbon substrate invokes in many organisms increased synthesis of enzymes or enzyme systems involved in the initial metabolism of the limiting nutrient, whereas in some organisms, the response may be synthesis of a high-affinity enzyme system that could involve a different metabolic sequence for the metabolism of the substrate. In either case, the physiological significance is that the response allows the organism to sustain a higher rate of metabolism of the growth-limiting nutrient.

Mixed Substrate Utilization

It is well established that when presented with more than one carbon and energy substrate under the nutrient-sufficient conditions of a batch culture, microorganisms usually metabolize the substrate that supports the highest rate of growth, whereas enzymes for the metabolism of the others remain repressed. Such a distinct preference for one compound to the exclusion of all others would be of little value in a nutritionally poor environment where selective

pressure would favor those organisms capable of utilizing different substrates simultaneously. Evidence is rapidly accumulating (reviewed in 43, 44) that many microorganisms do in fact simultaneously utilize a multiplicity of nutrients that serve a similar physiological function (e.g. carbon and energy source), provided the concentration of these nutrients is low. An example (L. Dijkhuizen, unpublished information) may serve to illustrate the general response observed. When *Pseudomonas oxalaticus* was grown in batch culture (substrate-sufficient conditions) on a mixture of acetate plus oxalate, diauxic growth was observed with acetate utilized first. However, in continuous culture under carbon and energy-source limitation at dilution rates below 0.15 h^{-1} , the organism utilized both organic acids simultaneously and completely. At dilution rates above 0.15 h^{-1} , an increasing proportion of the oxalate supplied to the culture remained unutilized, which was reflected in a decrease in culture density. In contrast, no residual acetate was detected in the culture supernatant, up to a dilution rate of 0.30 h^{-1} . Thus, in a continuous culture limited by oxalate plus acetate, *P. oxalaticus* can utilize the two compounds simultaneously provided their concentration is low, which is the case at low growth rates. At higher growth rates, when the concentration of the substrates becomes higher, a situation develops that is comparable to that observed in batch culture, namely, one of impaired utilization of oxalate. The molecular details underlying this general response observed have been discussed elsewhere (44). The ability of many organisms to concurrently utilize a mixture of carbon substrates during nutrient-limited growth must be considered as one consequence of the general tendency towards diminished catabolite repression under these conditions (Table 1).

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 the first compound when they are used simultaneously. Evidence indicates that under certain conditions the metabolism of each of the substrates may proceed completely independently, as if the second substrate were absent. However, instances have also been reported in the literature in which the metabolism of a certain compound is significantly influenced by the presence of another, even when both are utilized to completion (see 44). The few examples of this latter behavior currently available indicate that such studies deserve much wider attention in view of the potential practical applications (6). But equally importantly, the studies on mixed substrate utilization under appropriate mixed nutrient limitation have disclosed the significance of such processes in natural ecosystems. In fact, evidence is now accumulating that the ability of organisms to simultaneously utilize various substrates may confer a competitive advantage upon them (40), which indicates that such organisms could be of major importance in the cycling of nutrients in environments in which the turnover rates of substrates are comparable and low (see 44).

NITROGEN LIMITATION

When microorganisms are growing in the presence of growth-limiting concentrations of an essential nutrient other than the carbon and energy source, it seems reasonable to expect that they will be able to accommodate to such conditions through one or more of the adaptive responses listed above. Under these circumstances, however, the flux of the nonlimiting carbon source is generally not tightly balanced to biomass formation, and this may lead to a significant accumulation of intracellular reserve materials (26), extracellular polymers (114), or a variety of low molecular weight metabolites (91). These various processes have been extensively reviewed and are not considered here. As Tempest & Neijssel (118) observed, the general response to this type of nutrient limitation may be characterized as follows: The formation of cellular polymers that contain substantial amounts of the specific growth-limiting nutrient is highly constrained, whereas formation of those polymers and products that do not contain any of the growth-limiting nutrient is promoted. In the following, the effects of nitrogen limitation on the metabolism of nitrogenous compounds by the cell are considered. The regulation of the assimilation of nitrogen compounds has been reviewed recently (24, 122).

Metabolic Response to Ammonia Limitation

Nitrogen is an essential nutrient and, depending on the properties of particular organisms, may be supplied in the form of an organic nitrogen compound, ammonia, nitrate, or molecular nitrogen. Many organisms are able to utilize ammonia, which is mainly incorporated into glutamate or glutamine, and these compounds in turn serve as the main precursors for the synthesis of other cellular organic nitrogen compounds (41). In many gram-negative bacteria, two pathways lead to the synthesis of glutamate from ammonia (Figure 3) (12, 15, 55), whereas in most yeasts, only the pathway involving glutamate dehydrogenase is present (12). In some bacilli, ammonia assimilation is predominantly by the glutamine synthetase/glutamate synthase pathway (34, 84).

In microorganisms, uptake of ammonia from the environment is generally thought to be mediated by facilitated diffusion (60, 104), but unfortunately little is known about the way in which the properties of this $\Delta\psi$ -driven carrier-mediated ammonia transport system change in relation to changes in the external concentration of ammonia during growth of the organisms. In contrast, the adaptation of cytoplasmic ammonia-assimilating enzyme systems to environmental ammonia concentrations is well documented. Most yeasts (see 12) respond to ammonia limitation by synthesizing very high levels of NADPH-specific glutamate dehydrogenase. This enzyme only has a low affinity for ammonia, the apparent K_m values being in the region of 1–5 mM, and therefore cannot provide an effective mechanism for maintaining a low intracellular

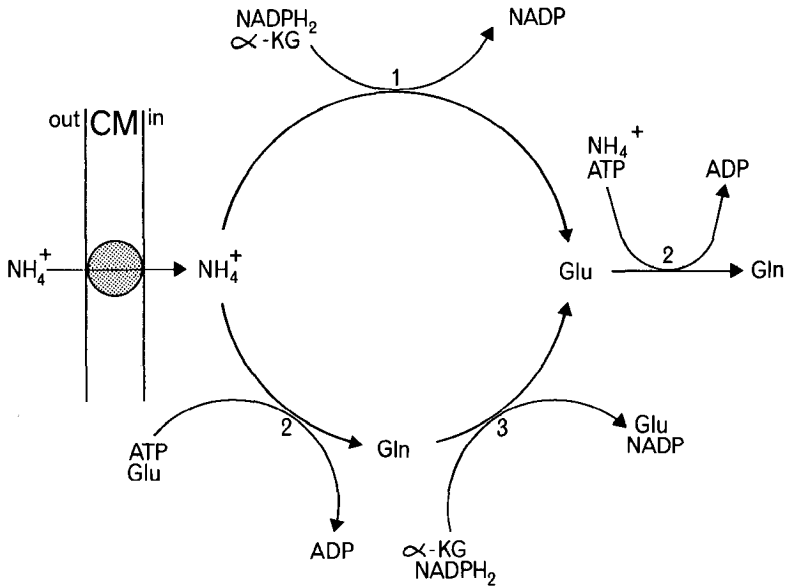


Figure 3 Two routes for the synthesis of glutamate. 1, Glutamate dehydrogenase (NADP); 2, glutamine synthetase; 3, glutamate synthase; CM, cytoplasmic membrane; $\alpha\text{-KG}$, α -keto-glutarate. concentration of ammonia (which would enable accumulation of ammonia from ammonia-depleted environments) unless it were synthesized to very high levels within the cell. However, a large number of bacteria, including enteric bacteria (84), nitrogen-fixing bacteria (76, 89), phototrophic bacteria (13, 14, 56), *Thiobacillus neapolitanus* (9), and *Pseudomonas aeruginosa* (55), responded in a different manner to ammonia limitation. These organisms possess the glutamate dehydrogenase pathway for ammonia assimilation under ammonia-sufficient conditions, but they synthesize glutamine synthetase and glutamate synthase under ammonia limitation, whereas glutamate dehydrogenase is repressed (Figure 3). The ammonia-scavenging potential that this alternative pathway confers to organisms is much higher and resides in the low K_m value for ammonia of the first enzyme (124). Thus, both an enhanced synthesis of the ammonia-capturing enzyme system as well as synthesis of a different pathway with a higher affinity for ammonia assimilation have been observed in microorganisms in response to ammonia limitation in their environment. In this respect, the response seen is basically similar to that observed for carbon substrates under carbon limitation.

Limitation by Organic Nitrogen Sources

A more complex situation arises when the nitrogen source is an organic nitrogen compound. Often such compounds can be used both as a carbon and

energy source and as a nitrogen source, and the synthesis of enzymes that play a role in their assimilation is generally controlled by induction, catabolite repression, and nitrogen control (78). In gram-negative bacteria, a number of enzymes involved in the catabolism of organic nitrogen compounds are known to be inducible, e.g. amidase (10), histidase (72), enzymes involved in the utilization of arginine (98), allantoin (100), and lysine (38). A similar situation has been reported in yeasts that can utilize methylamine as a nitrogen source (138). Catabolite repression of such enzymes is generally observed when, besides the inducer, a preferred carbon source and ammonia are available. In the absence of ammonia, catabolite repression can be partly overcome, thus enabling the utilization of the organic nitrogen compound at a rate sufficient to satisfy the cell's requirement for nitrogen (122, 139). This regulation by the availability of nitrogen, called nitrogen control of enzyme synthesis, was thought to be mediated by glutamine synthetase, which itself becomes derepressed under nitrogen limitation and supposedly acts as an activator protein (78). However, recently, doubt has arisen whether the activator protein is actually glutamine synthetase itself (81, 95). Irrespective of the precise identity of the activator protein(s), it is clear that metabolic control in many microbes operates in such a way as to override carbon catabolite repression of enzymes involved in the metabolism of organic nitrogen compounds that would otherwise impede the utilization of such compounds under nitrogen-limiting conditions. An interesting adaptation to nitrogen limitation was recently reported in *Neurospora crassa* (28). A mutant of this organism, lacking all three constitutive amino acid transport systems, responded to nitrogen starvation in the presence of arginine by synthesizing an exocellular enzyme that removes the α -amino group from the amino acid.

Under conditions of nitrogen limitation, many microbes do not only derepress the synthesis of enzymes involved in the scavenging of ammonia from their environment or produce enzymes involved in the utilization of organic nitrogen sources, they may also derepress transport systems for the uptake of various amino acids. This may be of importance for their susceptibility to those antimicrobial compounds that enter the cells by means of amino acid transport systems (20). For instance, it has been reported that ammonia-limited *Klebsiella aerogenes* cells were at least 10 times more sensitive to cycloserine than were phosphate-limited cells (112).

PHOSPHATE LIMITATION

Conditions of phosphate limitation are common in natural ecosystems, and it is therefore of considerable interest to study in which way microorganisms adapt to such conditions. Although specific transport systems for phosphate have

been demonstrated in many microorganisms (31, 39, 64, 105), virtually nothing is known about the phenotypic adaptation of these proton motive force-driven carrier systems in relation to changes in the external concentration of this nutrient. Similarly, little is known about the way in which the initial assimilation of inorganic phosphate, which proceeds via phosphorylation of ADP, is affected by phosphate limitation in the environment.

Detailed studies on the kinetics of phosphate transport, leakage, and growth in a marine strain of *Rhodotorula rubra* grown under phosphate limitation (18, 101) have shown that under these conditions *R. rubra* elaborated a high substrate affinity by forming a phosphate transport system with an accumulation capacity between 12 and 35 times that required for growth at saturating concentrations. Thus, the organism responded by synthesizing more of the already existing carrier system. As expected, evidence was obtained that under phosphate limitation the rate of growth of the organism was limited by the rate of transport of this nutrient. These studies also revealed that under phosphate limitation at low growth rates approximately 10% of the amount accumulated leaked from the cells, both in the form of inorganic phosphate and of phosphate-containing metabolites.

In the last decade, much has become known with respect to major changes in the chemical composition of the cell envelope of bacteria consequent upon environmental constraints by phosphate. Studies on the cell wall of gram-positive bacteria (130) followed an initial observation (115) that glucose- or magnesium-limited cultures of *Bacillus subtilis* contained non-nucleic acid phosphorous, which was virtually absent from phosphate-limited cultures. Subsequent work demonstrated (33) that this was due to the fact that in the cell wall the phosphorous-containing glycerol teichoic acids, which under phosphate-sufficient conditions accounted for over 50% of the cell wall of this organism, were almost totally replaced by teichuronic acids during phosphorous deprivation. Further experiments revealed that these changes were due to a phenotypic response rather than to selection of a different genotype, which led Ellwood & Tempest (33) to the conclusion that the cell wall of *B. subtilis* was a phenotypically highly variable structure. Essentially similar responses to phosphate limitation have been reported for a number of other gram-positive bacteria (33). The functional significance of these changes in the nature of the major wall anionic polymer clearly is that under conditions of phosphate constraints, the cells use the little phosphate that is available for the synthesis of essential phosphorous-containing polymers, notably nucleic acids. By doing so, their requirement for this essential nutrient is reduced, in the case of *B. subtilis* from 3.2 to 1.7 g/100 g (dry weight) of cells (116). The above phenotypic variation in wall composition has been exploited in a very elegant fashion by Archibald (5) in studies on phage binding and wall growth in *B. subtilis*.

The composition of the cell envelope of gram-negative bacteria, in particular the lipid composition of the membranes (32), also varies markedly with changes in the physicochemical composition of the growth environment. A particularly striking example of the effect of phosphate limitation on the phospholipid content of a marine strain of *Pseudomonas fluorescens* has been reported (87). Lipids extracted from phosphate-sufficient magnesium-limited chemostat cultures of this organism contained phosphatidyl-ethanolamine, phosphatidyl-glycerol, and diphosphatidyl-glycerol. However, under phosphate limitation at a dilution rate of 0.2 h^{-1} , no traces of phospholipids were detected in lipid extracts. They had been completely replaced by ornithine-containing lipids and an acidic glycolipid, indicating that in this organism acidic and zwitterionic lipids totally lacking phosphate may take the place of phospholipids in the cytoplasmic membrane under phosphate limitation.

Phosphate limitation may also have a marked effect on the production of exoenzymes by certain bacteria. This has, for instance, been reported for *Bacillus licheniformis*, which, when grown under such conditions, produced exocellular ribonuclease and alkaline phosphatase (137). More recently, Toda et al (121) reported an increased excretion of invertase and acid phosphatase in phosphate-limited *Saccharomyces carlsbergensis* cultures. The molecular details of these processes remain to be established, although the potential value to the organism of some of these responses is obvious.

LIMITATION BY OTHER INORGANIC NUTRIENTS

Potassium Limitation

Specific transport systems for potassium have been found in many microorganisms (7, 47, 108), and it is generally thought that this cation is taken up mainly electrogenically, although in addition, potassium/proton or potassium/sodium antiport systems have been implicated. It is not clear if and in which way the properties of the K^+ transport systems change in relation to changes in the external concentration of this nutrient during growth of organisms. In view of the fact that K^+ limitation rarely, if ever, occurs in natural ecosystems, microorganisms in the course of evolution probably never had to face the problem of potassium constraint on their metabolism. Consequently, adaptive responses as seen for other essential nutrients that are often in limited supply may not have evolved in the case of potassium.

Nevertheless, potassium is an important nutrient, not in the least because of its role in pH homeostasis (94), and although the intracellular concentration of this cation has been found to vary with the growth rate of organisms, the osmolarity of the environment, and the external pH, it is always in excess of 50 mM and may reach 0.5 M in gram-positive organisms (115). Potassium is

unique among the major nutrients in that it is contained within the cell in unmodified and mobile form. Since the environmental K^+ concentration is generally much lower than that present in the cytoplasm, the cells must expend energy to maintain a certain (often sizeable) transmembrane K^+ gradient. It is to be expected that this gradient will be dependent upon the extracellular K^+ concentration and will be maximized under conditions of K^+ limitation. When in a glucose-limited culture of *Klebsiella aerogenes* the effect of a decreasing (from 9 to 0.05 mM) potassium concentration was studied, a progressive increase in the respiration rate of cells was observed along with a corresponding fall in the yield with respect to both glucose and oxygen (54). Essentially similar observations were made in studies with *Candida utilis* (1), although the situation in this yeast was slightly more complicated because this organism, under conditions of K^+ limitation, had a much reduced potassium content when compared to K^+ -sufficient cells. When *K. aerogenes* was grown under K^+ limitation, a significant extracellular accumulation of products of the oxidative metabolism of the carbon- and energy-substrate glucose (namely, pyruvate, acetate, gluconate, and 2-ketogluconate) was observed (91). Konings & Veldkamp (66) have suggested the following interpretation for these observations. When a microorganism is confronted with a greatly decreased external concentration of this essential cation, the organism has to increase the proton motive force to maintain the internal concentration constant. Such a response, however, will also lead to an increase in leakage processes and this means that a relatively small increase in the proton motive force requires a considerable increase in the primary pump activities. The primary pumps that a cell can mobilize for this purpose are the electron transfer systems, and an increase in their activities requires an increased rate of dissimilation of the energy source. The predicted response of a cell to growth limitation by an essential cation, therefore, would be an increased rate of oxidation of the energy source and this is precisely what has been observed experimentally. Tempest & Neijssel (118) subsequently showed that there was a linear relationship between the specific rate of oxygen uptake by K^+ -limited cells and the electrochemical potential of the K^+ gradient.

The observation (1) that the potassium content of K^+ -limited *C. utilis* cells was more than 10 times less than that of K^+ -sufficient organisms posed the question of the effect of a reduced cytoplasmic K^+ concentration on the functioning of mitochondria. A comparison of mitochondria isolated from glucose- and K^+ -limited organisms grown at a dilution rate of 0.1 h^{-1} showed the K^+ -limited mitochondria to have a lower P/O ratio, consistent in the loss of one site of energy conservation (2), whereas glucose-limited organisms possessed mitochondria with a P/O ratio of 3. So far this effect of K^+ limitation has remained unexplained.

Magnesium Limitation

Specific carrier systems for magnesium transport have also been reported (63, 108), but as with potassium transport, little is known about a possible modulation of the activity or properties of these systems under conditions of magnesium limitation. There is evidence that magnesium-limited *Bacillus subtilis* cells excrete compounds that bind magnesium, thereby improving their ability to compete with other organisms for growth-limiting amounts of magnesium (82). Similarly, cell walls of magnesium-limited cells of this organism showed an increased Mg^{2+} binding affinity over magnesium-sufficient cells (83). The latter process indicates that certain organisms may respond to magnesium limitation by synthesizing binding sites in the cell wall at which these cations may be concentrated prior to their translocation. Magnesium is an essential nutrient for living cells and their basic requirement for it does not seem to vary much for widely different organisms (1, 2, 115). Under a variety of growth-limiting conditions, including magnesium limitation, the cellular magnesium content increased with increasing growth rate in parallel with the cell's ribosome content. This led Tempest (115) to the conclusion that most of the cell-bound magnesium was associated with the ribosomes. Magnesium is also present in the walls of bacteria and its abundance in the walls varies greatly with growth conditions (119). This no doubt is related to the observation that the susceptibility of magnesium-limited cells of *Pseudomonas aeruginosa* to lysis by ethylenediaminetetraacetic acid and polymyxin or to killing by rabbit phagocytes was markedly lower than that of carbon-limited cells (37). However, these phenomena are not yet clearly understood at the molecular level.

Magnesium limitation generally provokes an increase in respiration rate of cells (as is the case with potassium limitation). This leads to a decrease in the yield on the carbon and energy source and on oxygen.

Sulfate Limitation

Accumulation of sulfate from the environment is also mediated by specific transport systems (31, 63, 108). Its further assimilatory metabolism in the cell is well documented (41) and proceeds via cysteine into various important cellular components, particularly coenzymes such as thiamine pyrophosphate, coenzyme A, biotin, and α -lipoic acid. Only few systematic studies on the effect of sulfate limitation on microorganisms have been reported. In *Klebsiella aerogenes*, sulfate-limited growth conditions caused a reduction in the protein content of the cell envelope and into the sulfur content of the soluble protein. However, the sulfur content of the ribosomal fraction was not different, nor was there any effect on the ribosomal protein content of the cells (103). The external sulfate concentration also exerted a strong influence on the sulfur content of protein in two marine bacteria (21), but the distribution of sulfur in

major biochemical fractions was not affected. In *Alteromonas eutroviolaceus*, sulfate starvation led to a rapid development of sulfate transport capacity (22).

Sulfur is also an important constituent of non-heme iron proteins, and in mitochondria of sulfate-limited cultures of *Candida utilis*, the characteristic $g=1.94$ electron paramagnetic resonance signal of non-heme iron proteins was missing, whereas it was detected in mitochondria prepared from glucose-limited cells (73). As a consequence of this, mitochondria of sulfate-limited cells lacked site 1 phosphorylation and the growth yield of these cultures was markedly lower than those of glucose-limited cells. A similar conclusion was reached in studies on the effect of sulfate-limitation on *Paracoccus denitrificans* (126), *Escherichia coli* (97), and *K. aerogenes* (113).

Sulfate is an essential electron acceptor for growth of sulfate-reducing bacteria. Under conditions of sulfate limitation, *Desulfovibrio desulfuricans* is able to ferment pyruvate, malate, and choline, whereas *Desulfobulbus propionicus* can ferment pyruvate, lactate (133), and ethanol (70). Lactate and ethanol cannot be fermented by *Desulfovibrio* sp. unless it is co-cultured with hydrogen-utilizing methanogenic bacteria (16), which substitute for sulfate as a hydrogen acceptor.

Limitation by Trace Elements

The quantitative requirement of microbes for trace elements such as iron, manganese, zinc, copper, nickel, and cobalt is so low that except in certain anaerobic ecosystems, their availability generally does not lead to growth limitation. A notable exception to this is probably iron, and there is, for instance, evidence that mechanisms to ensure iron limitation of potential pathogenic microbes form part of the host-defense mechanism against microbial infections (131).

Limitation by iron in the environment has been shown to provoke the synthesis and excretion of iron chelating compounds in a number of organisms (71). These compounds include the fluorescent pigment of *Pseudomonas fluorescens* (86), the enterochelins of enteric bacteria (93), and the mycobactins of *Mycobacteria* (111). In enteric bacteria, iron limitation also results in the synthesis of specific envelope proteins that in conjunction with the enterochelin are thought to function as a high-affinity iron uptake system (11, 49). Since iron plays a major role in electron transport chains in microorganisms, iron limitation is expected to affect the synthesis and function of iron-sulfur proteins and cytochromes. In accordance with this was the observation that as with sulfur limitation, iron limitation caused a loss of site 1 energy conservation in *Candida utilis* (73). In contrast, oxidative phosphorylation in iron-limited *Paracoccus denitrificans* was not affected (126).

CONCLUDING REMARKS

Microorganisms possess a remarkable potential to adjust themselves, both structurally and functionally, to changes in their environment. This applies in particular to the behavior in response to the most common of environmental constraints that these organisms experience, namely, that of nutrient limitation. Chemostat studies carried out during the last two decades have shown that limitation by a major nutrient may invoke adaptive changes at three different levels. The principal objective of these phenotypic adaptations appears to be to allow uptake and further metabolism of the limiting nutrient even when its concentration is diminishingly low. The response may include changes at the level of transport of the nutrient across the cell envelope and cytoplasmic membrane, and changes in the initial metabolism of the nutrient in the cytoplasm and in the partitioning of the nutrient over metabolic pathways leading to cell polymers. Although our knowledge of these processes has sufficiently advanced in recent years to enable an appreciation of the significance of these mechanisms, there are major areas where our understanding of the molecular details of the various processes is only superficial or even almost non-existent. This is particularly true in the case of regulation and adaptation of the transport systems for various nutrients in response to nutrient limitation, and there is clearly a need for much work in this area.

Most of the studies carried out to date have been performed with organisms obtained by batch-type (nutrient-sufficient) enrichments. We have previously argued that the properties of these organisms may not be representative of those that permanently live in oligotrophic environments (44). Despite the fact that such environments are, by volume, by far the most important on earth (i.e. the ocean below the photic zone), very little is known about the way in which the organisms that inhabit such environments have adapted to life under conditions of permanent nutrient scarcity (96). The study of such organisms presents very much of a challenge and to us appears to be potentially very exciting and rewarding.

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