25 Inniss, W.E., and Ingraham, J.L., Microbial life at low temperatures: Mechanisms and molecular aspects, in: Microbial Life in Extreme Environments, pp. 73–104. Ed. D. J. Kushner. Academic Press, London 1978.

26 Kawamoto, S., Kojima, K., Hanada, T., Iokuyama, S., Yashima, S., and Eguchi, Y., Cold resistant mutants of Escherichia coli. Agric. Biol. Chem. *48* (1984) 1097–1101.

27 Law, B.A., Reviews of the progress of dairy science: enzymes of psychrotrophic bacteria and their effects in milk and milk products. J. Dairy Res. *46* (1979) 573–588.

28 Luisetti, J., and Gaignard, J.L., Gel de printemps et bactéries glaçogènes. Arboric fruit. *375* (1985) 46–48.

29 Maki, L.R., Galyan, E.L., Chang-Chien, M., and Caldwell, D.R., Ice nucleation induced by Pseudomonas syringae. Appl. Microbiol. *28* (1974) 256–259.

30 Malcolm, N.L., Subunit structure and function of Micrococcus cryophilus glutamyl transfer RNA synthetase. Biochim. biophys. Acta *190* (1969) 347–357.

31 Marr, A.G., and Ingraham, J.L., Effect of temperature on the composition of fatty acids in Escherichia coli. J. Bact. *84* (1962) 1260–1267.

32 McGibbon, L., and Russel, N.J., The turnover of phospholipids in the psychrophilic bacterium Micrococcus cryophilus during adaptation to changes in growth temperature. J. gen. Microbiol. *131* (1985) 2293–2302.

33 Morita, R.Y., Psychrophilic bacteria. Bact. Rev. *39* (1975) 144–167.

34 Nash, C.H., Grant, D.W., and Sinclair, N.A., Thermolability of protein synthesis in a cell-free system from the obligately psychrophilic yeast Candida gelida. Can. J. Microbiol. *15* (1969) 339–343.

35 O'Donovan, G.A., and Ingraham, J.L., Cold-sensitive mutants of Escherichia coli resulting from increased feedback inhibition. Proc. natn. Acad. Sci. USA *54* (1965) 451–457.

36 Olsen, R.H., and Metcalf, E.S., Conversion of mesophilic to psychrophilic bacteria. Science *162* (1968) 1288–1289.

37 Pacha, R.E., Characteristic of Cytophaga psychrophila (Borg) isolated during outbreaks of bacterial cold-water disease. Appl. Microbiol. *16* (1968) 97–101.

38 Piton, C., and Richard, J., Détermination du taux et de la nature des Pseudomonas psychrotrophes intervenant dans les deux sources principales de contamination microbienne du lait à la ferme: la peau des mamelles et le matériel de traite. Sci. Alim. *5* (1985) 13–16.

39 Potier, P., Hipkiss, A.R., and Kushner, D.J., Protein turnover in a psychrotrophic bacterium. Archs Microbiol. *142* (1985) 28–33.

40 Punsola, L., and Guarro, J., Keratinomyces ceretanicus sp. nov. a psychrophilic dermatophyte from soil. Mycopathologia *85* (1984) 185–190.

41 Rizk, J., Accidents transfusionnels d'origine bactérienne. Méthodes de prévention. Thèse Doct. Med., Lyon 1985.

42 Russel, N.J., The regulation of membrane fluidity in bacteria by acyl chain length changes, in: Biomembranes, vol. 12. Membrane Fluidity, pp. 329–347. Eds M. Kates and L.A. Manson. Plenum, New-York 1984.

43 Schmidt-Nielsen, S., Über einige psychrophile Mikroorganismen und ihr Vorkommen. Zentbl. Bakt. ParasitKde *9* (1902) 145–147.

44 Shaw, M.K., Effect of abrupt temperature shift on the growth of mesophilic and psychrophilic yeasts. J. Bact. *93* (1967) 1332–1336.

45 Stokes, J.L., General biology and nomenclature of psychrophilic microorganisms, in: Recent Progress in Microbiology, pp. 187–192. University of Toronto Press, Toronto 1963.

46 Svensson, B.H., Different temperature optima for methane formation when enrichments form acid peat are supplemented with acetate or hydrogen. Appl. environ. Microbiol. *48* (1984) 389–394.

47 Tai, P.C., and Jackson, H., Growth and respiration of an obligate psychrophile, Micrococcus cryophilus, and its mesophilic mutants. Can. J. Microbiol. *15* (1969) 1151–1155.

48 Tai, P.C., Kessler, D.P., and Ingraham, J.L., Cold sensitive mutations in Salmonella typhimurium which affects ribosome synthesis. J. Bact. *97* (1969) 1298–1304.

49 Ward, E.W.B., Temperature-induced changes in the hyphal morphology of the psychrophile Sclerotinia borealis. Can. J. Bot. *46* (1968) 524–525.

50 Westlake, D.W., Jobson, A., Phillippe, R., and Cook, F.D., Biodegradability and crude oil composition. Can. J. Microbiol. *20* (1974) 915-928.

51 Whitman, P.A., and Marshall, R.T., Isolation of psychrophilic bacteriophage-host systems from refrigerated food products. Appl. Microbiol. *22* (1971) 220–223.

0014-4754/86/11/121192-06$1.50 + 0.20/0

Life without oxygen: what can and what cannot?

by A. J. B. Zehnder and B. H. Svensson

Department of Microbiology, Agricultural University, Hesselink von Suchtelenweg 4, NL-6703 CT Wageningen (The Netherlands)

Summary. The basic principles involved in the biotransformation of organic carbon compounds in the absence of molecular oxygen (dioxygen) are presented in this paper. The role of various electron acceptors during the breakdown of organic compounds is discussed and the metabolic end-products expected are summarized. The different biochemical possibilities and strategies for the anaerobic degradation of organic matter and the metabolic response of some organisms to anaerobiosis are elucidated. Positive and negative effects of anaerobiosis on environmentally relevant processes and their influence on man and on animals are reviewed. Finally, some examples of the biotechnological application of anaerobic processes are presented.

Key words. Anaerobic metabolism; anaerobic environment; biotechnological application of anaerobes; redox sequences; evolution of oxygen.

1. Introduction

Although our ideas about the sequence of events in the evolution of primitive life have changed in the last 40 years, it is now commonly agreed that the first living organisms are most likely to have resembled present-day fermentative bacteria[7]. They lived in the absence of molecular oxygen at the expense of abiotically synthesized organic compounds (anaerobic heterotrophy). When anaerobic life became more firmly established, anaerobic photosynthesizing bacteria emerged. These microorganisms possessed a light-harvesting and energy-transducing substance, chlorophyll, which enabled them to use energy from light to form organic matter from carbon dioxide. Once established, these organisms could maintain and even increase the supply of organic carbon needed for the persistence and further development of heterotrophic life. Advancing evolution of the photosynthetic machinery (addition of photosystem II) led to the emergence of prototypes of cyanobacteria which could substitute water for hydrogen or hydrogen sulfide as electron donor for the assimilative reduction of carbon dioxide. The oxidation of water resulted in the release of molecular oxygen (dioxygen) into the atmosphere. Initially, the oxygen that evolved, most probably reacted rapidly with oxidizable inorganic matter such as bivalent iron, forming magnetite and hematite. These oxides probably led to the 'Banded Iron Formations' and later to the 'Red-Bed' deposits[7]. Eventually, about 2.3 eons ago[33], free oxygen began to accumulate in the atmosphere, changing the environment from reducing to oxidizing conditions (fig. 1).

The occurrence of free molecular oxygen represented a considerable hazard to the already existing heterotrophic life. This compound, which was 'new' for the environment, was very aggressive, and this was amplified especially when oxygen reacted chemically with various reduced biological electron carriers, such as NADH or ferredoxin. The reaction products were hydrogen peroxide or superoxide. At that time the organisms had two strategies for surviving these extreme life-threatening conditions; either they retreated to anoxic habitats or they developed enzyme systems able to destroy these reactive molecules. Organisms following the latter strategy succeeded in the production of protective systems such as superoxide dismutase, catalase and/or peroxidase. When some organisms 'learned' to use molecular oxygen for the regeneration of their electron carriers and in some cases also for synthetic reactions or for the purpose of degradation, evolution acquired a new dimension, which finally led to the very complex multicellular creatures which populate the present-day earth. However, even the most advanced aerobic organisms still possess the ability to catalyze many biochemical processes which were probably carried over from the ancient anaerobic time. These reactions, which are not always operative under oxic conditions, can be turned on when oxygen becomes scarce (e.g. fermentations, anaerobic glycolysis in eukaryotes, etc.).

In the following, we discuss the metabolic capacity of anaerobes and their wide range of alternative pathways, as well as the ability of some aerobes to utilize anaerobic pathways for energy conservation in the absence of oxygen. Further, we summarize the potential for the use of anaerobes in biotechnological processes, and the effects of anaerobiosis in some isolated habitats on the oxic environment.

2. *Principles of the anaerobic metabolism*

For the discussion of the general principles of anaerobic metabolism, it is helpful to divide the cellular metabolic processes into three parts (fig. 2):

(1) Oxidation reactions leading from the substrate to intermediary products such as pyruvate, acetyl-CoA, oxoglutarate etc., reduced co-factors and ATP.

(2) Reactions during which the reduced co-factors such as NADH or $FADH_2$ are reoxidized and thus regenerated. These processes are commonly referred to as fermentation and respiration. Some of them can serve for ATP production.

(3) Biosynthetic reactions used by the organisms to produce macromolecules, which constitute the major portion of the cellular mass starting from the intermediary products or directly from simple compounds present in the extracellular environment. These are generally ATP-consuming reactions.

Reactions (1) and (2) are termed catabolism or degradative metabolism and reaction (3) is often called anabolism.

In the following we would like to restrict our discussion to heterotrophic reactions, i.e. metabolic processes in which organic substrates serve as energy and main carbon sources. For an understanding of the many aspects of anaerobic metabolic processes it is helpful first to summarize briefly those reactions where molecular oxygen is directly or indirectly involved. Oxygen mainly serves two different functions in the metabolism of

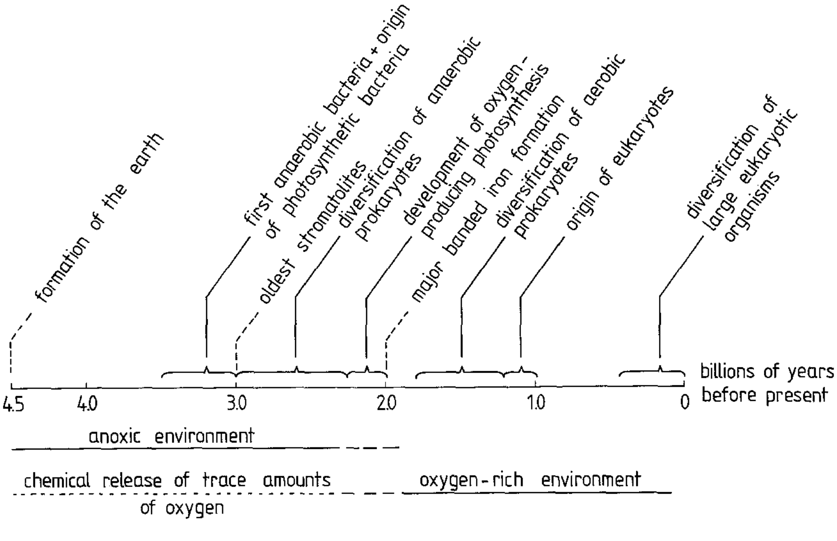

Figure 1. Major events in evolution in chronological sequence together with the history of dioxygen gas formation.

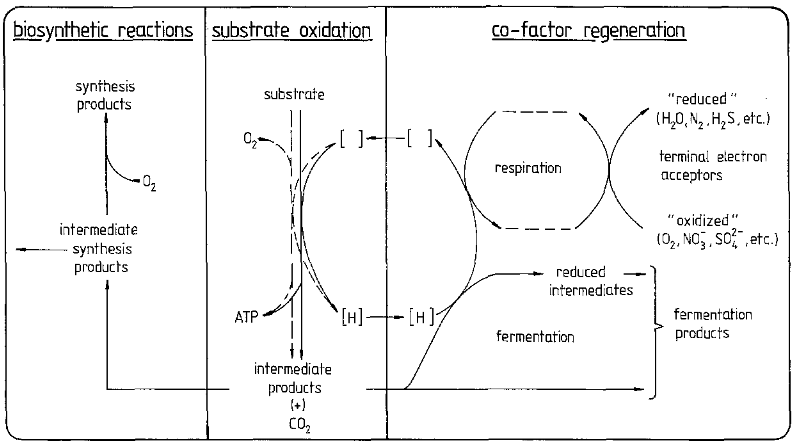

Figure 2. The three basic parts of metabolism together with the various possible modes of action of molecular oxygen during these processes. During anabolic reactions oxygen cannot be replaced by other compounds. However, there are alternative anaerobic reaction sequences for most of the oxygen dependent transformations. For the regeneration of the primary electron acceptors ([] = NAD, FAD, etc.) oxygen can be replaced by other oxidized inorganic or organic compounds, e.g. nitrogen-, metal- and sulfur oxides, carbon dioxide, protons, fumarate, pyruvate, etc.

aerobes; namely a) as terminal electron acceptor during respiration processes (in part (2)) and b) as a direct oxidant of organic and some inorganic molecules (in parts (1) and (3)). In the absence of oxygen the first function, i.e. the regeneration (oxidation) of the electron carriers (in most cases FADH$_2$ and NADH) can be fulfilled by other oxidized compounds such as oxides of nitrogen, oxidized metal ions, some organic compounds, sulfur and its oxides, carbon dioxide or protons. These alternative electron acceptors will yield less energy during electron transport phosphorylation as compared with molecular oxygen[45]. In its second function oxygen *cannot* be replaced by other compounds, since here molecular oxygen is inserted into the substrate molecule by the action of specific enzymes known as oxygenases (dioxygenase, mono-oxygenase and amine oxidase). However, reactions similar to those catalyzed by oxygenases can also be accomplished by strictly anaerobic bacteria. For example, hydroxylation reactions are possible in the absence of molecular oxygen, by a combination of dehydrogenation and hydration reactions such as during the synthesis of malate from succinate in the citric acid cycle (see below). There are many more such cases in the metabolism of anaerobes where oxygenation steps are either circumvented or accomplished by an oxygen-independent mechanism[24]. We would like to emphasize that almost all catabolic and anabolic reactions in organisms basically proceed as anaerobic processes and that the direct involvement of molecular oxygen in metabolic processes is still an exception.

2.1 Substrate oxidation

During catabolic processes the electron donor (substrate) is generally oxidized via various pathways. The most common is probably the glycolytic pathway (Embden-Meyerhof pathway) during which two molecules of pyruvate are produced from one molecule of glucose. A portion of the change in free energy between these two compounds is 'trapped' in the form of ATP. The four electrons liberated in the course of this oxidation are transferred onto two oxidized molecules of nicotinamide adenine dinucleotide (NAD$^+$) resulting in two NADH molecules. Other possibilities for the formation of pyruvate from glucose are the Entner-Doudoroff pathway and the pentose phosphate pathway. The latter is, in most cases, used for biosynthetic and not for oxidative purposes. Pyruvate is further oxidized to acetyl-CoA and finally to carbon dioxide via the tricarboxylic (citric) acid cycle. The electrons liberated are temporarily stored in the form of reduced dinucleotides, namely in three NADH, one NADPH and one FADH$_2$. All these reactions take place in the absence of oxygen, regardless of whether the organism can use oxygen or not.

The main purpose of the oxidative reaction sequences is to form common intermediates such as pyruvate, acetyl-CoA and, in some cases, also intermediates of the tricarboxylic acid cycle and ATP. This general statement is almost valid irrespective of the organism and the substrate involved.

In the following we would like to summarize some of the main anaerobic reaction mechanisms used by organisms to transform and/or oxidize their substrate:

Hydrogenation. During this process triple and double bonds, and aromatic compounds are reduced. In the case of aromates hydrogenation is necessary for anaerobic ring cleavage[44], a reaction which has only been observed in benzene rings substituted with groups containing an oxygen function, e.g. carboxyl-, hydroxyl- and methoxy-groups. The anaerobic degradation of nitrobenzenes and nitrophenols has been observed although the metabolism of these compounds has not yet been elucidated.

Hydration. In some unsaturated aliphatic ompounds the double bond can be hydroxylated, resulting in alcohol formation.

Hydrolysis. This is one of the most important processes in nature, since it makes insoluble biopolymers available for various metabolic reactions, e.g. hydrolysis of cellulose, xylane, protein etc.

Carboxylation. Formation of succinate from propionate is one of the few reactions know where carboxylation is of importance. However, there are some indications that this type of transformation might prove to be the primary activation of some compounds, still assumed to be inert under anaerobic conditions[46].

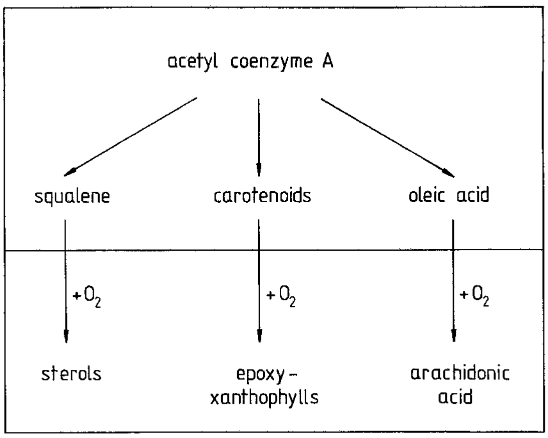

Figure 3. Schematic sequence for the biosynthesis of various compounds starting from acetyl-CoA. Oxygen-dependent steps can be carried out only by aerobic organisms which evolved comparatively late in the history of life. No alternative pathways are known for the anoxic biosynthesis of sterols, epoxy-xanthophylls or arachidonic acid and their derivatives.

Reductive dehalogenation. In recent years an increasing number of reports have been published about the anaerobic degradation of halogenated alkanes and aromatics. Compounds which are recalcitrant under aerobic conditions are subjected to dehalogenation by a reductive reaction in the absence of oxygen. Reductive dehalogenation plays an important role during anaerobic degradation of hexachlorocyclohexane[25], dichlorobenzoate[39], tetrachloroethylene[41] and formation of monochlorobenzene from trichlorobenzene[40]. The exact reaction mechanism of the reductive dehalogenation is not yet known. Other anaerobic reaction mechanisms of importance are *dehydrogenation, dehydration, decarboxylation, lyase reactions,* and *demethoxylation.*

During the anaërobic oxidation of organic compounds a variety of the reaction types mentioned above will be used by the organisms to produce common intermediates which generally are further oxidized via classical biochemical pathways. There are only a few natural and synthetic molecules which seem to resist anaerobic oxidation; namely, 1) saturated alkanes and unsubstituted aromatics or those with functional groups composed of halogens or carbon and hydrogen; and 2) molecules which can be synthesized only in the presence of oxygen[47]. The latter group consists of triterpenes (e.g. sterols), high molecular weight lignin and humic compounds (fulvic acid, humic acid, humins).

2.2 Co-factor regeneration

To allow the substrate oxidation to proceed, the reduced primary electron acceptors such as NADH, $FADH_2$, ferredoxins etc. have to be reoxidized. This regeneration is done by transferring the electrons to inorganic or organic terminal electron acceptors which are released into the environment after reduction (e.g. N_2, H_2S, CH_4, ethanol etc.). The reoxidation of the reduced primary electron acceptor basically proceeds by respiration or by fermentation. In respiratory processes, external oxidized compounds are used as terminal electron acceptors (e.g. nitrate, sulfate, carbon dioxide, fumarate, etc.) whereas during fermentation the electrons from the oxidation reactions (e.g. glycolysis) are transferred to an organic intermediate which was formed during the same process (e.g. pyruvate will be reduced to lactate, acetaldehyde to ethanol, etc.). As a result of fermentation, ATP synthesis is only possible by substrate level phosphorylation and in certain cases also by the efflux of a fermentation product.

If an organism has the choice between different respiration and/or fermentation pathways it will generally choose that which is energetically most favorable under the given environmental conditions. An exception to this rule is *Thiosphaera pantotropha;* this organism will respire oxygen and nitrate concomitantly[28]. In cases where a natural population has the possibility of choosing between different final electron acceptors, the population itself will follow the concept of electron free energy[38,45]; i.e. it will follow the redox sequence by first using nitrate (denitrification and nitrate reduction) followed by metal oxides, sulfate (sulfate reduction) and carbon dioxide (methane formation). For each acceptor there exists one or more specific bacterial species which catalyzes the corresponding redox reaction.

The goal of respiration or reduction of organic intermediates (i.e. fermentation) is in essence the regeneration of reduced co-factors. The fact that these reactions sometimes have the capacity to yield energy is only a very useful side-effect.

2.3 Anabolic reactions

Synthetic pathways that emerged early in the Precambrian are able to proceed in the absence of oxygen. Reaction steps presumably added at a later age might be expected with increasing frequency to have an intrinsic requirement of oxygen. The oxygen dependence has particularly been determined in detail for three synthetic pathways. One of these controls the manufacture of a class of compounds which includes the sterols, the other gives rise to the carotenoids and the last one is concerned with the synthesis of fatty acids (fig. 3). Two observations about the evolution of these biosynthetic pathways are appropriate. First, even in groups of organisms that have long been aerobic the first steps in the synthesis are independent of the oxygen supply. Molecular oxygen enters the reaction sequence only at later stages. The second observation is in accordance with this, namely that anaerobic bacteria are only capable of performing the early steps of the pathway and that they have no alternative routes for synthesizing sterols, epoxy-xanthophylls and fatty acids with more than one double bond per molecule starting from acetyl-CoA[20].

3. Biochemical adaptation to changes in the redox conditions

In the following section we want to emphasize some strategies by which various organisms may adapt their metabolism to changing redox conditions, i.e. to the presence or absence of specific electron acceptors. As a first example, *Escherichia coli* was chosen as a model, because it represents a versatile organism which is able to use a variety of respiration and fermentation pathways, depending on the environmental conditions. Besides bacteria some animals also have the capacity temporarily to survive oxygen depletion. They partially employ 'ancient' anaerobic fermentation pathways which they have included in their specific needs and biochemical potential. Some examples will be discussed in the second section. Finally, a unique response of the glyoxylate cycle to the switch from aerobic to anaerobic respiration in *Thiobacillus versutus* will be presented.

3.1 Features of catabolism of E. coli

In its natural habitat, the gut, *E. coli* is found mostly growing attached to the epithelium, and living at an interface of aerobiosis and anaerobiosis. From the epithelium side oxygen may reach the habitat of *E. coli* (oxygen is brought there by arterial blood), while on the gut side strictly anaerobic conditions prevail[13]. *E. coli* has adapted almost ideally to this situation by having the capacity to grow aerobically and anaerobically. Three different ways of energy conservation are available for *E. coli* (fig. 4). Firstly, in various modes of respiration, organic compounds are oxidized via glycolysis and the tricarboxylic acid cycle to carbon dioxide, water and reducing equivalents in form of reduced co-factors such as NADH and $FADH_2$. These are then regenerated by different routes of respiration, where oxygen, nitrate or fumarate may act as terminal electron acceptors[13].

In the absence of these acceptors *E. coli* has to rely on organic compounds formed during the degradation of organic substrates in order to reoxidize NADH originating from the glycolysis. When inorganic electron acceptors or fumarate are lacking, lactate is fermented to acetate, formate and ethanol. In this process a part of the intermediary pyruvate acts as an electron acceptor to obtain a balanced redox reaction with ethanol as the reduced compound. In the case of lactate, ATP will be formed only from the acetate formation of the remaining pyruvate. In this connection it must be mentioned that under certain limiting conditions *E. coli* can use both respiratory and fermentative metabolism concomitantly.

E. coli is able to reduce nitrate to nitrite (see above) but not further to nitrous oxide or dinitrogen[13]. However, nitrite can be used as an electron acceptor during fermentation[5]. In the presence of nitrite, lactate is converted to acetate and formate, and the electrons used before for the formation of ethanol are now disposed of onto nitrite which is reduced to ammonium (fig. 4). The reduction of nitrite to ammonium does not yield any additional energy. But because more pyruvate is now being converted to acetate per mol of lactate the organism will benefit energetically by the use of nitrite. In fact, Cole and Brown[5] have shown experimentally that the cell yield of *E. coli* in a continuous culture increased when nitrite instead of ammonium was added to the growth medium. This third possibility for energy conservation might be regarded as a transitional development between fermentation and respiration, as might have happened in the course of evolution. Here the reduction of nitrite still serves its pristine goal by exclusively regenerating the co-factor without being directly coupled to energy conservation.

The examples above emphasize the importance of electron acceptors to allow catabolic reactions to proceed continuously. The mode of coupling and efficiency of the reoxidations of co-factors obviously affect the extent of primary electron donor oxidation. The respiratory systems allow for complete oxidation of the organic substrate to carbon dioxide, while fermentations give rise to reduced compounds such as ethanol. The use of an extra electron scavenging system in fermentation will result in more oxidized products coupled to a net gain of ATP.

3.2 Metabolic changes in animals in response to anoxia

Some animal cells depleted in oxygen are able to maintain their cytoplasmic redox levels, i.e. oxidize the co-factors reduced in the glycolytic reactions, by pathways differing

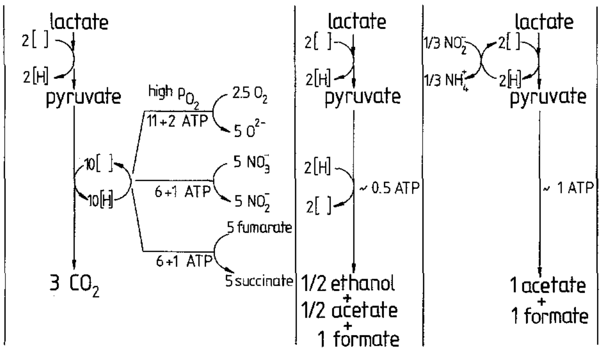

Figure 4. Different reoxidation possibilities for the reduced coenzymes resulting from the oxidation of organic substrates in *Escherichia coli*. Energy saved as ATP by the different pathways is also given. At low oxygen concentrations less ATP is probably gained than indicated. The ATP-formation by the use of NO_2^- acting as the terminal electron acceptor is uncertain. The figure is mainly based on the review by Ingledew and Poole[13] and results presented by Cole and Brown[5]. Lactate was chosen as a model substrate, since *E. coli* seems not to be able to respire nitrate in presence of glucose as the only electron donor.

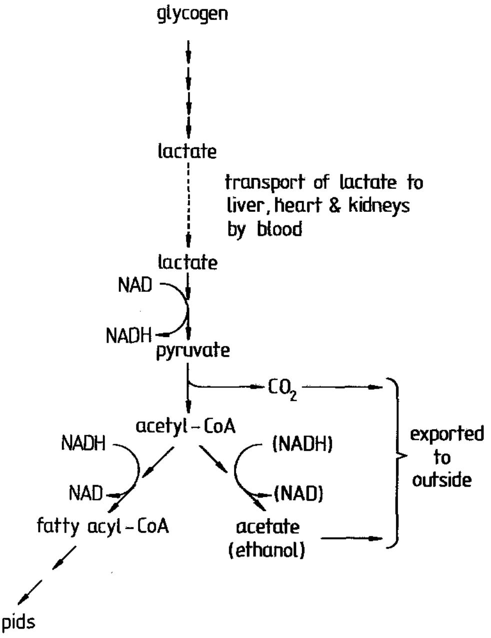

Figure 5. Pathways for maintaining the redox levels under anoxic conditions in fish, according to Hochachka[11].

form those discussed above for bacteria. Mammalian cells mostly utilize the lactate dehydrogenase reaction to reoxidize NADH by forming lactate from pyruvate. In cephalopods and bivalve molluscs the NADH from catabolic reactions may be regenerated during condensation of either pyruvate and alanine or pyruvate and arginine. These reactions are catalyzed by dehydrogenases and the products formed are alanopine or octopine and NAD[11]. The co-factor, reoxidized in this manner, is thus available again for utilization in catabolic oxidation processes. Up to 30% of glucose metabolized in the bivalve mollusc heart muscle may occur as alanopine, implying that this reaction is important during oxygen starvation. For some vertebrates the pathway shown in figure 5 is the only way to survive anoxia[11]. Here the glycolytic oxidation and subsequent NADH formation are balanced by the reduction of pyruvate to lactate as discussed above. The accumulation of this product may, however, result in a decrease in blood pH. Within liver, heart and kidneys lactate may be reoxidized to pyruvate and subsequently to acetyl-CoA. The two NADH which are formed during this process from one lactate will be oxidized by a reversed β-oxidation during which the acetyl-CoA moieties are used for lipid synthesis[11]. When the oxygen supply is not limited any longer these lipids may again serve as energy and carbon sources.

The production of alanopine, octopine and lipids are clear examples of how even higher animals are able to overcome periods without oxygen. Instead of disposing of valuable organic compounds and reducing equivalents form glycolysis in the form of fermentation products (ethanol, lactate, etc.), as is done by some prokaryotes and lower eukaryotes, these organisms store the 'waste products' from their anaerobic metabolism in molecular forms which are neutral with respect to pH and osmotic activity. These 'waste products' will be available again when the environmental conditions become more favorable.

3.3 Effect of the terminal electron acceptor on carbon assimilation

As mentioned above, the production of intermediary products and their conversion into biomass are functions generally independent of the co-factor regeneration. In *Thiobacillus versutus* a unique change in the carbon assimilation route was observed when the bacterium grew on acetate and with nitrate instead of oxygen as the terminal electron acceptor. Under aerobic conditions, *T. versutus* is lacking one of the two key enzyme systems in the glyoxylate cycle: the isocitrate lyase. If the organism is grown anaerobically with acetate as carbon and energy source and with nitrate as terminal electron acceptor, isocitrate lyase is synthesized after a short adaptation period and the glyoxylate by-pass becomes functional[4]. This is the only example known to us where the final electron acceptor induces or represses an enzyme in the early phase of a carbon assimilation pathway without being directly involved in the reaction or accepting electrons from this reaction.

4. Effects of anaerobic processes on the environment

The number and the volume of anaerobic environments have substantially increased in recent years. This is especially due to the interference of man's activity with several biogeochemical cycles. The disposal of nutrients (e.g. nitrogen and phosphorus compounds) and organic matter into the aquatic environment has resulted in eutrophication and enhanced sedimentation rates, which in turn has led to increased oxygen consumption rates. Since in most aquatic habitats with a high organic matter content oxygen input is slower than oxygen removal, anaerobic habitats have grown larger, and they have even become persistent where well-aerated conditions prevailed earlier. As a consequence more reduced compounds from anaerobic respiration (e.g. N_2O, H_2S and CH_4) have been liberated (table 1). Besides the pollution of aquatic systems, intensified agriculture has added to an increase especially of methane and nitrous oxide (increase in number of cattle with a subsequent higher methane formation and extensive fertilization of agricultural land giving rise to more nitrous oxide[37]).

The main sources of nitrous oxide are respiration processes performed by nitrifying and denitrifying bacteria in terrestrial and aquatic systems. The increase of nitrous oxide formation is not only due to the larger volumes of anaerobic habitats, but is also enhanced by the use of nitrogen fertilizer, and the resulting larger concentrations of nitrate and ammonia. Depending on the environmental conditions in soil (e.g. energy supply, oxygen tension, pH, etc.) nitrous oxide is formed by denitrifiers in varying proportions compared with dinitrogen, the main end-product of denitrification[16]. However, nitrous oxide may also be produced by means of nitrification by ammonia-oxidizing bacteria under microaerophilic conditions[14, 27]. Since nitrous oxide released into the troposphere is indirectly involved in the degradation of ozone in the stratosphere[6, 14], and since it also contributes to the greenhouse effect (about one sixth of the contribution of methane)[1, 17, 42], any increase of the concentration of this gas in the atmosphere is, on a global scale, of environmental concern.

The activity of denitrifying bacteria has been calculated to be responsible for a gaseous loss of 2–77% of nitrogen fertilizers applied to arable land[31]. Denitrification is thus of great economic concern for many farmers.

As a result of microbial activities and the subsequent creation of anaerobic conditions iron and manganese can be reduced in soil, aquifers or waterbodies to their soluble forms, either directly by the bacteria or as a result of environmental changes (production of reducing and complexing substances)[2]. If ground or surface water containing soluble iron and manganese is used for the supply of drinking water, the chemical autoxidation of these ions may lead to severe clogging and deposition problems.

Environmental problems can also develop from fermentation reactions, where anaerobic conditions are promoted by man. This type of reaction may also give rise to undesired effects, e.g. when organic compounds reach sites which are normally anaerobic e.g. deep soil, ground water and sediments. Acetic acid formed during anaerobic decomposition of straw has been shown to inhibit growth of seeds and roots of wheat and barley seedlings[19]. This phenomenon is probably important when no-till management practices are used, and seeds are sown on the surface relatively soon after harvest. The large amounts of organic matter then present will allow anaerobic conditions to occur if sufficient water is available. Since fermentation takes place rather easily under these conditions the inhibiting acids are readily formed.

Odor and bad taste in drinking water are problems associated with anaerobic and microaerophilic conditions. The anaerobic catabolic reactions occurring in manure feed lots give rise to various smelly organic nitrogen and sulfur compounds as well as ammonia[18]. The release of this kind of volatile reduced nitrogen compound may influence the nitrogen cycle locally or even regionally. The use of glycols as anti-freezing agents on airplanes before starting, and as carrier liquids in tubing for heat extraction from soils may lead to severe groundwater pollution (Torstensson, pers. comm.). When leaching into the soil at low oxygen tensions these substances may be transformed to malodorous compounds by fermentation processes, which renders the groundwater unsuitable for drinking water production.

Some xenobiotics are unaffected under aerobic conditions but will be decomposed when the soil environment turns anaerobic. The decomposition of halogenated aliphatic compounds is especially promoted under anaerobic conditions[35]. On the contrary, the breakdown of aromatic compounds not carrying substitutions containing oxygen seems to need the presence of molecular oxygen. These phenomena are of great concern since a lot of xenobiotics of these types are nowadays accumulating in soils, and are likely to be released into the groundwater.

Sulfate reduction and the concomitant sulfide production by anaerobic bacteria can be detrimental to fish and plants[26]. Killing of fish may occur after H_2S bursts into waters of heavily polluted lakes and may also occur as a so-called winter-kill, when rivers or lakes are covered with ice. Rice plants may suffer from sulfide produced in the water-logged soils. Sulfide formation in coastal sediments has been shown to be a potential source of sulfur oxides in the atmosphere[10]. Substantial amounts of sulfide evolution from shallow sediments were observed at night, when the surface oxygen production by algal photosynthesis had ceased. The source strength seemed to be two to three orders of magnitude higher than that expected by atmospheric chemists for northern Europe as a whole. Sulfate reduction by bacteria in sediments of acid lakes has been estimated to be of great importance in scavenging protons and thereby counteracting the acidification[15].

The activity of sulfate reducers has long been associated with corrosion in water pipes, oil drilling and oil storage[26]. The hydrogen film formed on iron by polarization (anodic reaction) in water acts as a protective layer on the metal. Slow oxidation of this film by oxygen causes rust under aerobic conditions. Anaerobically, in the presence of sulfate, sulfate-reducing bacteria may efficiently consume the hydrogen film and thereby cause depolarization. The sulfide produced can bind to the iron, and so severely damage metal pipes or other metal constructions[43].

Atmospheric methane concentration is estimated to have started to augment around the end of the 16th century[3] and is now growing at a rate of about one percent per year (the total content in the atmosphere is about 5000 million tons). The principal sources of methane appear to be ruminant animals, organic-rich sediments, and rice paddies[1]. Together with some other trace gases, methane absorbs radiation at the wavelengths where the earth's heat normally escapes. Like carbon dioxide, it therefore contributes to the greenhouse effect[1,36]. Apart from its involvement in the global warming, methane formed in sediments may in some cases be responsible for the depletion of oxygen in the water column of some parts of the oceans, in lakes and ponds[32].

5. *Applications of anaerobic processes*

Microorganisms were improving and spoiling the food and drink of mankind long before anyone realized that microorganisms existed. In time, but still without knowing what was happening biologically, man learned to exploit the fermentative activity of microorganisms in making cheese, yoghurt, wine and beer. Today, with microbial activity fairly well understood, fermented foods and beverages constitute a large and important sector of food industry (table 2). Cultured dairy products and alcoholic beverages are by far the most important (by sale and volume) biotechnological products. Besides improving the taste the microbial fermentation products (organic acids, alcohols) improve the shelf life of food and drink[30].

Fermentation processes are not only applied for the preservation of human food but also for vegetable fodder for ruminants. During ensilage lactic acid is produced by fermenting anaerobes. The resulting low pH will protect the fodder from putrefaction[22]. We should also keep in mind that the anaerobic processes in the rumen are the initial steps in milk and meat production and that the steaks have been synthesized by the cow starting from the microbial fermentation products acetic acid, propionic acid and butyric acid[12].

Table 1. Effect on the environment resulting from anaerobic microbial processes

Process	Compounds involved	Effects	Reference
Denitrification	N_2O	Greenhouse effect	1, 17, 31, 42
	N_2O via NO_x	Ozone depletion in the stratosphere	
	N_2O, N_2	Loss of nitrogen from agricultural systems	
Iron and manganese reduction	Bivalent iron and manganese	Mobilization of iron and manganese in groundwater	2
Fermentation	Organic acids	Growth inhibition of grain and seedling roots	19
	Xenobiotics	Increased degradation of substituted aliphatic compounds and decreased degradation of aromates	35
	Organic sulfur and nitrogen compounds	Taste and odor problems	18
Sulfate reduction	H_2S	Corrosion, winter kill, inhibition of rice plant growth, sulfate in the atmosphere	10, 26, 43
Methanogenesis	CH_4	Greenhouse effect, oxygen depletion in water bodies	1, 32, 36

Table 2. Some of the major applications of anaerobic micro-organisms and anaerobic processes

Process	Products or effects	Type of organisms
Production of food and beverages	Cheese	Mainly bacteria of the genera *Streptococcus, Lactobacillus, Propionibacterium* and molds of the genus *Penicillium*
	Yogurt	*Streptococcus thermophilus, Lactobacillus bulgaricus*
	Alcoholic beverages	Mainly yeast of the genus *Saccharomyces*
	Soya sauce	*Aspergillus oryzae, Saccharomyces rouxii, Torulopsis* sp., *Pediococcus soyae*
Ensilage	Preservation of vegetable fodder for animal feed	Lactic acid producing anaerobes
Industrial	Ethanol	Mainly yeasts of the genus *Saccharomyces* and *Kluyveromyces*
	Acetone-butanol	*Clostridium acetobutylicum*
Waste and wastewater treatment	Reduction of carbon and nitrogen content, detoxification, production of methane	Complex mixture of various anaerobic microorganisms

Apart from food production and preservation, microbial fermentations were and still are used to produce bulk chemicals. The best known are ethanol, acetone and butanol[23, 29]. Our relative ignorance with respect to the potential of various fermentations has prevented the development of new large-scale processes. The increased interest of microbiologists in new types of anaerobic fermentations in recent years will certainly lead to an augmented application of anaerobes for biotechnology processes in industry within a few years.

The anaerobic treatment of waste and waste water has already been in use for over a hundred years[21]. A complex mixture of many different anaerobic microorganisms break down the waste and reduce its volume or concentration. In most cases, methane is produced as a useful endproduct[9]. Anaerobic treatment processes also become more and more important in the detoxification of industrial wastes and waste waters[8, 34], and in the treatment of animal manure, which is produced in tremendous amounts by the bioindustry in some countries.

It would go beyond the scope of this article to describe all these processes in detail. The interested reader should refer to the literature on these subjects.

6. *Concluding remarks*

In the absence of molecular oxygen, organisms are capable of degrading, transforming or synthesizing almost all types of organic compounds by biochemical processes identical to or similar to the ones used by aerobes. In most cases anaerobes have even more versatile metabolic possibilities at their disposal than their aerobic counterparts. In recent years, a growing interest in anaerobes has led to the discovery of numerous new types of reactions some of which will certainly prove to be beneficial to mankind. It is true that a certain number of the endproducts of microbial activity in anaerobic habitats have a deleterious effect not only locally and regionally but also on a global scale. However, it has not to be forgotten that these effects are only the result of an excessive anthropogenic interference with natural processes. If we are ready to explore the whole potential of biological anaerobic reaction mechanisms, 'life without oxygen' will reward us with a storehouse of new possible biotechnological applications.

This paper is based on a lecture presented by A.J.B.Z. at the 45th Annual Meeting of the Swiss Society of Microbiology (April 10–12, 1986 in Buchs, SG, Switzerland).

Acknowledgments. The Dutch Ministry of Education and the Swedish Institute are acknowledged for financial support to Bo Svensson during his post doctoral studies at the Department of Microbiology at the Agricultural University of the Netherlands. The authors also want to thank Ans Broersma for typing the manuscript and Nees Slotboom for drawing the figures.

1 Abelson, P.H., Greenhouse role of trace gases. Science *231* (1986) 1233.

2 Alexander, M., Introduction to Soil Microbiology, 2nd edn. John Wiley & Sons Inc., New York 1961.

3 Chameides, W.L., Increasing atmospheric methane. Nature *301* (1983) 568.

4 Claassen, P.A.M., and Zehnder, A.J.B., Isocitrate lyase activity in *Thiobacillus versutus* grown anaerobically on acetate and nitrate. J. gen. Microbiol. *132* (1986) in press.

5 Cole, J.A., and Brown, C.M., Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle. FEMS Microbiol. Lett. *7* (1980) 65–72.

6 Crutzen, P.J., SST's a threat to the earth's ozone shield. Ambio *3* (1972) 201–210.

7 Ehrlich, H.L., Geomicrobiology. Marcel Dekker, Inc., New York/Basel 1981.

8 Fedorak, P.M., and Hrudey, S.E., Anaerobic treatment of phenolic coal conversion wastewater in semi continuous cultures. Wat. Res. *20* (1986) 113–122.

9 Gujer, W., and Zehnder, A.J.B., Conversion processes in anaerobic digestion. Wat. Sci. Techn. *15* (1983) 127–167.

10 Hartman-Hansen, M., Ingvorsen, K., and Barker Jørgensen, B., Mechanisms of hydrogen sulfide release from coastal marine sediments to the atmosphere. Limnol. Oceanogr. *23* (1978) 68–76.

11 Hochachka, P.W., Living without Oxygen. Closed and Open Systems in Hypoxia Tolerance. Harvard University Press, Cambridge, Mass. 1980.

12 Hungate, R.E., Anaerobic biotransformation of organic matter, in: Bacteria in Nature, vol.1, pp.39–95. Eds E.R. Leadbetter and J.S. Poindexter. Plenum Press, New York 1985.

13 Ingledew, J.W., and Poole, R.K., The respiratory chains of *Escherichia coli*. Microbiol. Rev. *48* (1984) 222–271.

14 Kaplan, W., Sources and sinks of nitrous oxide, in: Current Perspectives in Microbial Ecology, pp.478–483. Eds M.J. Klug and C.A. Reddy. ASM, Washington, D.C. 1984.

15 Kelly, C.A., and Rudd, J.W.M., Epilimnetic sulfate reduction and its relationship to lake acidification. Biogeochemistry *1* (1984) 63–77.

16 Knowles, R., Denitrification. Microbiol. Rev. *46* (1982) 43-70.

17 Lacis, A., Hansen, G., Lee, P., Michell, T., and Lebedoff, S., Greenhouse effect of trace gases, 1970–1980. Geophys. Res. Lett. *8* (1981) 1035–1038.

18 Luebs, R.E., Davis, K.R., and Laag, A.E., Enrichment of the atmosphere with nitrogen compounds volatilized from a large dairy area. J. Envir. Qual. *2* (1973) 137–141.

19 Lynch, J.M., Gunn, K.B., and Panting, L.M., On the concentration of acetic acid in straw and soil. Pl., Soil *56* (1980) 93-98.

20 Margulis, L., Origin of Eukoryotic Cells. Yale University Press, New Haven 1970.

21 McCarty, P. L., One hundred years of anaerobic treatment, in: Anaerobic Digestion 1981, pp. 3–22. Eds D. E. Hughes et al. Elsevier, Amsterdam 1982.

22 McDonald, P., The Biochemistry of Silage. John Wiley & Sons Ltd., Chichester 1981.

23 McNeil, B., and Kristiansen, B., The acetone butanol fermentation. Adv. appl. Microbiol. *31* (1986) 61–92.

24 Morris, J. G., The physiology of obligate anaerobiosis. Adv. Microbiol. Physiol. *12* (1975) 169–246.

25 Ohisa, N., Yamaguchi, M., and Kurihova, N., Lindane degradation by cell-free extracts of *Clostridium rectum.* Archs Microbiol. *125* (1980) 221–225.

26 Postgate, J. R., The Sulphate-Reducing Bacteria. Cambridge University Press, Cambridge 1984.

27 Poth, M., and Focht, D. D., ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea:* an examination of nitrifier denitrification. Appl. envir. Microbiol. *49* (1985) 1134–1141.

28 Robertson, L. A., and Kuenen, J. G., Aerobic denitrification: a controversy revived. Archs Microbiol. *139* (1984) 351–354.

29 Rogers, P., Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes. Adv. appl. Microbiol. *31* (1986) 1–60.

30 Rose, A. H., The microbiological production of food and drink. Scient. Am. *245* (1981) 95–104.

31 Rosswall, T., Exchange of nutrient between atmosphere and vegetation. Agro-Ecosystems *4* (1977) 296–302.

32 Rudd, J. W. M., and Hamilton, R. D., Methane cycling in Lake 227 in perspectives with some components of carbon and oxygen cycles. Arch. Hydrobiol. Beil. *12* (1979) 115–122.

33 Schopf, J. W., The evolution of the earliest cells. Scient. Am. *239* (1978) 110–139.

34 Schraa, G., and Zehnder, A. J. B., Biodegradation of chlorinated compounds, in: Organic Micropollutants in the Aquatic Environment, pp. 278–291. Eds A. Bjorseth and G. Angeletti. D. Reidel Publishing Company, Dordrecht 1986.

35 Schraa, G., van der Meer, J. R., van Neerven, A. R. W., Colberg, P. J., and Zehnder, A. J. B., Microbial transformation of micropollutants, in: Microbial Communities in Soil, pp. 315–326. Eds V. Jensen, A. Kjøller and L. H. Sørensen. Elsevier, London–New York 1986.

36 Seiler, W., Contribution of biological processes to the global budget of CH_4 in the atmosphere, in: Current Perspectives in Microbial Ecology, pp. 468–477. Eds M. J. Klug and C. A. Reddy. ASM, Washington, D.C. 1984.

37 Söderlund, R., and Svensson, B. H., The global nitrogen cycle, in: Nitrogen, Phosphorus and Sulphur – Global Cycles. SCOPE Report No 7. Ecol. Bull. (Stockholm), vol. 22, pp. 23–73. Eds B. H. Svensson and R. Söderlund, 1976.

38 Stumm, W., What is the $p\varepsilon$ of the sea? Thalassia Jugoslavica *14* (1978) 197–208.

39 Suflita, J. M., Horowitz, A., Schelton, D. R., and Tiedje, J. M., Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science *218* (1982) 1115–1117.

40 Tsuchiya, T., and Yamaha, T., Reductive dechlorination of 1,2,4-trichlorobenzene by *Staphylococcus epidermis* isolated from intestinal contents of rats. Agric. biol. Chem. *48* (1984) 1545–1550.

41 Vogel, T. M., and McCarty, P. L., Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. envir. Microbiol. *49* (1985) 1080–1083.

42 Wang, W. C., Yung, Y. L., Lacis, A. A., Mo, T., and Hansen, J. E., Greenhouse effects due to man-made perturbations of trace gases. Science *194* (1976) 685-690.

43 Widdel, F., Microbiology and ecology of sulfate- and sulfur-reducing bacteria, in: Biology of Anaerobic Microorganisms. Ed. A. J. B. Zehnder. Wiley, New York (1986) in press.

44 Williams, R. J., and Evans, W. C., The metabolism of benzoate by *Moraxella* species, through anaerobic nitrate respiration. Evidence for a reductive pathway. Biochem. J. *148* (1975) 1-10.

45 Zehnder, A. J. B., The carbon cycle, in: Handbook of Environmental Chemistry, vol. 1: Part B, pp. 83–110. Ed. O. Hutzinger. Springer-Verlag, Berlin 1982.

46 Zehnder, A. J. B., and Brock, T. D., Methane formation and methane oxidation by methanogenic bacteria. J. Bact. *137* (1979) 420–432.

47 Zehnder, A. J. B., and Colberg, P. J., Anaerobic biotransformation of organic carbon compounds, in: Microbial Communities in Soil, pp. 275–291. Eds V. Jensen, A. Kjøller and L. H. Sørensen. Elsevier, London–New York 1986.

0014-4754/86/11/121197-09$1.50 + 0.20/0

Full Paper

Epitheliophagy: Intrauterine cell nourishment in the viviparous alpine salamander, *Salamandra atra* (Laur.)

G.-D. Guex and P. S. Chen

Institute of Zoology, University of Zürich, CH-8057 Zürich (Switzerland), 9 April 1986

Summary. The intrauterine nourishment of the viviparous alpine salamander, *Salamandra atra,* has been demonstrated to consist of two phases: 1) oophagy – after hatching from the jelly membrane, the developing embryo ingests the remaining disintegrated, unfertilized egg mass. 2) Epitheliophagy – a special cranial portion of the uterus wall, the zona trophica, is stimulated by the presence of the embryo. After the yolk mass has been exhausted, the developing embryo is supplied with epithelial cells as nourishment until the end of pregnancy. The epithelial cells of the zona trophica are released into the uterus lumen by partial necrosis of the underlying connective tissue. Regeneration and detachment of the uterine epithelium occur simultaneously in different regions of the zona trophica. A special dentition enables the embryo, according to its position in the uterus, to feed directly on the zona trophica.

Key words. Salamandra atra; viviparous salamander; epitheliophagy; zona trophica.

Introduction

The female alpine salamander, *Salamandra atra,* produces about 100 eggs[1,8]. However, in general, in each uterus (the most caudal portion of the oviduct) only a single egg (according to Czermak[1] the so-called embryonic egg) is surrounded by a jelly membrane, is fertilized and develops into an embryo[8,21,25,32], in contrast to the