CAUSES AND CONSEQUENCES OF EUKARYOTIZATION THROUGH MUTUALISTIC ENDOSYMBIOSIS AND COMPARTMENTALIZATION

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ABSTRACT

This paper reviews and extends ideas of eukaryotization by endosymbiosis. These ideas are put within an historical context of processes that may have led up to eukaryotization and those that seem to have resulted from this process. Our starting point for considering the emergence and development of life as an organized system of chemical reactions should in the first place be in accordance with thermodynamic principles and hence should, as far as possible, be derived from these principles. One trend to be observed is the ever-increasing complexity resulting in several layers of compartmentalization of the reaction system, either spatial (of which the eukaryotic cell is an example), or functional (as in the gradually deepening distinction between metabolic, enzymatic and information-storing functions within the cell). One of the causes of this complexification of living systems will have been the changes in environmental conditions, particularly the geochemical impoverishment of the biosphere during geological history, partly brought about by living systems themselves, and partly by the trend towards increasing efficiency and specificity of the reactions that occur.

1. INTRODUCTION

In this paper we propose an alternative scenario for eukaryotization. A current scenario, here for convenience called the predatory endosymbiotic theory, assumes a period of mutual metabolic adaptation of two prokaryotes after one of the two phagocytosed another predated one. This process of mutual adaptation would have occurred whilst the symbiont was already inside its host or between the two membranes of its cell wall. As this scenario has grave shortcomings, both methodologically as well as from the point of view of the known structures and processes, an alternative one is needed; this alternative scenario is here for convenience called mutualistic endosymbiosis.

This alternative consists in the occurrence of a long period of mutualistic ecological adaptation during which the eventual symbionts remained outside of their

host and excreted their own wastes which in turn were consumed by their host. At this stage this may have already been mutually beneficial: the producer of waste not suffocating in its own waste and the consumer being certain of a constant supply of its preferred food. The host itself produced digestive secretions in a specialized cell extension consisting of a labyrinth of membrane invaginations, the vacuome. Gradually, the previous ectosymbionts will have become entangled in the membranous folds, eventually becoming encapsulated as endosymbionts. During all this time, and in certain respects even up to the present day, both the host and its symbionts remained metabolically and ecologically distinct to a large extent, their mutual independence only gradually decreasing and even this only up to a certain point.

Central to this conception is that eukaryotization cannot be viewed as a single, even momentous, event as in Margulis' (1981) scenario of predation without digestion (see also Guerrero, 1991; Guerrero *et al*., 1986). Instead, it represents a long-term, diffusely defined, drawn-out process, consisting of many elements possibly originating independently and operating at widely different times. What earlier had been an ecological symbiosis of free living bacteria, gradually internalized into one of the partners, which we recognize as endosymbiosis. The cluster of processes of endosymbiosis proper forms but one of those elements and can therefore only be understood within the context of the broader, much more complex framework of eukaryotization processes. The term eukaryotization merely integrates all the more elementary processes within one conceptual framework. The consequence is that we have to show the development of this framework, how endosymbiosis fits into it, and what the consequences may have been of adding one element after the other. From this perspective, causes and consequences are tightly intertwined and cannot be considered separately.

Defining eukaryotization as the result of endosymbiosis assumes occurrence at the level of the cell only. "Eukaryotization" though can also be used at higher levels of integration, such as that of communities, where a gradual substitution of the more ancient, prokaryotic life forms by eukaryotic ones is meant. In the present context it concerns intensifying interactions between prokaryotes such that they require a multitude of mutual adjustments with regard to endosymbiosis. In a sense one can consider the development of the eukaryotic cell as a miniaturization of a prokaryotic ecosystem down to the size of the cell.

2. HYPOTHESES, ASSUMPTIONS, AND PREMISES

Our alternative has methodological advantages as it accounts for a set of ten testable hypotheses. Their testing will be done diffusely in various parts of the text.

1) The process of biochemical evolution as a whole, including eukaryotization, can be reconstructed to a large extent from first principles.

2) The first principles are those from physical chemistry concerning both thermodynamic and kinetic stabilization processes. These processes are gradually differentiated spatially by an increasing functional compartmentalization of the internal and external environment of the cell.

3) The development of metabolic processes, together with their compartmentalization, follows developments in geochemical conditions.

4) Eukaryotization, including endosymbiosis, forms an integral part of long-term evolutionary processes covering most of the entire Precambrian.

5) Eukaryotization by endosymbiosis follows rules that are known from symbiotic developments in general.

6) The symbionts adjust their mutual requirements and waste products quantitatively.

7) Each of the guest components belongs to a taxon from which more members are prone to engage in symbiosis or parasitism.

8) The eukaryotization process as a whole follows the historical development of the biochemical traits involved with regard to the development of molecular structures. It also follows the gradual build up of the catalytic system of biochemical pathways and cycles and the tuning of processes with regard to their specific chemistry and timing.

9) Present-day cytological biochemistry and its underlying morphological structures still contain or reflect the various steps taken during this historical development.

10) Metabolic cascades in the recent eukaryotic cell recapitulate the sequence of events during the evolution of metabolic chains and pathways.

Of these ten hypotheses, particularly the first one concerning the historical derivation of the living system from first principles needs special mention. Often, the adoption of certain components from the environment, having been generated abiotically, does not fit this reasoning. In fact, the consumption or utilization of existing or ready-made compounds differs from their production by the system itself. Any reasoning connecting the two is based on some form of Lamarckism in which the function of some structure precedes this structure, rather than the other way round. This also applies to research concerned with questions of how to reconstruct certain macromolecular compounds such as RNA, etc. under laboratory conditions, although here the technicalities of the same Lamarckian methodology are more complex and hence less obvious. Of course, this reasoning does not need to apply to the very first stages of the origination of life when some amino acids having rained from outer space onto the early Earth or having formed on it according to Miller's (1953) experiments. Existing compounds may have reacted with each other forming some initial reaction system. But as soon as this system differentiated out chemically or spatially from processes in the environment at large, and this may have been very soon for many compounds, Lamarckian reasoning would enter. In this context one should also remember that the initial pool of usable compounds can quickly have become exhausted either by being used up in the pre-biological reactions or by natural decay (Shapiro, 1988, 1995). Moreover, they should also have been able to enter some initially closed space (along the lines Russell and co-workers developed and which we favour) in which such reactions would have been initiated. The only way to avoid this Lamarckian trap is by means of following the deductive, bottom-up approach.

Apart from the premise of the first two hypotheses, that all life phenomena should conform to first principles from physics and chemistry, we have made more methodological premises. Firstly, some eukaryotization "event" cannot be separated from general phylogenetic developments happening during early Precambrian times. Of course, so much mutual adjustment had to occur that eukaryotization cannot possibly be understood from a single "event" like the gobbling up of one bacterium by

another. The processes of rebuilding and mutual adjustment are so numerous, diverse, and far reaching that it is in fact impossible to single them out from main developmental lines starting right at the very origin of life. Dating the eukaryotization of life forms can therefore only be done in very broad and vague terms, thus losing its meaning.

Secondly, because of the extreme intricacy of the chemical interactions forming the essence of life at this level, existing mechanisms can hardly be altered or replaced, if at all, but are supplemented by other ones in order to preserve their continued functioning. Any replacement by other mechanisms could easily be destructive. Thus, $[FeS]_4$ cubanes in ferredoxins as electron transfer molecules seem to go back to structures allowing electron flows in the initial phases of mineral crust formation of $(FeS)_n$ (mackinawite) complexes as the earliest stage in the origin of life (Russell *et al.,* 2003). Moreover, not only is their functioning conserved but so is their place within the operation of the cell membrane as well as their place in the extended chemiosmotic apparatus. New, supplementary and compensatory elements have gradually been added at increasingly distant places on either side. The more recent photosystem II is therefore the first in the present photosynthetic reaction chains after which the older photosystem I is placed. This system is followed by the most ancient ferredoxins (e.g. Arnon, 1988), subsequently forming ATP from ADP which energizes the more recent NADP (Daniel and Danson, 1995). These two nucleotides in particular hand the energy released photochemically over to mechanisms of protein and carbohydrate formation. Broadly speaking the metabolic cascade may thus recapitulate biochemical evolution. As such, it is one of the consequences of system rigidity due to the fragility of a complex of a multitude of fine-tuned chemical interactions. Below we will meet with several other such consequences.

Thirdly, at this molecular level evolution progresses modularly whereby existing mechanisms are kept intact and are utilized as interchangeable modules of newly-formed mechanisms. The complexity of a large proportion of proteins appears to have been built up that way beginning at the most ancient of them, the ferredoxins in which the four protein strands of cysteine appear to have been added, and subsequently have undergone several duplications of the initial module (Eck and Dayhoff, 1966) possibly allowing in the first place some form of redundancy (Meyer, 2003). Dorit *et al*. (1990) (see also Clothia, 1992) even suggested that all extant proteins can be derived from no more than one thousand initial proteins now forming the modules of more extended and complex ones. In fact, at an even more basic level there are only twenty amino acids composing the proteins, and even less, five nucleosides being kept intact in energy transfer or in the structure of RNA and DNA strands. Thus, by changing the context in which the modules operate, flexibility can be obtained and at the same time the necessary biochemical rigidity be safeguarded. As said, this rigidity is needed not to destroy the intricate, fine-tuned interactive network of the biochemical processes happening within the cell. To a large extent, evolution involved changes in interactions between modules that are themselves rigid and unchangeable. Eukaryotic compartmentalization and multicellularity greatly enhanced this possibility of increasing and changing these interactions even more, thus speeding up evolutionary rates and allowing an endless variety in shape and functioning.

Fourthly, not only the functional relationships are kept or redefined in the modular rearrangements or in heterochronic shifts of gene expression but so are their spatial

relationships. Thus, the initial chemiosmotic activity of the initial mackinawite crusts still retains its place in the cell membrane as the pivotal energy processing organelle with which many other processes are connected even in the membrane system internal to the eukaryotic cell. Therefore, many structures and processes in these complex symbiotic cells can only be understood within their historical and functional context of spatial structures.

For a large part, evolution at the biochemical level is therefore a continuous redefinition, elaboration, extension, reorganization, and compartmentalization of the interactions between a relatively limited number of rigid modules. Life can only be understood in structuralistic terms in the biological sense (Piaget, 1968). In this, unlike in Leibniz' monads, the rigid modules are open to functional change depending on physical and chemical conditions and interrelationships, thus giving flexibility and ever new opportunities to the system they compose. Understanding a living system in structuralistic terms supplies the necessary biological mechanisms which analytical reductionism on the one hand and the more global vitalism and holism on the other are lacking. For example, phosphorylation on which all energy processing of the living cell depends cannot be understood from chemical reactions but should be framed within those of the supra-molecular structure of the cell membrane (Mitchell, 1961). Yet, structuralism also puts severe constraints on the analytical understanding of living systems, the more severe the greater the number of interactions (Elsasser, 1987).

3. DEFINING LIFE, AND TWO ENDOSYMBIOTIC SCENARIOS

Before developing our concept of eukaryotization, we first look briefly at problems arising from defining life according to its present-day features, as well as at those inherent to eukaryotization through predatory endosymbiosis. These problems are dealt with in the first two sections of the present chapter. After this we discuss those problems concerning processes which lead to endosymbiosis as an integral part of the broader development of eukaryotization from life's earliest steps onwards. In the final chapter we will discuss multicellularity in terms of a direct consequence of the origin of the eukaryotic cell.

Criteria used in defining life

Defining life according to present-day properties

Current analyses of life's origination often result from definitions of life as based on its present-day properties (Table 1) (see also Barbieri, 2003; Lahav, 1999; Rizzotti, 1996).

As predominantly present-day properties, many of these traits may in fact have originated long after the very first steps had been taken during the early evolution of life; they will therefore have been absent during the initial evolutionary stages. Life, for example, may not have started off with polymerized macromolecules, although these constitute present-day genomes, proteins, carbohydrates, and lipids. Thermodynamically, their early construction is even unlikely since the elimination of water, basic to their condensation, requires too much energy, particularly when the reaction equilibrium is shifted towards hydrolysis under water-saturated conditions (Van Holde, 1980). This constitutes the thermodynamic barrier limiting peptide length

to ca. six residues (Black, 2000; see also Kahne and Still, 1988). It is not even obvious that life could have started as a carbon-based metabolic system; this kind of metabolism, heavily dependent on specialized endergonic energy storage and therefore on an elaborate catalytic system, may only have been able to develop during later evolutionary stages.

Table 1. Criteria currently used in defining life

Functionality, upward causation, teleology (vitalism)
Organic versus inorganic compounds
l-proteins and d-sugars -> life force
Organic soup (Oparin's co-acervates)/outer space (astrobiology)
Fermentation; supply from outer space
Water
Inorganic minerals
Stoichiometric ratios
Level of polymer
Macromolecules: proteins, RNA/DNA, sugars, lipids
Cell, cell differentiation
Minimum cell size
Cell membrane: envelope preventing diffusion
Independence internal from external processes, environment
Thermodynamic disequilibrium between cytosol and environment
Internal organization
Level of organization: Eigen's hypercycles, Kauffman's complexity
Interactions (Elsasser's holism, biological structuralism)
Homeostasis, feedback control
(Auto-)catalysts
Cycles (metabolic/ DNA<->proteins)
Chemical networks
Metabolism/metabolic pathways
Material compounds vs. process: energy/material flow; entropic decay
Darwinian model for chemical/biological structures, processes
Ability to evolve
Replication plus natural selection
Replication/reproduction, multiplication
Growth, development, differentiation
Epigenesis (of the cell and the organism)
Genomes, (naked) genes
Information: hardware vs. Elsasser's software
Communication
Energy sources: solar energy, geothermal activity, electrical discharge, and chemi-osmosis

Nor should it necessarily be considered as an initially fermentive system (e.g. Broda, 1975; de Duve, 2002) dependent on the existence and continuous abiotic production of polysaccharides in the environment. Such a non-biological organic resource would have been chemically heterogeneous, fitting only a few primitive catalysts necessary for their decomposition. Also, under the reducing conditions of this period, the carbohydrates may have fallen apart quickly which reduces the size of this resource continually. Therefore, soon after their exhaustion, the early systems should have been able to form them themselves as an internal biological energy resource. This requiring an elaborate, specific enzyme system as well as a source and activating mechanism for the input of much energy but which they may not have developed at that early time. Moreover, the biologically produced carbon compounds should have been the same as those initially present in the non-biological resource, requiring either the reverse operation of the decomposing enzymes or a Lamarckian construction of a novel reaction system.

Defining life following a first-principles approach

Varying on Samuel Butler's "A chicken is the egg's way of producing another egg", Black (2000) stated "Energy uses an organism as a mechanism for self-dissipation." Accordingly, we conceive living systems as thermodynamically dissipative structures (e.g. Prigogine and Stengers, 1984), channelling energy such that they decrease their own entropic value; it is the entropy of their environment that increases. Two questions arise: 1) How did this energy flow start? and 2) How did it become structured such that this structure could reach ever higher levels of organization and compartmentalization of which eukaryotization and eukaryotic multicellularity are the most sophisticated, ultimate results yet maintain its basic, initial structure and identity? Table 2 contains the criteria of our approach to solve these problems.

Table 2. Ingredients used in the present approach of defining life

Inorganic minerals; water
Cell membrane: the essence of life
Thermodynamic disequilibrium cytosol - environment
Energy/material flow; entropic decay
Chemical networks of linear reaction chains
Tuning of proteins and nucleic acids
specific reactions
reaction rate control
Modular construction and evolution
Energy source: chemi-osmosis through redox reactions in cell membranes

Redox reactions and the initiation and functioning of life

This table shows that, in our opinion, the present thermodynamic approach starts from driving forces internal to the system as based on redox reactions. This is

subsequently supplemented by electron transport, stepwise releasing free energy. Also, both the sizes of the molecules concerned as well as their initial organization are assumed to be minimal (Trifonov and Bettecken, 1997). Only gradually and over long stretches of time could these self-sufficient, linear redox reactions of small molecules form simple networks of, by themselves, linear reaction chains (e.g. Morowitz, 1992). These networks eventually led to sophisticated chemical cycles and pathways and thus to thermodynamic stabilization. Only at a much later stage, covalent, endergonic carbon bonds, based on kinetic stabilization could have supplemented them and, as a buffer, stored the energy thus stabilizing the system as a whole against the hazards of a daily and seasonally variable environment. The latter type of stabilization driven by sunlight is only reached stepwise and by going through complex catalytic biochemical cycles releasing the energy contained in water molecules. Yet, the very processes of obtaining and processing energy are still based on redox reactions in the light reaction happening in cell membranes where initially energy processing had started. Particularly those organic reactions, concerned with energy storage and release and operating independently of the first, are based on strong covalent bonding although this type of bonding is not restricted to carbon only. Typically, as a phylogenetically secondary development not directly related to the actual energy acquisition, they are found in the cytosol. Moreover, it happens in sufficiently oxidizing, anaerobic environments where the reaction products are stable but which probably did not occur during the stages when life originated. Finally our approach puts the cell membrane central, as it is here that a thermodynamic disequilibrium is maximal at a discrete point, thus allowing its energy to be tapped and processed efficiently. It is here that the core processes of life started and where they are still concentrated and where life, as a consequence, shows one of its most fascinating evolutionary developments including those of eukaryotization. In the sense of an entropy-decreasing mechanism, rather than being a mere envelope, the cell membrane is the seat if not the unit of life.

All this concerns the feasibility of the build up, maintenance, and changeability of complex living systems. More central, though, are considerations as to its inevitability which follows from the physical and quantum chemical properties of the various chemical elements and compounds in the spatial temporal setting of a geologically dynamic environment; ultimately having given rise to the origin and further development of life (e.g. Pullman, 1972; Wald, 1962; Westheimer, 1987; Williams, 1981; Williams and Frausto da Silva, 1996). Often, inevitable conditions for life's origination are restricted to discussions of the availability of, particularly, water, together with a suitable temperature range. Liquid surface water will have been available at the prevailing temperatures on Earth since ca. 3.3 Ga (Wilde *et al*., 2001). Also its bipolar nature is emphasized, as well as its highest density at four degrees centigrade, making ice float rather than freeze life on sea beds. This however, is too narrow an approach although it is usually the only compound being searched for in astrobiology despite the fact that so much more can be known about the physical chemistry of other planets such as their elemental composition.

Thus in order to explain life as a supra-atomic phenomenon, we must know the physical properties of the individual elements within the spatial and temporal setting and the development of the general environment. Yet this requirement shows constraints to its origination and development presumably implying, in fact, rather a small window of phylogenetic development. In other situations elsewhere in the

universe with the same, small window, life could in principle have started up in a similar way although the chances are small being proportional to the small size of the initial physical and phylogenetic window. The time and direction its further development may have taken also depends on many chance events and processes in time and space making parallel or identical phylogenies improbable in the extreme if not practically impossible to realize. Moreover, whether life could have lasted that long as it did here on Earth depends on the direction its development will have taken and this because of its complexity and its probabilistic character will never be the same. We therefore can describe its development on Earth as an inevitable necessity without automatically inferring that it will ever be reproduced anywhere else in the universe.

Five consequences of the complexity of living systems

One consequence of the complexity of a living system is that the chance that it originated in exactly the same form twice is the smaller, the greater its complexity. As a rule of thumb living systems will be monophyletic (in the sense of consisting of organisms from the same branch of the tree of life) or biphyletic at most (Martin and Russell, 2003).

Secondly, the chances of horizontal gene transfer will have been so small that we can accept it only after having obtained strong evidence in favour of it rather than accepting it off hand. For example, photosynthesis or electron transport systems depend on multiple gene complexes parts of which cannot be transferred horizontally without fatal damage to their functioning (e.g. Dyer and Obar, 1994). This probably holds as well for the metabolic system of which they form a part.

Thirdly, ancient parts can be expected to have remained operational since their origin, being firmly embedded within the system. When changes happened in environmental conditions, additional mechanisms kept the original structure operating or improved its functioning. Also gene duplication, rather than large-scale gene mutability, can be considered a significant process for extending the biological functions of an organism.

Fourthly, overall the physical conditions under which the structure originated broadly remained the same so as not to disturb the operation of some of its elements and the interaction between all elements. For example, temperature conditions must have remained more or less the same since life originated (Bada and Lazcano, 2002; Russell and Hall, 2003), just because of the functional efficiency and specificity of proteins under particular conditions of temperature or pH. Moreover, the tightly knit network of functional interactions requires that all proteins keep operating optimally rather than dropping out the one after the other under changing conditions, or starting malfunctioning. In fact, many individual enzymes or their systems are found to be ancient, or form protein families with ancient roots (Baymann *et al*., 2003).

Finally, complexification is neither a trend independent of the identity and function of the mechanism concerned nor a process happening independently of those of its elements. Life started up from elements or compounds with particular properties, making them operate within their specific chemical context. Gradually, the system they formed grew more efficient and specific without becoming detached from its roots, and without, therefore, losing its initial core elements or compounds and their

functioning. A process of complexification independent of the identity and functioning of these mechanism or its parts is biologically unthinkable, although perhaps being mathematically more attractive (e.g. Kauffman, 1993). Throughout the text, we will see several examples of the basic tenets of biological complexification allowing us to trace back the origin of the energy metabolism, or of RNA and DNA to their nucleotides, their pre-RNA roots, etc. Central to these types of compounds are the various chemical elements with their specific physical properties or initial pre-biotic abundances. Evolution's tinkering progresses through processes such as addition, rearrangement, or duplication of previous building blocks, all of them still showing their identifiable, initial functions, following on from their specific properties.

Any of these five consequences contain strong clues for recognizing the possible origin of the eukaryotic cell. From this perspective, sudden, drastic changes in these systems, as those according to the predatory endosymbiotic scenario, are unlikely to have occurred. The present approach therefore adopts a more gradual development of entire systems, based on the continuity of previously existing sub-systems and semes, as well as on the proneness to a certain behaviour common to members of a large taxon, similarly to the fourth and fifth methodological principles we followed, respectively. Here too, as with the specific properties of elements and chemical components, it is the physiological and hence ecological identity that counts, a fact not accounted for in the predatory endosymbiotic theory of eukaryotization. If, for example, none of the members of a bacterial taxon shows any proneness to interact with other bacteria, it is an unlikely candidate for engaging in symbiosis. We also have to find out if the potential symbionts take part in the symbiosis as a response to (altered) ecological conditions. Ecological conditions are essential in driving an evolving symbiotic system (Kooijman and Hengeveld, in press); symbiosis is a biological response of organisms of two taxa, co-adapting out of necessity and therefore only under certain conditions of ecological stress.

This process of eukaryotization through co-adaptation involved the development of many new traits independently probably starting at different times and happening at different rates. In fact, the predatory endosymbiotic scenario is based on a monothetic process definition, not leaving any space for historical understanding. The mutualistic scenario, in contrast, follows a polythetic definition allowing historical explanations of often discordant variation in the origin and operation of its process elements.

Eukaryotization through predatory endosymbiosis

For several reasons, the endosymbiotic process as based on growing mutualistic relationships between two or three bacterial taxa seems more likely than that according to the current predatory endosymbiotic scenario Margulis (1970, 1981) once proposed. Firstly, predatory endosymbiosis holds that one bacterium engulfs another one through phagocytosis, after which their respective metabolisms and genomes would co-adapt. In some scenarios along this line, the host cell is assumed to shed its cell wall completely which implies shedding its mechanical barrier that so far was integrated with its metabolism. For this to happen the rigid cell wall of the host must be opened temporally for which, under experimental conditions, extreme temperatures, pressure and electric conditions are required (Vellai *et al.*, 1998). These harsh conditions should, of course, not have damaged the cell structure and function; it should even

have allowed the cell to phagocytose another bacterium as a coincidental step. This process, although unlikely, should even have occurred quite often, to give the next even less likely process, a chance to happen.

During this next phase of the process the phagocytosed cell should neither be consumed, nor damaged metabolically in any way, but it should have been maintained under the ecologically novel living conditions defined by the metabolic chemistry of its host. Moreover, this cell too should have lost its cell wall. Thus, neither the host cell nor its phagocytosed prey should in the least have been affected negatively by each other's continued, close presence, despite all the consequences of their individual metabolic and energetic demands and their mutual waste products. Ecologically this implies that the prey would have had to survive and replicate normally although suddenly occurring in an utterly different chemical environment than before. For its part, from then on the host has to keep two metabolic systems running, each with different energetic and nutritional requirements. In order to survive, from that moment onwards, it should simultaneously have altered its surface-to-volume ratio and membrane functioning for the altered rates of nutrient uptake and waste excretion. This mere enlargement of the host cell in fact requires its metabolic processes to be reorganized rigorously (Kooijman, 2000) and at once.

Meanwhile, both cells should have performed their normal life history functions, such as growing and dividing, and therefore have metabolised sufficiently, as if still living under their initially preferred conditions, although now in concert. This should therefore have happened at equal rates between them so that their cell cycles remain in tune keeping the co-evolving cells operating conjointly. As the symbiotic phase of metabolic and genetic integration is only the end product of the process, the tricky situation before proper integration should have lasted millions of years in which during this period both cells will only gradually have adapted their initially distinct, highly intricate metabolic systems to each other.

This process of predatory endosymbiosis, therefore, makes two basic assumptions: 1) Both cells have lost their cell wall: the phagocytosing host just before taking up its prey. 2) The two cells could metabolise sufficiently for millions of years of co-adaptation, despite their initial differences in material and energy demands and despite the fact that the phagocytosed prey found itself in a different chemical environment. Neither of these assumptions has passed critical testing favourably so far. Basically, therefore, the theory of endosymbiotic eukaryotization, as based on predation without prey digestion, does not satisfactorily reconstruct the processes of mutualistic development from its initial stages onward. In the next chapter we first look at the early development of life and build up our picture from there in order to develop an alternative scenario.

4. THE THERMODYNAMIC BASIS OF LIFE

Within this framework a purely thermodynamic definition of life is compulsory since the build up and maintenance of any structure, whatever its nature, requires energy. Another advantage of deducing life processes from thermodynamic principles is that oxidation-reduction processes, once common in the environment, can be thought of having become biologically functional in the cell membrane (Russell and Hall, 2003). As the environment changed during the subsequent geological periods,

these initial processes remained essentially the same, although almost hidden amongst other ones keeping them operating under the altering conditions. Finally, for such reactions to occur, the pertaining elements require particular physical properties and in chemical compounds, particular quantum chemical ones.

Considering living systems as thermodynamically dissipative structures, of course, is not new (e.g. Schrödinger, 1944), also in the context of life's origination and early development (e.g. Russell and Hall, 1997). Yet, most studies analysing supposedly initial chemical or biological compounds or metabolic structures do not take their thermodynamic basis explicitly into account (see, for example, Brack, 1998; or Zubay, 2000, among many others). At best such approaches, though, take thermodynamics as a marginal condition to the chemistry of life rather than allowing it its central position. Just as in the case of carbohydrates as energy-storing molecules having started up life, we do not assume an early development of information-storing and processing, even before some primitive form of carbon metabolism developed. Instead, in the following, we concentrate on the thermodynamic function of the cell membrane right from the start of life.

The cascade of electrons and protons

Electron transfer from electron-rich elements to electron-deficient ones as the material basis of the energy processing of biological systems forms the root of their origin. Electrons can either be gained, a process called reduction, or be lost, called oxidation. In many cases however, not only are electrons transferred, but so are hydrogen ions, H^+, or protons. Similarly to electron transfer, gaining protons is called reduction and losing them oxidation. As reduction of one atom or molecule implies the oxidation of another one, reactions based on electron or proton transfer are called oxidation-reduction or redox reactions. In contrast, there are also reactions hardly requiring any electron transfer, if at all, but in which the atoms share their electrons when forming a compound, resulting in covalent bonding. This kind of bonding is strongest in carbon. Here, many reactions require activation energy and are therefore usually achieved catalytically. Also, they allow an almost kaleidoscopic interchange and redistribution of atoms resulting in a myriad of chemical combinations, the carbohydrates.

The first type of reaction, implying redox reactions brought about by electron or proton transfer, starts from a thermodynamic non-equilibrium condition and results in thermodynamic stability, whereas the reactions of the second type start from no such condition and result in kinetic stability. The first ones release free energy - they are exergonic - and therefore run spontaneously, in contrast to endergonic reactions which require energy. After taking up much energy during their specific catalytic formation, carbohydrates store it until they are broken down catalytically again. As they store more energy than other macromolecules, like proteins, lipids, or polyphosphates do (e.g. Kooijman, 2000), they have become the chemical energy stores of living systems *par excellence*. This only happens, of course, when there is sufficient energy available for their complex catalytic formation, itself also involving the evolution, formation, and maintenance of catalysts specific for each reaction and operating under specific conditions of temperature or pH. These two conditions obviously will not have been met during the time that life originated.

Because an electron, e^-, can be conceived of as a hydrogen atom without a proton, the redox coefficient Eh is a measure of electron activity, electricity. Conversely, a proton, H^+, is a hydrogen atom missing an electron, and pH as a measure of proton density – proticity – is another important determinant of many redox reactions. Therefore, graphs spanning the Eh/pH space contain relevant information on the thermodynamic stability range of many chemical compounds. Also, with the x-axis representing pH and the y-axis Eh, the flow of electrons from the lower right-hand corner towards the one at the upper left-hand represents chemical oxidation. The lower right-hand corner supplying electrons or protons represents reducing environmental conditions able to oxidize molecules in the upper left-hand corner with oxidizing ones gaining the electrons or protons. Biologically, such a graph may therefore even contain information about the direction in which metabolic systems (Russell and Hall, 1997), such as nitrogen metabolism (Beaumont, pers. comm.) and life forms may have evolved, partly as a response to the gradual exhaustion of the initially reducing environment in terms of transferable electrons. The evolution of not only metabolic pathways, but also that of enzymes, as well as of energy processing in the membranes may reflect, as we will see, this general shift from reducing conditions at low, negative redox potentials towards those of oxidizing ones at relatively high, positive ones. It can also be expected that life started at a neutral pH and that, if change occurs, it broadened slightly towards the left with a higher protonicity; that is towards lower pH values or higher acidity. Biological evolution can thus be expected to show an upward broadening or a shift of the area a metabolic system or a taxon occupies in this space. Electron or proton transfer in chemical reactions forms the physical basis of the thermodynamic dissipative system any living organism constitutes, or of the thermodynamic decay of matter at large, channelled through the tiny dissipative membrane systems constituting life on Earth.

Redox reactions and the initiation of life

Electron or proton transfer from one atom to another, defining redox reactions, occurs when an electron or proton donor comes into contact with an acceptor, that is when the redox potential falls outside the stability range of the compound. This means that within those areas net electron or proton flow is minimal in relation to that compound, resulting in its stability, whereas outside it this compound easily falls apart; the components reacting with other ones. Within Eh/pH space life may in fact have started at low Eh values – reducing conditions – and roughly at a neutral pH, which allows protons to be donated to acceptors at higher values; oxidizing conditions.

Chemical reduction may have happened under natural conditions when alkaline water seeped from basaltic ocean bottoms into the surrounding metal-rich, mildly oxidizing water of the ancient, Archean ocean (e.g. Russell and Hall, 1997; Martin and Russell, 2003). Thus sulphur in the seepage, reacting with iron that was abundantly available in the surrounding seawater at that time, would form $(FeS)_n$ films (mackinawite crust) in the chemical gradient enclosing the seepage plume (Russell and Hall, 1997). The system as a whole can therefore be considered an electrochemical cell or more exactly, a photoelectrochemical cell, since the electrons gained by ferrous iron, Fe^{3+}, giving ferric iron, Fe^{2+}, are oxidized by energy-rich UV light turning it back into Fe^{3+}. This film or crust therefore primarily operated as a semiconductor

(Morowitz, 1978; Russell and Hall, 1997) and only secondarily as a semi-permeable membrane. In fact, Fe^{3+} is still an electron acceptor among some micro-organisms (Kashefi and Lovley, 2003). Contrary to the black and white smokers, which violently spout hot water into that of the ocean (e.g. Van Dover, 2000), seepage through very fine pores in the basaltic ocean floor went gently, which leaves initial, tiny and brittle chemical structures intact.

Significantly, electrons can freely flow through these $(FeS)_n$ films from the enclosed alkaline water inside the crust into the ocean water because of the steep thermodynamic disequilibrium between the two sides. In the very initial stages, energy thus dissipated into the cavity, only gradually being carried from the film inside and subsequently channelled through primitive chemical pathways. This may have initiated a natural steep charge gradient. Moreover, the ocean may have been slightly oxidizing because of relatively high concentrations of HCO_3, CH_4 and NH_4. Because of the higher proton density inside, this could have resulted in a natural proton pump keeping the redox reactions of the early systems inside going. At some stage, though, this film broke up forming Fe_4S_4 cubanes or rhombs to which short cysteine strands attached (e.g. Eck and Dayhoff, 1966; Russell *et al*., 2003). Thus, they resulted in primitive iron-sulphur proteins, situated in the cell membrane and transferring energy across (Cammack, 1983). A couple of duplications in the protein strands gave rise to the ferredoxins ($[FeS]_4[SR]_4$) which can be thought of having operated with the same function of electron transferring in the cell membrane ever since (Eck and Dayhoff, 1966).

The metabolic function of these inorganic films or primeval cell membranes, therefore, is not only to delimit or envelope a cavity within which particular reactions can take place nor primarily to prevent certain reaction products or systems from diffusing away into the surrounding water. Its primary result is the formation of a steep chemical gradient at which free chemical energy and protons were tapped from the environment. In this, it is similar to a power plant typically situated at an artificial dam at which, in this case, potential energy can be tapped efficiently. Thus, the chemiosmotic basis of the energy processing systems in the membranes could have started right at the beginning of life itself fuelling the initial reactions and dynamically maintaining the early structures. Photosynthesis as the ultimate, most refined energy-processing system could be conceived as an elaboration of this initial way of tapping energy, dissipating from the environment into the cell, and subsequently through its pathways and cycles.

Redox reactions in a chemical system in thermodynamic disequilibrium typically situated in and around membranes keeping the disequilibrium intact thus became life's driving forces. The energy entering the tiny cavity could condense simple phosphor compounds – pyrophosphates – into dimers or trimers that, after transferring this energy over some distance, could hand it over to other elements or compounds activating them. On the one hand the phosphate bonds are energy rich but they can on the other hand easily be formed on the inside surface of the mackinawite crust and broken up again by hydrolyzation, which is thermodynamically favourable in the enclosed water off this crust. This could have been the origin of energy transfer by the ubiquitous energy carrier ATP in which some nucleotide NTP (a nucleoside, here adenine, with a ribose sugar, together forming the nucleoside adenosine) is attached to the active part formed by the pyrophosphates. Thus nucleotides may have developed

either transferring energy along the membrane and possibly into the cytosol as well or forming long nucleotide strands as in RNA and DNA. The initial polymerisation of the phosphates may have happened on the inside of the crust, thus overcoming the thermodynamic problems they would have had in free water before the crust would have formed. In such a case, they would have needed some other surface (see Cairns-Smith, 1982; Ferris, 2002), together with some unknown future function. Instead, the pre-RNA world can be thought to have happened encapsulated within an iron-sulphur crust. The principle, and even the thermodynamic basis, of these processes of energy acquisition and transfer have remained the same ever since. ATP remained instrumental, first with respect to the iron-sulphur film and later to the ferredoxins in the membrane into the cytosol. Similar processes may have happened in the formation of high-energy bonds of sulphur and nitrogen making these probable compounds of living systems (e.g. Wald, 1962; Westheimer, 1987).

After a next evolutionary step, ATP could have activated a newly evolved carrier, NAD. This molecule is chemically related to ATP as it consists of two esterified purine nucleotides with the substitution of one adenine moiety with nicotinamide. These molecules transfer the energy typically to peptides for their condensation into proteins and, probably still later, to carbohydrates with other, related nucleotide-linked oxido-reductive dehydrogenases. The initial nitrogen-based molecules on the inside the mackinawite membrane could have been energy-hungry because hydrolytic decay within a matrix of water made them unstable (Black, 2000). Under a scenario, such as the one Black (2000) suggests, hydrophobic mantles around the short peptides could have reduced their hydrolytic decay or even have enhanced their elongation. The thermodynamic disadvantage of the condensation of such large molecules might have turned into an advantage on surfaces, such as clay (Cairns-Smith, 1982) or pyrite surfaces (Wächtershäuser, 1998a). However, the clay minerals have a negative charge and in pyrite the electrons are firmly bound and therefore do not work. In contrast, Russell's mackinawite crusts do better in this respect, being positively charged, and transducing electrons (e.g. Russell and Hall, 1997). It might be possible that negatively charged (poly)phosphates, condensing macromolecules such as proteins and carbohydrates by dehydration, for example, may have attached onto such surfaces, later developing into DNA and other nucleotide electron carriers and dehydrogenators. In very early stages, apart from maintaining the disequilibrium in existence at the cell side of the mackinawite film, the continuous inflow of energy may thus have been spent for a relatively great deal in chemical maintenance, rather than in the build up of energy-storing macromolecules, or to that of elaborate chemical networks or cycles.

It is relevant to realize that the often-made distinction between metabolism and genetic information storage may not have been realized in the emerging systems. For example, ATP carries energy-rich electrons both to metabolic as well as to information-storing systems and even this molecule once had to evolve. This initial development occurred during the pre-RNA world during which even the proteins could have been missing (e.g. Joyce, 2002). It is conceivable that life started with the condensation of pyrophosphates and thioesters (Baltscheffsky and Baltscheffsky, 1994), transferring energy along the mackinawite film, or from this inward into the tiny trickle of seeping alkaloid water this film encapsulated. At that time, the early chemistry of life could thus have been dominated by elements like hydrogen, nitrogen, sulphur, and phosphorus as abundantly available electron acceptors, and iron and

nickel as main electron donors, for example. These are the elements that still dominate the energy processing and basic information-storing and transferring systems. Only much later, in the carbohydrates, carbon and oxygen entered the scene as major players of life.

At some stage the pyrophosphates connected with a ribose ligand and this in turn with a nucleoside, such as adenine, both of which consist of molecular rings that enhanced their catalytic function. By electronic resonance, these ring structures enhanced by their heterocyclicity act as charge stabilizers of the energy-rich phosphate bonds by delocating electrons (e.g. Pullman, 1972). Heterocyclicity implies that at one or two locations in the ring an element other than carbon is included, like nitrogen or oxygen, because they form double or triple bonds which can delocalize the electrons. These single or double ring structures, the pyrimidines and purines respectively, may thus be remnants of an early, primeval metabolism (Benner *et al*., 1989; White, 1976, 1982) even occurring before RNA. RNA could, at some stage, have been formed from them by esterification of the phosphates by forming hydrogen bridges between the configurationally best fitting nucleosides (Pullman and Pullman, 1961; Pullman *et al.,* 1966). A number of apparently ancient oxido-reductases all transfer energy, electrons or groups, i.e. coenzymes like ATP, NAD, NADP, FAD, FMN, or CoA, all following roughly the same pattern of pyrophosphates connected with adenine as a purine nucleoside. This also applies to coenzymes containing nucleosides other than adenine. Moreover, their resonance stabilization through heterocyclic structures could have had an important stabilizing function during times of strong high-energy radiation in the early solar system (Pullman and Pullman, 1962; Pullman, 1972). Lipids, as early hydrocarbon compounds that either make the mackinawite crusts less rigid and brittle, or cause charge insulation, similarly contain many double bonds in their fatty acid chains (Pullman, 1972). In fact the phosphates as essential intermediates in many biochemical syntheses and degradations are themselves negatively charged as well, surrounded by mobile electrons. It may therefore be supposed that the pyrophosphates were the initial electron carriers which catalyze reactions by delocalizing or polarizing the electrons in the molecule concerned. Later, they were either stabilized and functionally enhanced by purines or pyrimidines and ribose rings or had grown out to lipids.

The same nucleosides can also be found in the nucleotides building up the tiny threads of RNA that are still abundantly found in eukaryotic cells, as well as from in the longer RNA strands, and also in miRNA, in mRNA or in rRNA. Moreover, their operation could have been made more efficient and specific by peptides, which later after their polymerization, added to their enzymatic function as proteins, which can to some extent also store energy (e.g. Kooijman, 2000). In turn, their building blocks, the amino acids, also evolved from an initial few to the present twenty (Trifonov, 2000). Furthermore, more than half of the active sites of proteins, the cofactors, consist of nucleotides or RNA as coenzymes (e.g. White 1976) without which the remaining apoenzymatic part of the protein does not work. Ribosomes, the organelles forming the proteins, similarly consist of an enzymatically active site of RNA with the protein facilitating and buttressing it (Cech, 2000). The same seems, again, to apply to the spliceosomes, little organelles of the size of ribosomes splicing mRNA together (Maniatis and Reed, 1987). In fact, RNA splicing could have been more ancient that the evolution of proteins (Reanney, 1979). In turn, the early nucleotides as well as

RNA could have had broad-range enzymatic functions, the effectiveness of which was facilitated by amino acids which condensed into proteins and are often regulated by miRNA. Thus, this apoenzyme serves to facilitate the catalytic function of the nucleotide, and may as such have evolved later thereby leaving the initial function of the nucleotide intact. Strictly speaking the protein rather than the nucleotide should be considered a cofactor (e.g. Altman, 1984).

As RNA often consists of single strands and contains oxygen at the 2'-OH site in the ribose ring, it is an unstable molecule. Its stability is improved by the formation of double-stranded DNA which also lacks oxygen at that site. Finally, uracil is replaced by thymine in DNA. Interestingly though, DNA is still formed from RNA precursors (Brewin, 1972) and in bacteria it is similar to part of the protein-forming rRNA that is attached to the cell membrane. Therefore, the basis of most enzymatic functions still consists of ancient (inorganic) redox reactions in which energy, electrons or protons are exchanged.

As mentioned, initially proteins built up by energy released through ATP hydrolysis can also have stored energy, releasing it again by deamination and dehydration, respectively, thereby condensating ADP into ATP. Subsequently, the more stable lipids (Wächtershäuser, 1998b) and the even later (and hence independently) evolving carbohydrates took over the mechanical and energy-storing functions enabling the system to bridge periods of low energy inflow thus stabilizing it over time.

Part of the system of polymerized nucleotides stabilized into DNA and as such attained an improved standardizing function, necessary for orderly fine-tuning of metabolic and as a consequence for replication. Thus, the genetic system is separated from an initially enzymatic apparatus operating within the early metabolism. Yet, the instructions it receives and sends out, often controlled by various forms of micro-RNA, still form part and parcel of the general homeostatic metabolic system of the cell. It does not take some independent (vitalistic) position within it directing this metabolism. The cell is an integrated system in which no priority can be given to metabolic or replicative functions neither in time or origination nor in function.

Thus, during the pre-RNA world and subsequently during the RNA world, the function of storing energy and information could have remained indistinguishable for a long time only later crystallizing out into "compartments" of functionally distinct yet integral, chemical systems. Of these, the molecules processing and transferring energy in the membranes may have been earlier than the nucleotides with their complex structure and hence complex construction. These, in turn, after forming together specific energy and group carriers like ATP and Co-A, and with the still later evolving derivatives NAD, NADP, FAD and FMN constituted vital factors in the metabolism of the cell. The same applies to the less ubiquitous nucleosides guanine, cytosine, uracil, and later thymine. All these molecules and their functional interactions may still be remembrances of the initial, pre-RNA metabolisms connected with the $(FeS)_n$ films to which, for their part, the ferredoxins ($(FeS)_4R_4$) may still relate directly as oxido-reductive electron transducers. Central in these molecules though phosphorus was found particularly in the initial pyrophosphates, its operation due to its specific elemental, pentavalent properties (Westheimer, 1987).

Step by step, both the efficiency and specificity of the reaction system may have increased, first by constraints from the properties of the elements and molecules

present, later by the chemical interrelationships within the fluid. By lowering the reaction thresholds and by making them reversible, the enzymes not only smoothed the dissipative flow of energy but they also added to the homeostasis of the system (Kooijman *et al.*, 2003). This implied that pathways formed stochastically (Fothergill-Gilmore, 1986; Ronneberg *et al.* 2000). These could easily be reverted and sidetracked, eventually resulting in highly efficient and specific reaction systems we now recognize as the metabolic energy-processing and storing system, the information-storing system and later the mechanical support system (Kooijman and Hengeveld, in press). As such, it is the outcome of ever-tightening constraints to reactions resulting from ever-higher efficiencies and specificities, tuning them and together stabilizing the system in its operation. Gradually, first RNA and later DNA may have received the function of a reaction norm without however losing their place in the general homeostasis of the cell; they are not independent instructors of the cell metabolism, and could not possibly have formed independently and prior to it. Complexification increases through the addition of protective or improving mechanisms and their functional splitting out, rather than through altering their basic structures. For a large part, it will in the end be the result of adaptation to ever-changing environmental conditions, requiring different reactions mechanisms and pathways or entirely novel systems for storing energy or information, and eventually giving life new evolutionary opportunities. Still, it does not result from a general, biologically unidentified drive towards complexification as Kauffman (1993, 2000) insisted but results from many individual biologically functional adaptations.

At the time the first traces of eukaryotes were found, ~2.7 Ga, living systems had been in operation for some one billion years, which means that their dynamic structures had been firmly consolidated. Together with the observation that the initial metabolic mechanisms were still in place, this means that little if anything other than tuning of these mechanisms to each other could be done during the eukaryotization process. The potential symbionts must largely have remained the same both physiologically and ecologically. Yet, this tuning process must have taken a long time given the fact that the symbiontic fungi and algae are still independent after the first appearance as lichens almost half a billion years ago, only constituting their symbiotic life form under stressed conditions.

With the gradual exhaustion of electrons in the sea water or with an environmental shift towards higher pH values due to a continuous seepage of alkaline water from the ultramaffic sea floor into the reducing ocean water, some life forms became dependent on energy sources additional to or using the initial mineral ones like photochemical hydrolysis based on direct electron transfer. Moreover the resulting biological, adaptational shift could only happen at a later evolutionary stage when a sufficiently highly organized biochemical system had evolved utilizing biological catalysts. Yet in their core reactions the metabolic systems remained dependent on the initial membrane-based redox reactions of electron transfer. At some point these reactions were only supplemented by kinetic stabilization reactions of carbon-based covalent bonding resulting in energy storage in the cytosol.

Thus, living systems continued operating but from now on largely independently of their initial physical energy source from mineral reactions. The principle of tapping a thermodynamic disequilibrium at membranes was thereby maintained, only the initial causes of this disequilibrium were replaced by one initiated by solar energy,

whereby H_2O was oxidized. The protons and electrons thus released kept up the transmembrane proton and charge disequilibrium artificially, that is biologically. The free energy now became so abundantly available, though, that it was stored in carbohydrates by a different, independent process of CO_2 reduction, the Calvin cycle. Also, the earlier ones reversed now breaking carbohydrates down, thereby releasing the energy stored in them (Kooijman and Hengeveld, in press). This meant that the initial process, eventually resulting in the gradual exhaustion of mineral resources of energy and matter, came to function as one based on the recycling of in principle the same elements; the mechanism of electron flow into the cell though became driven by an energy source independent of them and recharging them continually. As conditions in the chemical environment kept changing, particularly under the increasingly higher levels of free oxygen, further substitutions in elemental uses happened. The switch to an independent and "biologically" maintained disequilibrium had unexpected consequences leading inevitably to the more complex, eukaryotic and multicellular forms of life as we know them today.

The new metabolisms

The present consensus is that photochemical reactions appeared after autolithotrophic chemosynthesis. But with chemical energy sources in excess for a long time there might not have been a need for photosynthesis to take over dominance in the bacterial floras. There may have been no selection in this respect as there still is no such need in large parts of the biosphere dominated by non-photosynthetic prokaryotes. Photosynthesis may therefore have been a relatively late evolutionary development. Another argument for a relatively late origin of photosynthesis is that initially the solar luminosity was low, in the early Archean being approx. 30% less than it is at present. The young, active Sun will have radiated more high-energy radiation, such as UV, the spectrum only gradually shifting towards the longer wavelengths relevant to photosynthesis. Also, the atmosphere was dense and thick being saturated with water vapour. The consequence of such a relatively late development of photosynthesis would have been that organisms decomposing carbohydrates by fermentation or respiration would have kept at a low key, if they could have developed at all. This is important to realize in connection with this aspect of eukaryotization.

The catching and processing of photons, as the first stage of photolytic hydrolysis, happens in a great number of chlorophyll units that together form an antenna complex. This complex concentrates their energy, eventually enough for releasing an electron into a photochemical reaction centre. There are two types of reaction centres which, together with their own antenna complexes, are called photosystems I and II. Of these, photosystem I seems to be the oldest, from which photosystem II may have been derived from a mutated genetic duplicate of system I. This oldest photosystem I is also the less efficient of the two and operates at a shorter wavelength, reminiscent of the slightly earlier radiation conditions on Earth, and a lower redox range than photosystem II. It is also interesting that this older photosystem I connects directly with ferredoxin, which hands the electron it receives from ferredoxin over to the nucleotide electron carriers FAD and $NADP^+$. Moreover, photosystem I utilizes sulphur compounds, such as H_2S, as a donor of one electron whereas photosystem II

uses two electrons from a water molecule, H_2O, possibly after having split CO_2 as a reductant in an earlier evolutionary stage (Dismukes *et al*., 2001). Utilizing solar energy, photosystem II splits water and oxidizes it to molecular oxygen thereby releasing electrons and protons with which it maintains the steep chemiosmotic gradient around the membrane. Taking up the protons releases energy with which a pyrophosphate moiety is added to ADP, giving ATP that in turn either carries it off directly into the cytosol or hands it over to NAD, reducing it to $NADH^+$ which then carries it away.

However, photosystem I does not contain an enzyme able to split water which is confined to the more recent photosystem II. The energy thus generated by photosystem II in connection with the hydrolytic enzyme are passed on to photosystem I, after which the latter system generates NADPH instead of ATP. This process whereby the two photosystems operate in series is known as non-cyclical photophosphorylation. They are connected by non-nucleotide electron carriers called plastoquinones, which transfers the electrons to the so-called b6-f complex. This complex is a proton pump transferring protons to the initial outside of the membrane, and is also found in the mitochondrial membrane, for example. The principle is to lift electrons from a high, positive redox potential to a low, negative one comparable to those that may have existed when life originated. This is a two-step process, photosystem II operating at the highest positive value of ca. +1V and lifting an electron to ca. 0V. From there, it flows "downhill" to photosystem I at an Eh value of about +0.4V which lifts it to ca. –0.5V. From here, they flow downhill again via ferredoxin and nucleotide electron carriers to CO_2 and to other electron acceptors in the cytosol. Together, the two connected steps constitute the Z scheme of photosynthesis after the shape of the path followed.

Thus, the conditions under which the membranes started operating are biologically reproduced under the present oxidizing conditions, thereby leaving the initial system intact. The principle of photosynthesis is that solar energy is used for the reproduction of the reducing conditions under which the energy processing membranes of living systems originated. The two individual steps at different redox potentials may be reminiscent of the changing environmental conditions, developing from reducing to oxidizing. Furthermore, under the final oxidizing conditions H_2O can be more easily oxidized than under lower redox conditions making it a more likely candidate for photolysis by photosystem II than it was for photosystem I which still uses H_2S. The longer wavelengths under which photosystem II operated optimally relative to photosystem I are also in agreement with this development. These two systems seem to have been superimposed on an earlier electron chain still operating in, for example, the membrane of mitochondria, of *Escherichia coli*, etc. The overall structure of the reaction chain therefore not only matches the altered conditions but it seems to reflect the subsequent stages of this gradual alteration.

Photosystem I is still utilized by the green sulphur bacteria and purple bacteria. These bacteria use light energy for transferring hydrogen atoms obtained from H_2S at a lower redox potential than for the oxidation of H_2O in photosystem II. Both in the green sulphur bacteria and in the cyanobacteria, the protons having crossed the membrane into the cytosol, are transferred to $NADP^+$ thereby reducing it to NADPH. In photosystem I, cyclic photophosphorylation can occur, implying that one proton is pumped across the membrane and only one molecule of ATP is generated without

however transferring its energy to NADP and thus further into the cell. Thus, by switching photosystem II on or off, the plastoquinones connecting the two systems can also function as a redox buffer within the membrane. In the oxidation of H_2S by the green sulphur bacteria, sulphur is released whereas the cyanobacteria operating with both photosystems I and II release O_2 when oxidizing water. Among the bacteria, these two photosystems can only be found operating conjointly in cyanobacteria which were the first to hydrolyse the energy-rich water molecules for the endergonic reduction to condensate CO_2 into carbohydrates and to produce dioxygen as a waste product.

Significantly, the ancient ferredoxins still take a pivotal role in the processing of energy, now being supplemented by reaction systems on either side. On the energy-gaining side within the membrane they connect first with the most ancient photosystem I which in turn connects further away with the more recent and more efficient photosystem II. On the energy spending and storing side of the cytosol they connect with FADH and NADPH, the latter carrying the electrons into the cytosol. Here, the energy obtained by photochemical hydrolysis and transferred into the cytosol reduces CO_2, thus condensing it into glucose by the citric acid (Krebs) cycle. Glucose can, in turn, be condensed into starch as an energy-storing polymer. These energy-storing processes, happening in the cytosol and known as the dark reaction of photosynthesis, operate independently of those in the membranes generating energy. These processes may have evolved at a later date since cyanobacteria, as the first bacteria utilizing both photosystems in series, produced so many oligosaccharides that apart from storing them as glycogen they have to excrete them in the form of sheaths enveloping the cells, for example. Apparently, they hardly developed a complex energy-storing and releasing system so soon after starting to exploit their new and highly efficient energy source of photolytic hydrolysis.

Thus together with the dark reaction another type of chemical reaction was added, with its own reaction conditions, enzymes, reaction products and place of operation. Initially, life processes depended critically on redox reactions happening in the membranes and tending to thermodynamic stability represented by the stability areas in the Eh/pH space. All these reactions were exergonic, meaning that electron transfer releases free energy. Under the newly added regime, the energy released by the photolytic oxidation of water was mainly deposited in the formation of carbohydrates which are not bound to occur in the membranes but which are found in the cytosol. From here, they interchange their energy through electron carriers with the electron-processing mechanisms in the membranes. All this happens starting with the endergonic reduction of CO_2 in which the catalytic activity of proteins is prominent. In effect, these biological catalysts lift the reaction across some threshold at a minimum cost of energy. Without them, the carbohydrates can neither be formed nor be broken down, the energy thresholds for spontaneous reactions to occur being too high on both sides. This results, rather than in thermodynamic stability, in kinetic stability of the reaction products that is typical for the covalent bonds of carbon compounds. Because of their kinetic stability, they are specialized in energy storage and release as well as in mechanical functions; they are locked in their stable position by catalysts as long as this is functionally – homeostatically - required.

The new reaction products, the carbohydrates are usually labile under the ancient, anaerobic, reducing conditions and fall easily apart under the influence of UV

radiation. However they are stable under the presence of dioxygen. This means that, for physical-chemical reasons, carbohydrates could not have taken their position of metabolic dominance under the initial conditions of the early Earth. Biologically, their early dominance is also unlikely because their formation depends on the existence of an elaborate system of specifically operating proteins. The stability and tuning of this system of proteins depends, in turn, on the existence of a complex, stable genome. Moreover, carbohydrates as typically energy-storing molecules had no function before large amounts of energy could be generated to be stored. All this requires an enormously elaborate and intricate organization at the cellular level which together with its material basis could only have evolved gradually taking long stretches of time. It seems likely, therefore, that the carbon metabolism will have arisen only late in the evolution of life relative to other forms of metabolism. In turn, the decomposition should also have been developed later by the mere lack of polysaccharides in the initial stages of life's evolution. It may even be speculated that this developed in concert with the evolution of photosynthesis. If so, this would be significant in connection with understanding the process of eukaryotization.

Before this time, the membrane-bound metabolism in the primary mackinawite crust may have depended on some form of nitrogen metabolism, basic to, first, the nucleic acids and, later, the amino acids, mutually catalysing their formation. This would explain the ubiquitous and metabolically central occurrence of nitrogen, phosphorus and sulphur, reflecting their abundant availability during the initially anaerobic conditions. Moreover, proteins are produced by RNA attached to the membrane and operate within the cell membrane whereas in prokaryotes the DNA is also attached to the membrane, replicating from the point of attachment. Under the initial, anaerobic conditions, nitrogen may have become available from the oxidation of ammonium, NH_3, which was released by volcanic activity. Although under present aerobic conditions, nitrogen is difficult to obtain from N_2 or nitrates, the ancient ferredoxins are known to catalyse reactions breaking the strong, triple bond of N_2, in this way also making nitrogen available to living systems. Molybdenum, at present associated with the polymerization of proteins but inactive in the ancient past, may have taken the place of vanadium, iron or even tungsten as an initial catalyst (Frausto da Silva and Williams, 1991; Williams and Frausto da Silva, 1999).

Environmental and biological consequences of the new waste products

The release of carbohydrates, oxygen, and hydrogen as waste products of photolytic hydrolysis may have had two important consequences for the immediate surroundings of the early photosynthesizers as well as for that of the environment and biosphere at large. Firstly, dioxygen will not have escaped into the surrounding water and, from here, into the atmosphere straightaway but it will first have been caught in the mats of interwoven cyanobacterial filaments themselves, producing it. This still happens, causing such mats to float unless anchored to a fixed substrate. Within these mats, the concentration of O_2 will therefore have been very high, in contrast to that of the general environment. Since calcium carbonate ($CaCO_3$) is thermodynamically stable under strongly oxidizing conditions, it may have precipitated as small, sharp crystals in the intercellular space within those mats. As a consequence, calcareous

stromatolites were building up even under the ancient, generally still anaerobic, reducing conditions.

Secondly, the early photosynthesizers may have attracted heterotrophic fermenting and respiring bacteria, utilizing their waste products. Their sugars may have been useful for the fermenting archaebacteria decomposing them through – anaerobically operating – glycolysis, from which these obtained their energy. In their cytosol, sugars are broken down through glycolysis involving a series of reactions identical to those in the carbohydrate-forming Calvin cycle but operating in reverse. In glycolysis glucose is oxidized, resulting in pyruvate, which is sometimes further converted into lactate or ethanol. Both the Calvin cycle and glycolysis are more ancient than photosynthesis. Chemolithotrophic bacteria like the sulphur, iron and nitrifying bacteria also fix CO_2 via the Calvin cycle and the reverse glycolytic pathway to synthesize glucose can be found in archaebacteria (Brocks *et al*., 1999). After glucose had been fermented, pyruvate and lactate remain as energy-rich end products of glycolysis.

The lipids, in turn, are broken down into glycerol and fatty acids, the shorter ones of which together with pyruvate and amino acids are broken down in the reactions of the citric acid cycle in the eubacterial mitochondrial matrix. FADH and NADH carry the electrons thus released to the inner membrane of the mitochondrion in which they are transported by the oxidative phosphorylation chain. Here, they are finally accepted by molecular O_2 after which H_2O is formed, releasing energy that as a next step hydrogenates ADP into the energy-rich ATP. The longer fatty acids, though, cannot pass the inner mitochondrial membrane and are fermented in the cytosol of the host by way of the glyoxylate cycle, which can be thought of as a primitive citric acid cycle, which also uses oxygen as the final electron acceptor. In this case of the proto-mitochondria, NADH is used, whereas in photosynthesis NADH is formed. Contrary also to the photosynthesizers too, which generate molecular oxygen in the build up of carbohydrates in their cytosol, they use oxygen and form water by their decomposition and ultimately release free energy in the form of ATP. The remainder of this energy is released as heat.

Thus, by using oxygen released by the photosynthesizers into the same community, the respiring eubacterial proto-mitochondria released energy left over after their host fermented the superfluous carbohydrates in the form of ATP and as heat. ATP may have been produced in such amounts that, in turn, these respirers excreted it together with proteins. This time this happened to the benefit of the fermenters, their future hosts, which, in terms of energetics, formed the least efficient link in the chain.

The generation of dioxygen from the oxidation of water therefore automatically restricted cyanobacterial nitrification and, hence, their nitrogen metabolism. The consequence of this is an imbalance between nitrogen and carbon metabolism and their products which is usually solved by expelling carbohydrates into the environment (Kooijman and Hengeveld, in press). This, in turn, opened the way to the development of fermenters as the first biologically heterotrophic life forms. These fermenters produce H_2 and CO_2 as waste products of which, according to Martin and Müller (1998), H_2 may have been utilized by an autotrophic archaebacterium. In plants, photorespiration occurs among others under relatively high O_2 concentrations, thereby reducing photosynthesis and thus excessive carbohydrate and oxygen production. The mechanism is that a central enzyme of the Calvin cycle, RubisCO, binds with dioxygen instead of with CO_2, thus preventing further carbohydrate production.

Instead, carbohydrates are now oxidized into CO_2 by the reverse of the Calvin cycle, i.e. by glycolysis. Thus, from this perspective too, the various ingredients for eukaryotization followed directly from the origin of photosynthesis by photolytic hydrolysis. This could have started a mutually beneficial relationship, first between the two heterotrophic types of bacterium, followed by the first oxygenic photosynthesizers. From data on protein sequences it has also been suggested that the nuclear genome is in fact a chimera between an archaebacterium and a eubacterium (Gupta, 1998; Gupta and Golding, 1996).

Interestingly, initially the citric acid cycle could have operated in reverse (Dyer and Obar, 1994) which seems to apply to all major pathways and cycles that originally will have built up carbohydrates, whereas at present they break these down (Kooijman and Hengeveld, in press). This can be the result of a simple change in the reaction equilibrium because of different concentrations in reaction products relative to each other. On the other hand, the presence of traces of dioxygen can also have been important. Whichever the reverting mechanism, the mere fact that reaction pathways and chains can easily be reverted may indicate that under variable environmental conditions of the ancient past, reversibility was once of adaptive significance either because of oxygen tension or of carbohydrate concentration.

This means that because of the alternative energy supply through the breakdown of the photosynthetic products, heterotrophic, fermenting bacteria will have been found in the immediate surroundings of, for example, cyanobacteria as the initial photosynthesizers, with two photosystems integrated, generating a superabundance of carbohydrates. In their turn, the respiring bacteria performing oxidative phosphorylation by utilizing the oxygen from the photosynthesizers, as well as glycolytic waste products from the fermenters, could have supplied the latter with additional energy in the form of ATP. The fermenters needed this extra energy for housing their enlarged enzymatic apparatus of proteins required for glycolysis, as well as for enlarging their genome to produce these proteins (Vellai *et al.*, 1998). Gradually, a symbiotic community of three different yet interdependent types of metabolisms may have formed this way. These symbiotic relationships will have intensified over time, eventually resulting in the eukaryotic cell as we know it today. This implied that even parts of the genome of the various metabolic structures and functions were gradually exchanged between the still largely independently operating partners.

According to Cavalier-Smith (1987), the three bacterial cell types together could recycle oxygen and carbon dioxide in the light. Moreover, in the light, the cyanobacterial component could photosynthesise under both anaerobic and aerobic conditions. The non-sulphur purple bacteria respired phagotrophically or photosynthetically obtained metabolites in both the light and the dark under aerobic conditions. And, finally, their host could ferment phagotrophically obtained food particles under anaerobic conditions. The three of them could therefore live together in symbiosis under widely different conditions of food, light and oxygen concentration and sufficiently recycle part of their nutrients internally without losing part of them into the environment. Of course, using each other's waste products is but one side of the coin, the other one being that the producer is freed from it. This may hold in the first place for the cyanobacteria overproducing carbohydrates and dioxygen, which were both expelled. Of these, dioxygen could in principle easily have escaped in the

environment but it could also have been held back to some extent in the less diffusive sugars surrounding the cells, together suffocating these. To have both waste products being removed could therefore have been beneficial. All this will certainly have been advantageous especially under the stressed conditions prevailing at the time (see below).

Summing up

Living systems are dissipative structures (e.g. Prigogine and Stengers, 1984), the complexity of which greatly speeding up local entropic decay. Being defined by thermodynamic principles, they ought to be considered in the first place as dynamic physical systems. These systems are determined by the "choice" of elements (Williams, 1981; Williams and Frausto da Silva, 1996), the quantum structure of the molecules, and the chemical reactions taking place. The chemistry of life is therefore secondary to its physics.

In living systems energy dissipates much faster than in non-living ones, because in living systems many reactions happen to occur in conjunction, in some way having obtained higher reaction rates than those in non-living systems. The question is, therefore, how these dissipative structures may have originated and developed. Here, we considered that energy dissipates in living systems by way of energy-rich electrons cascading through sequences of specialized molecular structures and biochemical systems and tapped at a steep thermodynamic gradient, the cell membrane. This membrane would date back to ancient iron-sulphur crusts precipitated at the interface of oxidizing ocean water and reducing seepages from the ocean floor. This gradient has remained intact ever since. First in a natural, geochemical way and then artificially as a biological one. The transition between these two happened when its operation refined, up to the point where water was hydrolysed photochemically under changed environmental conditions. At that point, it became possible to build carbohydrates in which the dissipative electron flow slowed down or halted for a while so that the energy became stored, becoming available again at times of energy shortage. Despite this temporary halt though, the living system is highly dynamic; each type of molecule having a specific turnover rate requiring a particular amount of energy for its statistical maintenance as a working unit. The living system as a whole, down to its simplest molecules as building blocks is in a permanent state of dynamic equilibrium maintained by a continuous flow of energy running through it.

This view has serious implications, first of all that life has to be analysed in terms of partly coupled electron and proton flows rather than principally in chemical terms. Rather than a chemical stoichiometry, the reaction equations and pathways require one in terms of electrons being transferred, and these, in turn, in terms of the free energy they contain. As we saw, this itself has consequences for the type of reactions we have to concentrate on in the first place, redox reactions. These happen in specific structures, particularly in the cell membrane where biologically available energy is generated, as well as in the nucleotides found both in metabolic systems and in that for storing information. With these reactions, in the first place happening in the cell membrane, our approach redirects from one concentrating on organic chemistry to an approach in which inorganic chemistry is central. Organic chemistry is the chemistry of energy storage in the cytosol which necessarily came after its processing in the

membrane. Biologically this means that life would not have started up with fermenters but chemolithoautotrophically from reactions between minerals and this, in turn, means that various metabolic pathways must have run the opposite way relative to the present one. Moreover, as a direct consequence of this the fermenters and respirators must have been in regular contact with the generators of organic molecules and for the respirators of dioxygen as well. This also means that the catalytic function of metals and nucleotides is primary going back to the initial phases of living structures, the spliceosomes and ribosomes consisting of RNA buttressed by protein and themselves building proteins for facilitating this catalytic function. And, finally, it means that from the beginning the three components of the eukaryotic cell must have occurred closely together perhaps even evolving their fermentive and respiratory traits in relation to the producers of the organic material they were breaking down for their own energy requirements.

This is, indeed, the stance we take in the next chapter discussing the mutualistic endosymbiotic theory of eukaryotization. Eukaryotization is one form of complexification of structures and processes expressing the response of living systems to stress due to changes in its environment life created itself.

Of course, without fossil evidence all this is speculation. But it intends to be speculation based on first physical principles, and checked continually by knowledge of the structure of partaking elements, and of the processes generating the structure and interactions of molecules arranged in biochemical configurations and pathways. Indeed this deductive explanation derives from a single set of physical, thermodynamic rules and these, ultimately, directly from only one principle; that of the universal increase of entropy. Our explanation does not derive from a plethora of biological compounds or functions often used as criteria for defining life which in fact had to evolve step-by-step over long stretches of geological time. Furthermore, biological explanations based on organic soups, whether or not of extraterrestrial origin, or on those adopting an initial distinction between metabolic and replicative functions, etc., unavoidably explain structures and processes with the knowledge of hindsight. After all in Miller's (1953) experiments, not only biologically relevant compounds were formed but the majority of compounds forming an amorphous tar (e.g. Shapiro, 1986) is not found in living systems. Similarly, the vast majority of compounds in the ancient waters will have been alien to any living system so that the relevant ones would have been selected from them so as to form some working system and without polluting it from its initial stages onward. This is difficult to imagine, however encouraging Miller's findings were from a retrospective, Whig point of view. Although we may be mistaken in the exact route followed in the autopoiesis of life, the explanatory principles applied in this paper suggest that a deductive approach is not only compulsory but that it seems feasible.

5. A MUTUALISTIC ENDOSYMBIOTIC SCENARIO OF EUKARYOTIZATION

As mentioned, the main metabolic pathways may have run in reverse relative to their present direction due to the geochemical conditions of the early Earth; the citric acid cycle and the glycolytic pathway building organic molecules rather than breaking them down. As later under different conditions the enzymes became more efficient,

leading the reaction thresholds in both directions, the difference between catabolic and anabolic processes diminished and metabolic homeostasis could grow. At the same time, organic molecules could be taken up from the environment, introducing first fermentation and then in the presence of oxygen, oxidative respiration.

Thus a community of photosynthesizers, fermenters and respirators formed all exchanging matter and energy. The switch to fermentation of the future hosts required significant morphological adaptations which will have developed stepwise, and this over a long period of time. They had to develop secretory enzymes, breaking down the carbohydrates outside their membranes, which may have required that they dropped their rigid cell wall, if, as archaebacteria, they had one. This naked cell had always been surrounded by a bilayer of (phospho-)lipids and sugars, which as complex carbon-based macromolecules are formed enzymatically. These lipids must, therefore, also have developed after the initial $(FeS)_n$ film had formed within which proteins had settled, as those attached to the $(FeS)_4$ cubanes, or those formed on the inside of the mackinawite crust. This membrane was extremely flexible and followed physical rules basic to the diffusionary dynamics of two-dimensional phospholipid bilayers. This also allowed for charge separation (Morowitz, 1981) by a charge-impermeable membrane (Mitchell, 1961, 1979) essential for its operation. Moreover, in order to take up macromolecules or even food particles this flexible membrane had to develop endocytosis requiring clathrines. Finally, this cell membrane had to extend relative to the cytosol thus keeping the surface-to-volume ratio constant as required for sufficient food uptake and the excretion of waste products. The resulting invaginated membrane eventually developed into an extensive "internal", for the largest part digestive membrane system comprising the Golgi apparatus, the endoplasmatic reticulum and the nuclear envelope (e.g. Rizzotti, 2000).

Eventually, the invaginations, retaining the original membrane function thus formed the vacuome as an intracellular digestive system concerned with the uptake and breakdown of food as well as the excretion of waste products (de Duve, 1984). Part of these invaginations could even be pinched off forming vesicles inside the cytosol that, apart from food particles, can from now on also contain accumulated elements like copper or calcium "within" the cell (e.g. Hopkin 1989). Within the vacuome, the endoplasmatic reticulum specialized in producing carbohydrates as well as in that of lipids for membrane synthesis and in producing digestive proteins. Many ribosomes are still attached to the membrane and located on the original cell side, forming these proteins through the membrane into the cavity of the reticulum, that is on the original outside of the invaginated membrane. From here the proteins arrive in the Golgi apparatus which is another part of the same complex of invaginations although more closely located to the present cell surface where they are extensively modified and thus ready to be used. After this, they are sorted, and then packaged in vesicles pinched off from the Golgi apparatus and sent to lysosomes with which they merge and where their contents hydrolyse the food being brought to there from outside through endocytosis.

The lysosomes, being pinched off continuously from the Golgi apparatus, are particularly involved with digestion. Waste products leave through the same mechanism and are released at the cell surface by ectocytosis. Carbohydrates are "excreted" inside the volume of the endoplasmatic reticulum as well: that is, at the former outside of the earlier evolutionary stages of the cell.

Part of the system of invaginated folds of the vacuome, as an inward extension of the endoplasmatic reticulum, even came to surround the genome, thereby forming the nuclear envelope. As such the eukaryotic cell is not distinct from a prokaryotic one by having developed a novel nuclear membrane "out of the blue". Rather this membrane too is part of the system of invaginations of the membrane originally enveloping the cell. Typically the periplasm between the inner and outer membranes is continuous with the cisternae of the endoplasmatic reticulum and contains proteins produced by ribosomes on the outside of the outer membrane; similar to and continuous with those of the endoplasmatic reticulum. The vacuome therefore appears to penetrate deeply into the cytosol, to the extent that it temporally shields the nucleoplasm off from the cytosol. Viewed this way, the nuclear envelope is the final extension of the system of membrane invaginations; with the Golgi apparatus at the end closest to the external environment and the endoplasmatic reticulum at an intermediate position. During mitosis both the Golgi apparatus and the nuclear membrane are fragmented and the fragments are taken up by the endoplasmatic reticulum.

All these functions are carried out as if still on the outside of the cell surface with which the various compartments are homologous. Strictly speaking they are therefore not part of the cytosol and actually have a higher redox potential, Eh, and a lower pH. These vesicles also came to carry out the functions of storage of elements, compounds or particles without direct contact with the cytosol and of their transport function through the cell. Thus these vesicles also serve as stores for elements expelled from the cytosol, such as calcium and sodium but which are retained in this way by the cell for possible external functions. These vesicles are transported within the cell along the tubuline filaments of the cell endoskeleton which appear to be derived from a protein originally constricting the bacterium during cell division (see Martin, 2002).

This part of the uptake, digestion and secretion apparatus of the cell, together with its storing capacity is in fact an internalisation of previously external digestive functions, initially occurring on the outside of the cell surface. By internalizing external functions, the cell seems to have grown considerably although the volume occupied by the original cytosol has obviously grown much less; it is particularly the intricately folded surface area of the metabolically active membrane that has extended significantly.

So far we have dealt with processes giving rise to the structure and functioning of the potential symbionts. Although our account still contains only the main phenomena, framed in the context of broad evolutionary lines, so much is clear that the various structures were soon after their origination already that complex and so much integrated with other components of the developing system that they could only be supplemented rather than changed. This means that only further developments such as those concerning eukaryotization are constrained in the same way and that, as a consequence, they can only be understood in terms of these constraints. Furthermore, the supplementary compounds of the system concerned adaptations to changing living conditions allowing it to persist. Symbiosis, and in the case of eukaryotization endosymbiosis, ought to be viewed in a similar way: that of an adaptation of certain life forms to conditions changing in a particular way. Such life forms could persist, retaining their own identity and individuality, due to their intimate containment under otherwise stressed conditions. Part of our problem will therefore be 1) to understand which changes may have happened, and 2) how much they needed to change, only

altering quantitatively by adapting their values of certain parameters or altering qualitatively by parameter change. Another part of the problem is how far the symbionts retained their individual population dynamics in their mutual interactions.

Mitochondria, chloroplasts, and their host

The respiring symbionts probably occurred in close contact with their fermenting host because it is there that the concentration of diffusing waste products is highest. As a consequence they will soon have inhabited some of its membranous invaginations. At some time they even became endocytic when the folds they inhabited pinched off forming a vesicle. From that moment onwards, or from even earlier stages of symbiosis they dropped some of their now redundant genes as did *Rickettsia* (Müller and Martin, 1999; Ogata *et al.*, 2001), or transferred them to their host nucleus (Gray, 1998; Martin and Herrmann, 1998) possibly contributing to its origin (Lang *et al.,* 1999; Gray, 2000). What happened to the proto-mitochondria, occurred to cyanobacteria as well: the latter eventually forming photosynthetic chloroplasts with their incorporation into the membranous digestive system of the host cell.

The mitochondria are distinct in performing the aerobic process of oxidative phosphorylation, expelling superfluous ATP into the cytosol of their archaebacterial host. This host, for its part, still carries out the less efficient process of anaerobic glycolysis. In turn the mitochondria consumed the end products of glycolysis, pyruvate and lactic acid breaking these down effectively with the citric acid cycle operating in their cytosol, the mitochondrial matrix. This produces, apart from CO_2, high-energy electrons that are fed via NADH and $FADH_2$ into the respiratory chain in the membrane eventually combining dioxygen molecules to produce water. The chemiosmotic processes in the membrane producing ATP, therefore, represent another process happening in the cytosol. The thermodynamic disequilibrium resulting in the proton-motive force of the mitochondrion, though, is maintained by expelling protons into the periplasm between "its" two membranes. This works efficiently because of a reduced proton diffusion within this confined space and is shown by a slightly higher pH. The same applies to chloroplasts, having a much higher pH within their thylakoids than in the surrounding stroma, whereby the inside of the thylakoids is homologous with the original outside of the proto-chloroplast cell.

The energy budget of both the mitochondria and the chloroplasts is positive: both release energy in the form of ATP to the benefit of their fermenting host. In chloroplasts, though, net production of ATP is reduced because of the production of glucose from CO_2 and H_2O in the dark reaction. Again, at the expense of ATP, glucose can be condensed into starch within the chloroplast. This compound typically serving as energy storage, its energy being released again at night. Thus, apart from their immediate release of ATP and glucose to the host cell, chloroplasts also serve as energy buffer on a longer, daily term as well as on those of more years when starch is stored in roots or seeds, etc**.** This early symbiotic system thus depended heavily on sugars and oxygen expelled as waste products by photosynthesizing bacteria. It is therefore likely that from the start, proto-chloroplasts must have formed part of this coherent community, eventually evolving into plants and into animals and fungi by losing the chloroplasts after eukaryotes containing them had evolved.

Besides for allowing for carbohydrates, photosynthesis also supplied electrons with which nitrite, NO_2, is reduced to ammonia, NH_4. This provides the cell with nitrogen for the synthesis of amino acids and nucleotides, a possibility also known from cyanobacteria under anaerobic conditions.

Thus, the outer membrane of the "host" cell envelops the symbionts similarly by invagination such that these continue operating as they did previously. However, at the same time, they are excluded from direct contact both from the cytosol of their host as well as from each other. As such, they remained distinct metabolic entities, freely co-adapting and evolving, and only when this fitted their mutual benefit. Co-adaptation was, therefore, neither qualitative nor instantaneous as it should have been when one cell phagocytoses another one without digesting it. In phagocytosis, the chances are slim that bacterial cells with finely-tuned, complementary metabolic interests fuse particularly when two different cell types, those of proto-mitochondria and of proto-chloroplasts have to be swallowed simultaneously to make the potential symbiosis viable. In contrast, according to the mutualistic endosymbiotic scenario, the three symbionts will each have started off with their mutual, historically anchored interests of their own only gradually adjusting the rates of their existing processes of uptake and release to each other without altering these processes as such.

Therefore, apart from certain gradually evolving co-adaptations as to ecological tuning, many things remained the same such as the exchange of compounds and energy, their membrane structure and operation or the anaerobic nature of their respective cytosolic metabolisms. Symbiosis implies ecological interchange and tuning rather than metabolic reconstruction although within this framework genetic interchange is, in principle, feasible. To a large extent the mitochondrion kept its individuality, moving about, growing and reproducing within the cell. From its side, the host keeps destroying mitochondria autolytically, a lysosome surrounding them as foreign bodies, thereby killing the mitochondrion and digesting it. Thus, a population-dynamic equilibrium is reached within the eukaryotic cells; too low reproduction rates of the mitochondria would result in cells soon lacking them, and too high ones in their overabundance.

Taxonomic proneness to symbiosis and ecological similarity

According to the scenario of predatory endosymbiosis through phagocytosis, the potential symbionts could in principle have been recruited from any bacterial family the members of which, by chance, came together as predator and prey. According to the scenario of mutualistic endosymbiosis, in contrast, they were from the beginning prone to relate metabolically to certain other, related bacteria, a phylogenetic trait expressed by their taxonomy and mutual ecological requirements. The near identity of the complex biochemical pathways concerned, together with the specific enzymes involved and their genetic background, could not have developed independently. The fermenters, later forming the hosts encapsulating the mitochondria and chloroplasts, partly have the same structure of the outer mitochondrial membrane and porins as gram-negative bacteria and partly that of archaebacteria (e.g. Gupta and Golding, 1996). From one lineage, the cyanobacteria with which they formed symbiotic relationships, they may have received energy and matter generated by photosynthesis. The resulting plastids, typical for algae and plants, are similarly to mitochondria

organelles with a double cell membrane. The same energy-processing system as is found in the membranes in the cyanobacteria as in those of mitochondria also occurs in another proteobacterium, *Escherichia*, although even less complete than in mitochondria. The enzymes in all these three groups are similar showing ancient common roots of these membranes. Moreover, symbiotic and parasitic bacteria related to the mitochondria belong to the same division of gram-negative bacteria as well, the α-proteobacteria, i.e. *Rhizobium* and *Agrobacterium* as plant symbionts, and *Rickettsia* as endocytic animal parasites (Gray, 1998). It is therefore interesting that the mitochondria are closely related to *Rickettsia*. These are endocellular parasites that live part of their life outside cells, the genomes of which show a high degree of similarity (Gray, 1998) although these are in both cases incomplete. The present mitochondrial genome consists of only 1% of the original genes it contained as a bacterium (Fenchel, 2002).

A comparative analysis of physiological and phylogenetic systems in prokaryotic communities from different biotopes shows that close relationships are realized by phylogenetic unrelated taxa. For instance, methanogenic archaebacteria are trophically connected to fermenting eubacteria. One may suppose that very much the same could have happened in the symbiogenesis of the eukaryotic cell; the potential endosymbionts may have been trophically and phylogenetically distant.

This ability to engage into some form of symbiosis therefore occurs in various forms within the same taxon and is therefore taxonomically fixed. This is unlikely for the scenario of phagocytosis without digestion but expected for that of mutualistic endosymbiosis. The development towards mitochondria seems to have taken place only once although hydrogenosomes show some similarities (Embley *et al*., 1997; Van der Giezen *et al*., 2002). Endosymbiosis of photosynthetic eukaryotic cells occurred repetitively at several, subsequent occasions leading to plastids with up to four sets of nested, bilayered membranes (e.g. Delwiche and Palmer, 1997).

Despite their taxonomic differences, the symbiotic components should be ecologically similar. This is, of course, not self-evident as shown in first courses in microbiology. Consequently, there are huge differences at short distances and in time in the composition of the bacterial soil flora as well as in their activity. Therefore, their ecological overlap should be large enough to bring them permanently into active contact, whereas they still differ in food choice, the one's waste being the food of the other. Moreover, their ecological preferences should be safeguarded as endosymbionts, such that their membrane systems keep operating unhampered. In this connection we have to realize that their ecological adaptability has strict limits, as shown by the taxonomic proneness on whether or not to engage in a symbiotic relationship of some sort, although within these limits there remains room for some evolutionary change (e.g. Tielens *et al*., 2002).

Timing of the eukaryotization processes

The idea behind the discussion about the processes eventually leading to the eukaryotic cell is that we have to view them in the perspective of the development of life from its very origin onwards. We cannot understand its origin by singling out a few processes just before and at the time of a presumed eukaryotization event. Many things had to be changed if not added to the systems. For example, as mentioned, the

tubuline endoskeleton was derived from an existing protein constraining the bacterium during cell division (Martin, 2002; van den Ent *et al*., 2001). Apart from this, the circular bacterial chromosome was subdivided into several rods allowing fast cell division and easy meiotic crossing over. Cells of the same type had to recognise each other for fertilisation to take place requiring special proteins in the cell membrane, for which a complete mitotic and meiotic apparatus had to be added. And so on. The development of each of these properties or processes must have taken much time and have lasted different periods and may have been initiated at different stages in eukaryotic evolution.

Thus, we started with describing the possible inorganic origin of an initial $(FeS)_n$ crust (compare Koch (1985), who inferred the same process based on phospholipids), accounting for a steep drop in ion density inside and outside the crust. The resulting thermodynamic disequilibrium accounted for a primeval proton pump as well as for a difference in voltage feeding the processes happening inside. This basically physical process of generating energy from inorganic reactions was kept intact in the most complex cell membranes as it still functions in the photosynthetic and respiratory bacteria. In the early cytosol, some anaerobic metabolism must have occurred based on mainly C, H, O, N, S, P, Fe, and, possibly, Ni, plus a good number of catalytic trace elements (Baymann *et al*., 2003). These elements were once abundantly available under the initial anaerobic conditions and had specific properties, making them biologically functional (Williams and Frausto da Silva, 1996). After the development of photosynthesis, particularly that of oxygenic photosynthesis based on photolytic hydrolysis, all this changed radically although the same elements remained active in the actual processing of energy. This particularly holds for heavy metals such as W, Co, Ni, Mo, Fe, etc., all having catalytic functions and as accelerators of metabolic processes taking an active part in the catalytic working of many metallo-proteins. The large amount of extra energy obtained from photolytic hydrolysis was essential for all the endergonic processes of the build up of the energy-storing carbohydrates, as well as building and maintaining the extensive enzymatic apparatus of the complex protein macromolecules with their high turnover rates. The same process of building the carbohydrates which now operates in reverse, although the same enzymes are utilized, served for their glycolytic breakdown. The citric acid cycle in the mitochondria or their counterparts in the peroxisomes in the cytosol of the fermenting host, the glyoxylate cycle, accounted for a more efficient decomposition of their reaction products and fatty acids. In this case, the molecular oxygen that is liberated during photolysis is used while water and CO_2 are produced

Eukaryotization as a highly complex process of symbiogenesis was the consequence of this transition to conditions of a super-abundance of energy; it allows temporary storage and eventual utilization of large amounts of energy. On the other hand, it still hinged very heavily on the energy-processing mechanism built up in the membranes since the very beginning of life. Yet, endosymbiosis seems not to have been feasible in the green sulphur and purple bacteria as their metabolism is based on the relatively inefficient reduction of sulphur in photosystem I only. This is insufficient as it neither produces carbohydrates nor oxygen which are required for respiration and thereby for the energy needs of the complex eukaryotic cell. After all the consortia of green sulphur bacteria, although probably as ancient as the stages before their initial radiation (Overmann and Schubert, 2002), are still exosymbiotic.

But the host could not have accomplished this by itself either, having an inefficient metabolism, the glyoxylate cycle, for breaking down the fatty acids from lipids. In fact, its high metabolic rates depended on energy in excess from the collaboration of the two other symbiotic bacteria. It is, moreover, the collaboration of cyanobacteria with proto-mitochondria that must have tuned their metabolisms in the first place; the cyanobacteria would suffocate in oxygen and carbohydrates and the proto-mitochondrion requiring these ingredients. The one could not have persisted without the other for long. Indeed, the cyanobacteria had to exude carbohydrates in order to balance their carbon-nitrogen metabolisms (Kooijman *et al.*, 2003) and they had to expel dioxygen to keep their cytosol anaerobic. These exudates were used by the proto-mitochondrion which produced ATP. The symbiotic relationship of cyanobacteria with proto-mitochondria tuned their high-energy metabolisms to each other to which the still low-energy metabolizing archaebacterial fermenter attached, being interested in the high-energy ATP that the mitochondria produced.

Despite the fact that chemolytic hydrolysis produces so much energy, the inner membrane of the cyanobacterial cell, as well as the membrane of the chloroplasts derived from them, are enlarged by invagination into the cytosol. These invaginations eventually pinched off their tips, which became the chloroplasts' thylakoids. They lie stacked one on top of the other in their stroma. The inner layer of the inner membrane of the mitochondria similarly enlarged by invagination into the matrix of these organelles, these invaginations being known as cristae. Thus, the membranes of all three symbiotic partners increased their metabolic output by invagination although in different ways.

These developments were feasible after the build up of a highly intricate energy processing membrane which is still operating inorganically through redox reactions, as well as after the build up of a highly integrated and intricate system of enzyme reactions both in the membranes as well as in the cytosol. This enzyme system, in turn, could only build up after a reliable, equally sophisticated information-storing system had developed, each requiring much energy for their continuous formation and maintenance at high turnover rates. Moreover, an elaborate enzymatic system required, in turn, a more elaborate information-storing system for which the initially circular DNA was replaced by a set of rod-shaped ones. These rods operated, divided, and replicated simultaneously, processes which obviously operated at high energetic and material costs.

Apart from those costs, these processes also pose problems of timing of the various components of the tightening symbiosis which, initially, would still have been mutualistic. For ectosymbiosis to happen, some form of recognition and signalling between cells as well as of mutual metabolic adjustment has to occur (Overmann and Schubert, 2002). Also, symbioses can be profitable under certain environmental conditions only, not under all of them. Overmann and Schubert (2002), for example, mention that it may have meant that all three components grew interdependent simultaneously as these symbiotic consortia drew tighter. The host not being able to evolve as such without the proto-mitochondria and these not without the proto-chloroplasts. The fact that, nevertheless, it is commonly assumed that the chloroplasts were the latest to have become engaged in the symbiosis may result from the fact that, in order to remain operating, they had to keep most of their functions whereas the opposite holds for the host with the mitochondria taking an intermediate position. The

only alternative would be that the proto-mitochondrion had not lost its photosynthetic functions initially but only later during its endosymbiotic phase (Fenchel, 2002). This would mean that initially it either combined photosynthesis with respiration, or that it assumed respiration only after the chloroplasts took over their photosynthetic activity. Both these scenarios look unduly complicated.

What seems to have happened as an overall process, therefore, is that some purple non-sulphur bacteria, with their highly sophisticated energy-processing membrane and a Calvin cycle operating in their cytosol, reversed the course of its energy processing system. Experiments showed (Lehninger, 1975, p.521) that, indeed, the reaction chain of oxidative phosphorylation can easily revert when the electron flow to oxygen is blocked. This may have happened in one of its descendants, the proto-mitochondrion, once this had adopted the citric acid cycle for the decomposition of the smaller sugars that were added to their initial decomposition of fatty acids. A partial, reversed Calvin cycle, the glycolytic pathway, together with the also partial citric acid cycle, the glyoxylate cycle, allowed a second descendant, the proto-host, to decompose the longer sugar and fatty acid molecules for the uptake of which it dropped its redundant membrane function. The host would have been relatively irrelevant concerning the amount of energy being processed, as a fermenter, it must have lived anaerobically being unable to supply the (proto-)mitochondrion with the oxygen it needed. The limited amount still entering it, was made innocuous in special organelles, peroxysomes, in which its glyoxylate cycle operated. Initially, Cavalier-Smith (1987) even suggested a simultaneous and synergistic adoption of chloroplasts and mitochondria, a suggestion he had to take back at the moment that more genomic data on ancient taxa became known (Cavalier-Smith, 2002). To make the uptake of molecules even more efficient, as required by the low efficiency of the two decomposition processes operating, it extended this membrane greatly by invaginations thus forming a pseudo-internal digestive system, the vacuome. By only partially breaking down glucose it supplied the proto-mitochondrion with part of the fuel it needed, pyruvate and the smaller fatty acids. Together, they effectively removed the waste products of the super-effective energy-processing system of the photosynthesising cyanobacterium.

Biochemically and also ecologically, the cyanobacteria may have been the first to have evolved and after them the respirators from which the mitochondria stem consuming their waste products. Only after these two conjoined the less efficient fermenter, the future host, may have entered the scene. After all, including a most efficient mitochondrion into a much less efficient fermenter would have greatly upset its internal system and feeding capabilities. And not having immediate and unrestricted accessibility to abundant food from the cyanobacteria could only have worsened this problem. We therefore have to revert the commonly held view. Because of their proximity as well as because of the tangle of invaginations of the proto-host, their initially external, ecological symbiosis gradually evolved, over millions of years, into the biochemically integrated endosymbiotic form of the eukaryotic cell as we know it today.

Another aspect of the intricateness of the processes involved is whether eukaryotization can have happened more than once, in other words, whether the origin of the eukaryotic cell can be polyphyletic. Given the fact that bacterial symbiotic consortia appear to be rare (3.5 per cent of all symbioses) and also that the interactions

required to be highly intricate and complex, the probability of this happening more than once seems remote. The fact that consortia between host cells with green sulphur bacteria are highly specific and ancient (Overmann and Schubert, 2002) accords with this suggestion.

Ecologically, these processes may have happened only in a certain corner of the total biosphere at the time, leaving other parts of this biosphere untouched. For example, the non-photosynthetic bacteria could have lived under darker conditions than the photosynthesizers and could therefore have been found in deeper waters than the latter. Also, for their energy supply they were dependent on mineral availability for their chemolithotrophy from which the photosynthesizers were freed, the latter in principle continually recycling the same elements. The photosynthesizers could, therefore, have occupied relatively nutrient-poor conditions or even flourish during geological times when nutrients were deficient whether due to their own activities or not. Although they did affect their mineral nutrient supply by forming dioxygen reacting with several of those minerals thus affecting their availability, in the long run they prepared, in a sense, their own success. Their success saw the real breakthrough in when oxygen concentrations reached a certain level allowing cells to form conglomerates with particular internal organizations, metazoans and metaphytes, arising particularly towards the end of the Proterozoic. Of these, all oxygen-hungry metazoans together used up so much of the oxygen the plants produced that the concentration of this element stabilized to some extent at a high level. The problem is therefore what changes in the environment could have brought organisms together when they were initially living in different parts of it? Could different bacterial floras have telescoped into each other during times of nutrient stress?

The changing chemical environment

Since the first chemical systems of life took shape, distinguishing themselves from those outside the mackinawite crusts, much had happened. Firstly, the initial reactions concerned were hardly fed by energy from outside, only the loss of an electron by Fe^{2+} to Fe^{3+} was replenished continually by the addition of another one under the influence of UV, supplying the energy needed. Other aspects of the initial redox reactions concerned a continuous loss of available electrons, of protons, or both, thus exhausting the original resource, and therefore reducing the disequilibrium between the conditions on both sides of the crust. This changed with the advent of photosynthesis, whereby another source of energy, light, split water which released large quantities of free energy to be used directly in chemical reactions. So much came free that a new metabolism started up based on carbon chemistry for storing energy which, in turn, opened up oxidative respiration and fermentation by other bacteria releasing the energy again. Respiration would eventually be forced to evolve to animal life and to an increasing heterotrophy in general, a form totally dependent on primary, photosynthetic producers supplying for the carbohydrates and oxygen needed. But animal life implies the operation of highly complex systems of organization. This organization comprises entirely new systems of operation, those of genetic organization, of developmental, anatomical and physiological systems, and those underlying motility and interactive behaviour. All these new systems were

superimposed on chemical reaction systems that were basically the same as those developed under the ancient conditions under which life had started up initially.

But of course ecologically not everything had remained the same. As said, the environment will have been exhausted to some extent both in terms of protons and electrons, as well as in those of minerals supplying them. For example, the nitrogen used in the building of nucleic acids and proteins may partly have been obtained from nitric oxide, NO, or have originated from reactions between atmospheric dinitrogen and carbon dioxide under the influence of electric discharges of lightning. As the amount of carbon dioxide decreased this abiotic nitrogen source dried up gradually, possibly causing a crisis during the Archean at 2.2 Ga ago (Navarro-Gonzalez *et al*., 2001).

Yet, the really great change came from its waste products, first of sulphur and later from dioxygen, either liberated from the splitting of H_2S or from that of H_2O respectively. With the latter reactions two main waste products were formed: dioxygen and various kinds of carbohydrates. At that time, most of the carbohydrates remaining insufficiently recycled by oxidative respirators were buried, leaving a remnant of unused dioxygen behind. The resulting rise in oxygen concentration coincided with the evolution of non-photosynthetic sulphide-oxidizing bacteria (Canfield and Teske, 1996). This remnant of dioxygen reacted with various elements essential to living systems which changed their environment drastically. One of these elements was ferrous iron, which reacting with dioxygen formed huge sedimentary layers, Banded Iron Formations or BIFs. The concentration of iron thus dropped with a factor of 1,000 (Anbar and Knoll, 2002)! In this process sulphur was released again as sulphide. To these compounds, in turn, phosphorus adsorbed to such an extent that this element too decreased greatly in abundance and hence in biological availability (Bjerrum and Canfield, 2002).

The fact that the BIFs were formed until 1.8 Ga, though, did not mean that from then on the environment became oxic. In fact much of the sulphate ($-SO_4^{2-}$) in the oceans formed by oxidation with dioxygen of part of the organic material had been reduced by bacterial sulphate reducers to sulphides (-S) from ca. 3.47 Ga onwards (Anbar and Knoll, 2002). This implying that metals were bound to sulphur, their precipitation reducing the availability of both (Canfield, 1998). As iron, which necessary for N_2 fixation, became short in supply for this reason as well and molybdenum having the same function in biological systems still reached only low levels, the restricted nitrogen cycle reduced the primary productivity of the biosphere (Anbar and Knoll 2002). This, in turn, would have led to biological innovations.

Bjerrum and Canfield (2002) mention that 2.7 Ga ago, the oxygen production by cyanobacteria depended on the availability of nutrients like phosphorus. However, with the adsorption of orthophosphates on BIFs, living conditions deteriorated because of phosphor limitation resulting in a reduced oxygenation of the ocean. One wonders if the eukaryotization of life occurring at this time (Brocks *et al*., 1999) relates to this ecological shift, allowing this new life form to use oxygen "internally" without suffering from heavy diffusionary losses into the environment in which it would eventually bind abiotically. On the other hand, multicellular, metazoan life could impossibly develop under the oxygen-stressed conditions having to wait till free oxygen was building up almost two billion years later, from ca. 800 Ma. As animals they fully depend on free oxygen for the respiration of carbohydrates whereas their

structure can be kept up by collagen, an extra-cellular protein, which is stable only under aerobic conditions (e.g. McMenamin and McMenamin, 1990). Respiring the carbohydrates with free oxygen, they produce carbon dioxide which can be recycled by Protista and algae and subsequently by metaphytes. Eventually a new geochemical cycle, based on the exchange of dioxygen and carbon dioxide between eukaryotic organisms, was added to those of other elements. The formation of the eukaryotic endosymbiotic life form constituted both an historically and ecologically pivotal role in this long-term process, covering aeons of time. Conversely an understanding of this life form can only be understood as part and parcel of these processes.

Dioxygen built up as a free molecule in the atmosphere after this period and subsequently in the oceans as well, gradually penetrating into the deeper strata. Towards the end of the Precambrian, from ca. 1.5 Ga onwards, we find thick layers of phosphorites (Lambert *et al.*, 1992) suggesting that, again, the phosphorus concentration will have decreased because of increasing oxygen levels in the ocean water. At this same time, metals like copper, cobalt and zinc forming insoluble sulphides under anaerobic conditions became available under the new aerobic ones. These metals could be incorporated in the metabolism as coenzymes, be stored in intercellular vesicles or they had to be excreted after special detoxification mechanisms had done their work (e.g. Hopkin, 1989). One of the metals, calcium, after its excretion as calcium carbonate, $CaCO_3$, precipitates extra-cellularly on collagen, thus forming exoskeletons of shells or carapaces, or endoskeletal bone. When dioxygen levels became sufficiently high by the end of the Precambrian, oxygen bound to carbon and calcium formed huge layers of sedimentary rock during the Phanerozoic. Thus all three elements were extracted from the mineral geochemical cycles, which lowered the atmospheric CO_2 concentration during the Phanerozoic. A further lowering of this CO_2 content resulted during this period from burial of carbohydrates in the form of coal, oil or gas.

As a result of all these changes, “extinct sediments” occur that are typical for the early geological history only, such as uranium and gold bearing conglomerates, Banded Iron Formations, laminated copper deposits in clastic rocks, sedimentary Mn ore and abundant phosphorites (Schopf and Klein, 1992). Many authors point to a decrease in Mg and an increase of Ca in the composition of carbonate sediments in the Proterozoic-Palaeozoic transition, a phenomenon possibly related to a growing biological control over carbonate precipitation in the ocean.

We can also mention silicon as one of many examples of a growing biological control over the geochemical cycles in biosphere. In the Precambrian ocean silica simply precipitated from the seawater forming layers or nodules in the sediments. The rise of organisms such as sponges, radiolarians, diatoms, and silicoflagellates, capable of absorbing the dissolved silica from the water and to use it in the construction of their skeletons, converted the ocean into an almost silica vacuum; the recent seas are under-saturated with regard to this element. Of course, the fact that it previously had an even greater abundance than it had after this time does not mean that it could, under slightly different conditions, have substituted carbon as a major metabolic element. This is not possible for various reasons such as the weak silicon-silicon bonds relative to the carbon-carbon ones, or the large inert super-molecules it forms with oxygen (Ponnamperuma, 1972; Wald, 1962).

There were also differences between the early Archean atmosphere and the subsequent ones, which show up from comparing the composition of volcanic gas and normal air. These two extremes encompass the main trend in the evolution of the atmosphere over the last 4 Ga. Living organisms are the principal actors in this dramatic change. Photoautotrophs, in particular, are responsible for decreasing the concentration of carbon dioxide and increasing the concentration of free oxygen in the atmosphere. The concentration of both these two gases determines the rate of weathering of rocks and the availability of some chemical elements in the biosphere. The rate of weathering essential for fertilizing the ocean and for the recycling of the biophilic elements in the biosphere has also been influenced by increasing rates of erosion and sedimentation. This was due to the increasing contrast of the planetary relief and the shift in composition of the major feeding provinces of the sedimentary basins from dark, basic rocks to the less resistant acidic and sedimentary rocks (Fedonkin 1996, and references therein).

All these factors and many more, though heterogeneous, have had a great impact on the evolution of the environment and thus on the rate and direction of the development of life.

Compartmentalization

What was new in the development from the prokaryotic to the eukaryotic cell was that this new unit started to perform different biological functions side by side, integrating them, as if the symbiotic partners were components of the same cell. In fact, by an intricate way of internalizing part of its own external, digestive functions, as well as by literally incorporating different metabolisms of proto-chloroplasts and proto-mitochondria, the process differed from one of a simple functional diversification. The formation of compartments with specialized metabolic functions and therefore each with different internal conditions of their own, accomplished this novel type of functional diversification. These compartments were formed by several sections of the invaginated membrane or by vesicles split off from them. Thus the metabolic performance of the cell was greatly enhanced and extended, not only internally but even relative to its external environment.

Thus, often different, chemically excluding reactions can now be found next to each other, just because they occur in separate, functionally different compartments. Another aspect is that particular concentrations, required for performing a certain function, need to be reached only locally without raising the concentration level throughout the cell and thus without introducing negative effects for other functions. Thus, high concentrations of manganese are particularly found in chloroplasts, and those of iron only in mitochondria. On the other hand, dioxygen is expelled from the cytosol as well as from the matrix of mitochondria and the chloroplast stroma which remain anaerobic and is only utilized in the mitochondrial membrane during oxidative phosphorylation.

There are yet other aspects connected with compartmentalization through membrane invagination. Although a large part of the membrane communicating with the external environment became internalized it is still functioning as an external membrane. This is reflected by the conservation of the inward/outward orientation of the proteins in the membranes. Thus elements or compounds being excreted from the

cytosol are still kept in vesicles from where they can be utilized for the cell's external relationships with other cells or for structuring its environment. For example, the endoplasmatic reticulum produces, among other things, the lipids needed for building and maintaining the extensive system of membranes. Also the polysaccharides and glycoproteins excreted by the endoplasmatic reticulum and the Golgi apparatus are related to export processes, to the sending of messages to other cells, and to biomineralization. Furthermore, the Golgi apparatus splits off vesicles with digestive proteins to the lysosomes, where digestion of phagocytosed food takes place or that kill and digest foreign bodies such as mitochondria. Other vesicles specialize in preserving elements expelled from the cytosol, such as Ca^{2+}, Mg^{2+}, H^{+}, or compounds as HPO_4^{2-}, which are utilized for other, external functions. Moreover each type of vesicle has its own complement of proteins, small molecules, or free ionic elements connected with its individual activities, spatially separated from the cytosol or other parts of the vacuome. Chloroplasts reduce NO_2 and CO_2, obtaining the protons and electrons required from NADPH, H_2, H_2S, or H_2O, with stored or excreted elemental sulphur, excreted sulphur compounds such as sulphates or oxygen as by-products. Photosynthesis itself is an essentially anaerobic process. Despite the fact that it produces dioxygen the mitochondria subsequently require for oxidative phosphorylation. The mitochondria, on their part, produce large amounts of ATP for the energy-hungry host cell. The storage of energy in carbohydrates, lipids and proteins depends mostly on photosynthesis.

This spatial differentiation of various functional aspects of an increasingly more complicated metabolism was repeated by the multicellular organisms forming a multitude of cell types each with a specific function and arranged in specialized organs. One can even hold that societies of many insect species such as in the Hymenoptera or in the vertebrates, especially in mankind, are structured functionally, each social compartment having its specific task. At the other extreme, at the time life emerged certain chemical processes happening were also compartmentalized into an internal reducing part within the mackinawite crust and were surrounded by an oxidizing external environment. It is through this compartmentalization of the ancient environment that fluxes of energy-rich protons and electrons started up, thereby in turn starting up the initial phases of life and having remained basically the same ever since. Over time several groups of fundamentally different types of components were thus formed: coenzymes operating as electron carriers; lipids closing off the cell from the external environment; nucleic acids for information storage; proteins for enhancing enzymatic functions; and carbohydrates for energy storage. They were all compartmentalizing various cell functions in a chemical way. Through various sorts of compartmentalization living systems became more and more complex and perfected, and, as mentioned, compartmentalized themselves several times in a number of successive rounds up to our social institutions.

Compartmentalization is also of interest in relation to external systems necessary for communication between eukaryotic cells; together forming multicellular complexes or organisms. Therefore, even the origin of multicellular organisms should be understood within the framework of the development of life from its initial stages onwards. One theme of the evolution of life is enhancing its flexibility whilst retaining the rigidity of its building blocks. One answer is the formation of modules and the other, as a special form of this, compartmentalization either internally culminating in

the eukaryotic cell or externally by multiplying the number of these cells and arranging them into organs constituting multicellular organisms. After modularity at the molecular level then compartmentalization seems to be a universal principle of the evolutionary development of the complexification of life. In turn, multicellularity as yet another form evolved many times independently in all domains of life.

6. TOWARDS MULTICELLULARITY

The role of continental growth and oxygen in the development of multicellularity

The origin of continental plates must have had an enormous impact on the development of life on the early Earth. Vast, stable continents became a trap for a great volume of chemical elements and sedimentary rocks thus removing them from intense recycling of sediments in the biosphere. This process plus that of the activity of organisms inhabiting the vast epicontinental basins became the two main factors driving the chemical evolution of the oceans and the atmosphere.

There is some evidence about the early origin of the ocean and the continents (Wilde *et al.,* 2001). Accretional growth of continents through the fusion of smaller fragments into large landmasses went through three major episodes (Lowe, 1994). Approximately 5% of the continental crust was formed 3.3-3.1 Ga ago, 58% between 2.7 and 2.3 Ga ago, and 33% of this crust 2.1-1.6 Ga ago. Therefore, ca. 96% of the present continental crust was in place by the Middle Proterozoic.

This extensive continental crust created ever new situations in the oceans. Large-scale upwelling became a factor enhancing the recycling of biophilic elements in the biosphere. The vast shallow, benthic biotopes opened up ecological opportunities because of enormous diversification of its microenvironments in contrast to the ecologically more uniform pelagic realm. To appreciate the importance of the shallow marine habitats, one has to remember that in the recent ocean, shallow-water environments of 0-200 metres deep occupy less than 8% of the ocean floor, whereas the total biomass of organisms living there constitutes 82.6% of the total biomass of benthic organisms in the ocean. This disproportion relates to the nutrient supply from the depths of the ocean through up welling as well as to that from land erosion, and to the intense recycling of organic matter in the shallow-water environment.

The intensification of nutrient recycling stimulated first of all the primary production by phytoplankton and phytobenthos, including the stromatolite-forming, photosynthesizing cyanobacterial and other prokaryotes that consumed carbon dioxide and released oxygen into the atmosphere. The removal of carbon dioxide from the geochemical cycle due to the burial of organic matter and the consequent formation of carbonate rocks on vast continental expanses may have been critical for a decreasing greenhouse effect during the Proterozoic. Already in the Early Proterozoic we recognise a remarkably small number of carbonate platforms and the first known extensive glacial event, chronologically coinciding with a maximum accumulation of Banded Iron Formations fixing enormous amounts of oxygen in the form of sulphates and iron oxides. Approximately 850 Ma ago the burial of carbon in sediments of the stable continental plates reduced the greenhouse effect further down to the very

sensitive balance and from this time onwards glacial periods became more or less regular events during the geological history of the Earth.

Usually, the origin of animals is thought to be related to the rise in oxygen content of the atmosphere, up to a level that allows aerobic metabolism to develop (Briant, 1991; Magnum, 1991; Knoll, 1996). Indeed an oxygen concentration of roughly 5% PAL (Present Atmospheric Level) is the minimum needed to sustain the activity of most eukaryotic microbes. Animals require oxygen concentrations of minimally approximately 50% PAL (Knoll, 1992). Most recent models on the evolution of the Earthly atmosphere suggest a dramatic rise in oxygen content at ca. 2 Ga ago. Holland (1994) estimates this change to have been from <0.1% PAL to >15% PAL. Knoll (e.g. 1996) suggests another increase in oxygen content of the atmosphere of up to >50% PAL by the end of the Proterozoic, an event that may have caused the Cambrian explosion (see however, Farquhar *et al.*, 2000).

The formation of the eukaryotic cell through symbiogenesis and the subsequent evolution towards greater biological complexity represented the response of some prokaryotes to the growing oxygenation of the environment to keep their metabolic pathways running. In this respect, the evolution of both the eukaryotic cell organelles and that of multicellularity look like two phases of one single process, together leading to the creation of a semi-isolated, compartmentalized and controllable internal environment. The increase in biological complexity was the only way to sustainable persistence under conditions of the chemical impoverishment of the aerobic environment. The progressive development of heterotrophy during the late Proterozoic and the early Palaeozoic eons can be seen as a consequence of the same causal chain (references in Brasier and Lindsay, 1998).

The early metazoans

In the initial eukaryotic protist cell, the cell membrane, lacking an electron chain of any sort, allowed contacts and interchange among the cells of the same type eventually enabling complexes of cells as an upstart of metazoans and metaphytes. The resulting cell developed a division of labour eventually leading to a new functional unit, the individual organism. This new unit needed mechanical structures for keeping it together as well as for connecting disjunct parts. In plants, expelled carbohydrates were used forming extra-cellular supporting tissue, such as cellulose, pectine, and lignine. They also needed tissues for the material interchange between two functionally different parts of the individual: the root for supplying water, minerals and nitrogen to the assimilating above-ground part in exchange for oxygen and energy, stored in carbohydrates by the latter. Thus, because of these two radically different and spatially separated metabolic functions, a single plant individual can be considered as functionally symbiotic (Kooijman and Hengeveld, in press). These two metabolically divergent parts cover a supportive and connecting dead tissue of the plant with a thin film of living tissue. Leaves for improving the photosynthetic functions developed much later during the late Devonian and the early Carboniferous, when both temperature and the level of atmospheric CO_2 dropped (Beerling *et al*., 2001).

In many respects the metazoans developed in a very different way. Firstly, being heterotrophic, they had to move in order to find food. The very first metazoans may have been attached forms rather than floating or vagile ones. Sessile life does not

require a complex locomotory system, nor a nerve system related to it, sense functions related to spatial orientation, nor interactions between locomotion and substrates. The sessile way of life has advantages in terms of predictability of environmental variables and is energetically advantageous because food and oxygen are brought in and waste products are removed by water currents. The attached way of life of larvae of most metazoans and the dominant sessile way of life among lower metazoans (sponges, cnidarians, ctenophorans) are consistent with the hypothesis of a primarily sedentary way of life among the first metazoans. However, it is movement and locomotion that created the immense diversity of the metazoans. Secondly, as multicellular heterotrophs they also needed a spatially structured system of food uptake, waste disposal, and internal communication, all together requiring an elaborate genetic system allowing for such a complex organization and its ontogenetic development. This system is built up hierarchically, earlier parts of the ontogenetic process starting up cascades of subsequent ones (e.g. Carroll *et al*., 2001). A complex system of Hox genes is basic to this hierarchy both in animals and in plants, in the latter of which they organize flower formation (e.g. Howell, 1998). A growing differentiation of body functions goes parallel with one at the level of the cell.

Also in animals, the head formed the entrance of the digestive tract and contained, as a consequence, searching or hunting functions requiring that the animal moved in that direction and that at that end sense organs for prey detection and food uptake concentrated. These sense organs had to be co-ordinated both among each other, as well as with body parts related to all those functions. Thus, a nervous system based on electric information transmission arose for quick responses. For responses operating on longer terms, both a hormonal connective system as well as an immune system developed, unifying various body parts in connection with sexual, defence and repair activities. Chemicals were transported via a circulatory system which also transported food and waste products between various body parts. For mechanical support, body protection, and muscle attachment, extra-cellular proteins formed long fibres, collagen or chitin. After some geochemical stage calcium carbonate precipitated on these fibres, thus forming exoskeletons in Protostomia or endoskeletons in part of the Deuterostomia, the vertebrates. Originally, in both animals and plants, the differentiation of sexual cells started only late in the lifetime of an individual. As the influence of somatic mutations is proportional to time the somatic line was separated from the germ line with the protected sexual cells and gradually reduced. This meant a stabilization of the adult body form, the younger developmental stages of the more ancient phyla being the most variable ones (Buss, 1987).

In fact the number of interactions within the living system, be they between different parts of the genome, etc., or between different life forms as in eukaryotes with all levels in between, may have formed the major source of phylogenetic development. The various metabolic systems having in principle remained the same since their rapid origination in the ancient Archean past often only running backwards. In other cases, patterns of interactions were added or shifted. A central principle was that, because of the complexity of the system, its constituent parts could not be dropped, altered, or supplemented freely without doing harm to the system; they were instead left intact as much as possible. This happened, for instance, in the development of photosynthesis described above. Occasionally, chemical elements could be substituted as long as the general structure in which they operate is maintained. Thus,

in crustaceans, copper substitutes iron in the haem part of their haemoglobine when this element became available and when iron had been depleted. This precipitating out as Fe_2O_3 in BIFs under increased oxygen concentrations at the end of the pre-Cambrian. In plants magnesium takes the place of iron in the haem group of chlorophyll.

Another important process is that existing structures can shift their function so that, again, no major change is required that would necessitate a restructuring of the system. For example, photosynthetic structures could have evolved from pre-existing porphyrines used earlier for the protection of the bacterium against light at various wavelengths, particularly the shorter ones. Also gene duplication fits this process or the modular build up of proteins, many of which are "living fossils" having been conserved for billions of years (e.g. Eck and Dayhoff, 1966). Similarly, the active site of proteins and ribosomes appears to be constituted by nucleosides, RNA, or by metals, the more recent protein part only facilitating their operation (see above). Changes in the genome by means of mutation are, in fact, counteracted actively by many means such as by histones, by an elaborate - and hence costly - repair system by strand duplication, by crossing over during the sophisticated and complex process of meiosis (e.g. Bernstein and Bernstein, 1997) and by the consequent mutual recognition of mating partners resulting in speciation (Paterson, 1985).

7. CONCLUSIONS

Step by step, the chemistry of life distinguished itself from that happening in the outside world. The elements taking part came to differ and so did the reaction rates, the types of their networks, pathways and cycles, and the whole morphological and functional contexts. In this paper we tried, as many have done before, to find out how certain of these steps were made.

Our aim was to follow a deductive, bottom-up approach as much as possible, trying to avoid the inclusion of relatively late, biological properties characteristic to present-day life and supposedly forcing its origination and development. Instead, the most basic, still non-biological initiating process should have been the general entropic decay of matter, dictating which primary exergonic reactions could have happened and releasing the largest amount of free energy. This energy could have been released most effectively at a steep thermodynamic gradient and carried off from there by means of energy-rich electrons or small compounds being released in any further reactions. Living chemical systems are therefore in principle entropically dissipative structures which cannot be separated from those outside. This unity of inside and outside processes, communicating through some steepened thermodynamic gradient, defines our ecological approach to the problem of the nature of life and hence of its origination.

Initially the processes were dictated by electron or proton transfer leading, by definition, to redox reactions within and around some inorganic crusts that later evolved into the cell membrane in which the principal energy-processing reactions still occur. Initially, too, there was no distinction between metabolic and information-storing processes and structures but both separated out gradually; the latter being distinguished by their standardizing function within an, on the whole, homeostatic structure. Thus, both metabolism and the "information-storing" system are based on

the same, ancient electron transferring and catalyzing metals and nucleotides, the apoenzymatic parts of the proteins and ribosomes facilitating and specifying the reactions. Within the membranes, the ferredoxins take a special position also dating back to the initial iron-sulphur, mackinawite crusts that were basic to any further developments.

The more complex a system becomes, the more vulnerable to any disturbances it will be. Therefore, this system will be rigid in some respects if it is to maintain itself. It is therefore not surprising to find the initial structures and processes still in operation at central positions of the living system. Yet, it had to be flexible as well in order to cope with any variation and changes in the environment. One possibility, still apparent in the cysteine strands of the ferredoxins, was by polymerization through duplication. In the most basic structures the initial modules are still apparent such as in the nucleotides or in the amino acids, or in larger composite molecules they constitute. Later gene shuffling and recombination were added, continually redefining patterns of interactions. Mutations are more risky, as they may introduce novel reactions alien to the system and therefore potentially disturbing. Less disturbing too, must have been the reversal of the direction of operation of the electron transport chains in the membranes and of the metabolic pathways and cycles in the cytosol. Another possibility for coping with environmental changeability was by protecting the initial system by additional, more elaborate structures leading to complexification. The elaboration of the energy-processing system in the membranes eventually leading to photolytic hydrolysis and respiration is the ultimate development in this respect. At yet a later stage compartmentalization added to the increasing complexity in which functionally different structures, operating under different conditions, were put side by side.

A major step in this latter direction was made by eukaryotic endosymbiosis of three bacterial components, to a large extent making the new unit independent of mineral and energy sources. Moreover, the possibility of storing energy in carbohydrates and releasing it again under more stressful conditions was a major next addition. Thus the cytosol became the seat of this aspect of cell energetics based on organic chemistry. The three components, unified in the eukaryotic cell, combined the photochemical generation of energy, its subsequent respiratory release, and fermentation of food taken up from the environment by scavenging. The unit as a whole could have originated under ecologically stressed conditions as a highly effective internally and externally recycling one. This may have been a long-term discordant process in which several evolutionary lines eventually combined to make it functional.

One of the new traits was that of meiosis which only later, among the protists, came to full fruition and for which the circular genome had to have been replaced by a number of rods. Another requirement was that cells of the same type had to recognize each other, a trait also necessary at a much later stage when the eukaryotic cell combined into multicellular organisms. These again may have originated under ecologically stressful, cold, oxic conditions and of changed mineral availability. On the other hand, multicellularity opened the way to greater mobility and an unsurpassed flexibility based on the still ancient metabolisms. The real development of this new form of life came during the Phanerozoic, falling outside the scope of this paper, but still following directly out of its contents.

AFTERWORD

This paper develops the theory of eukaryotic endosymbiosis along the lines discussed in Section 7. Key publications for our considerations have been: Williams and Frausto da Silva (1996) for the significance of inorganic chemical elements and processes during the historical build up and functioning of cells, and for the significance of compartmentalization herein. Mike Russell's work on the first, thermodynamic steps towards the formation of $(FeS)_n$ crusts as initial boundaries separating and thereby linking internal and external processes. Mitchell's (1961, 1979) papers on chemiosmosis obviously supplied the thermodynamic significance of cell membranes (see also Morowitz, 1978). Rizzotti (2000) and Dyer and Obar (1994) gave an explicit morphological picture of the initial steps of compartmentalization, particularly with respect to the double membranes of mitochondria and chloroplasts. De Duve (1984) supplied the concept of the vacuome in terms of a biological functioning of part of the compartments. Finally, Kooijman *et al.* (2003) supplied the elaboration of steps to be taken during symbiogenesis in general.

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