# The Notion of a DNA Minimal Cell: A General Discourse and Some Guidelines for an Experimental Approach

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Dedicated to the memory of *Francisco J. Varela*

The staggering complexity of even the simplest living microorganisms on Earth elicits the question of whether such complexity is really necessary for life, or whether, instead, the basic functions of cellular life (homeostasis, reproduction, and evolution) can be, in principle, expressed by much simpler unicellular entities that contain only a few dozen genes. This suggests the notion of a minimal cell, *i.e.*, the (potential) cell having the minimal sufficient molecular components to be defined as alive. The conceptual and practical implementation of such minimal cell(s) for our understanding of the notion of life, and also for possible biotechnological applications, is discussed here.

**Introduction.** – As is well known, even the simplest unicellular organisms on Earth display today a staggering complexity. *Escherichia coli* K-12 has a genome size of *ca.* 4.64 Mio base pairs [1] and *Bacillus subtilis* of 4.2 Mio base pairs [2], to give examples of well-known *Gram*-negative and *Gram*-positive eubacteria, respectively. The moderately halophilic eubacteria *Halomonas halophila* is endowed with the smallest known genome (1.45 Mio base pairs) of a free-living eubacteria [3], while the simplest known prokaryotic cell, the obligate cellular wall-less parasite *Mycoplasma genitalium*, has a genome size of 580074 base pairs, and contains 517 genes with only 470 predicted coding regions [4]. A report from *Douglas et al.* refers to the nucleomorph chromosomes from the cryptomonad *Guillardia* that has a 551 kb genome [5], and, according to *Moya* and co-workers [6][7], *Buchnera* species have even smaller genomes that can be reduced down to 450 kb. Aside from the actual value of the minimal extant genome, the question here is whether such complexity is a necessary attribute of cellular life, or whether, instead, cellular life could, in principle, also be possible with a much lower number of molecular components.

What would a minimal cell look like, *i.e.*, a cell that contains the minimal and sufficient number of components to perform the basic functions of cellular life? (Here, for the sake of simplicity, we will define cellular life as the capability to display three main properties: metabolic homeostasis, reproduction, and evolution). This question is interesting from the conceptual point of view, *i.e.*, for defining and understanding the logics of cellular life, and is also germane to the issue of the origin of life. In fact, in this field, it is generally accepted that the extant cellular complexity is the outcome of a lengthy process of evolution, in which intermediates were primordial cells that must have been genetically much simpler. How simple were these constructs at the time when they started to display the properties of cellular life? Moreover, the question of 'minimal cells' is potentially important also from view of biotechnology.

How can one approach the question of the structure of these simpler cells? Here, we will proceed by way of '*Gedankenexperimenten*', *i.e.*, imagening cells at decreasing degrees of complexity, so as to arrive, eventually, at the minimal cell, *i.e.*, at a situation characterized by the minimal possible genetic complexity compatible with cellular life. It is clear that this notion of a minimal cell cannot be sharply and univocally defined. As summarized in the proceedings of a recent workshop [8], different chimeric minimal cells can be conceived, depending upon the working hypothesis and theoretical bias. Nevertheless, as we hope to show in this paper, the elaboration of this notion can be a fruitful issue for discussion and advance in the field.

The notion of the minimal cell is not new, and, actually, with different emphasis and aims, the subject has been discussed several times in the literature, as shown by papers by *Venter* and co-workers [4][9], *Woese* [10], *Oró* and *Lazcano* [11], *Jay* and *Gilbert* [12], *Morowitz* [13], *Dyson* [14], *Varela et al.* [15], *Ganti* [16], and *Luisi* and co-workers [17][18]. We will discuss some of this work later in this paper. Recently, the notion of a minimal RNA cell containing only a couple of RNA genes in a self-reproducing vesicle has been presented [19]. Although, in the present paper, we will deal with minimal DNA cells, the link with the simple RNA cell will be considered.

**The 'Free Diffusing' Cell.** – The first '*Gedankenexperiment*' is to consider a cell that does not have the enzymes (or the corresponding genes) needed to synthesize low molecular-weight compounds, with the additional drastic assumption that substrates and other low-molecular-weight compounds, including all nucleotides and amino acids are available in the surrounding medium and able to permeate the cell membrane into the interior of the cell. This cell is able to perform protein biosynthesis by a modern ribosomal system, but it is limited to the following families of enzymes: *a*) enzymes that catalyze the synthesis of RNA and DNA; *b*) enzymes involved in the synthesis of ribosomes and other components of the protein synthesis machinery, such as aminoacyl-tRNA synthetases, elongation and initiation factors; and *c*) enzymes that catalyze the synthesis of cell-membrane constituents.

In the *Table* (left-hand column), the gene set of a minimal cell (based on *M. genitalium*) that meets the definitions indicated above is listed. As also shown in the *Table* (middle and right-hand columns), several enzymes and factors involved in protein biosynthesis are not taken into account. They have been eliminated under the assumption that they, although important for protein biosynthesis to regulate processes and improve the efficiency of cell activity, are not essential for the synthesis of proteins, nucleic acid components, or the cell membrane. We are aware that this choice is somewhat arbitrary.

Based on the list in the *Table*, this fully permeable minimal cell would have *ca.* 25 genes for the entire DNA/RNA synthesis machinery (without modifications of nucleotides), *ca.* 120 genes for the entire protein synthesis machinery (including RNA synthesis, 54 genes for the ribosome itself), and 4 genes enabling the synthesis of primitive cell membranes. We end up with a total of *ca.* 150 genes.

What would this cell be able to do? With the assistance of the outside supply, it should be capable of self-maintenance (leading to homeostasis) and also of self-reproduction. Self-maintenance would be implemented because all the activated biomonomers used for protein or nucleic acid synthesis are continuously furnished

Table. *A List of Genes That Define Minimal Cells According to the Definitions Used in this Paper, Sorted by Functional Category*

| Gene function | Number of genes | | |
|---|---|---|---|
| | Minimal DNA cell[a]) | 'Simple-ribosome' cell | Extremely reduced cell |
| *DNA/RNA Metabolism* | | | |
| DNA Polymerase III | 4[b]) | 4[b]) | 1 |
| DNA-Dependent RNA polymerase | 3[c]) | 3[c]) | 1 |
| DNA Primase | 1 | 1 | |
| DNA Ligase | 1 | 1 | 1 |
| Helicases | 2–3 | 2–3 | 1 |
| DNA Gyrase | 2[d]) | 2[d]) | 1 |
| Single-stranded-DNA-binding protein | 1 | 1 | 1 |
| Chromosomal replication initiator | 1 | 1 | |
| DNA Topoisomerase I and IV | 1+2[d]) | 1+2[d]) | 1 |
| ATP-dep. RNA helicase | 1 | 1 | |
| Transcript. elong. factor | 1 | 1 | |
| RNases (III, P) | 2 | 2 | |
| DNases (endo/exo) | 1 | 1 | |
| Ribonucleotide reductase | 1 | 1 | 1 |
| *Protein biosynthesis/translational apparatus* | | | |
| Ribosomal proteins | 51 | 0 | 0 |
| Ribosomal RNAs | 1 operon with 3 functions (rRNAs) | 1 operon with 3 functions (rRNAs) | 1 operon with 3 functions (rRNAs), self splicing |
| Aminoacyl-tRNA synthetases | 24 | 24 | 14[e]) |
| Protein factors required for protein biosynthesis and synthesis of membrane proteins | 9–12[f]) | 9–12[f]) | 3 |
| tRNAs | 33 | 33 | 16[g]) |
| *Lipid metabolism* | | | |
| Acyltransferase 'plsX' | 1 | 1 | 1 |
| Acyltransferase 'plsC' | 1 | 1 | 1 |
| PG Synthase | 1 | 1 | 1 |
| Acyl carrier protein | 1 | 1 | 1 |
| *Total* | 146–150 | 105–107 | 46 |

[a]) Based on *M. genitalium*. [b]) Subunits a, b, y, tau. [c]) Subunits a, b, b′. [d]) Subunits a, b. [e]) Assuming a reduced code. [f]) Including the possible limited potential to synthesize membrane proteins. [g]) Assuming the third base to be irrelevant.

from the outside environment; thus, their concentration in the cell would remain constant. Self-reproduction of this hypothetical cell would be driven by the existence of the machinery for replicating the cell's components – from the cell membranes to its macromolecules. However, no provision is made in the *Table* for the biochemical processes involved in cell division. In other words, the process of division is here not foreseen as a precise biochemical clock event due to the expression of manifold genes, but to a mere statistical process: as the cell components replicate themselves, and the cell membrane grows, the system would tend to divide by physical forces, as it happens in vesicles [20][21]. In this case, the process of division, being a statistical process,

would not always provide two compartments endowed with the same genetic information, but compartments where the distribution of macromolecular components would also follow a statistical distribution, with the result that only a fraction of the daughter cells could contain all necessary components for life. This poor efficiency of self-reproduction may have been a feature of early protocells.

To conduct this free-diffusing cell experimentally, one would need to construct a spherical membrane (*e.g.*, a liposome) that is nonselectively permeable to all low-molecular-weight compounds, but that does not permit the macromolecular components to leak out. This has not yet been realized, but, in principle, it is not impossible, as biomembranes are generally more permeable to low-molecular-weight compounds than to large macromolecules. Nonselective pores may be created, since it is known that these can form both in mixed lipid bilayers [22] and when phosphatidate is present in the lipid mixture [23]. Moreover, nonenzymatic facilitated diffusion of complexes formed between aldehydes, amines, and metal ions with amino acids, sugars, and nucleotides, respectively, may also take place [24–26]. One may ask whether this kind of free-diffusion model might have been relevant in the early evolution of protocells. It is, indeed, conceivable that, during early evolution, physical forces rather than fine biochemical processes might have governed the mechanisms of protocell division and metabolite permeability. (This is, generally speaking, our working hypothesis in this theoretical construction of minimal cells.)

**'Simple-Ribosome' Cell.** – In the previous model, the ribosome-based protein synthesis machinery requires the largest fraction of genes. As is well-known, this is an extremely complex system, encompassing more than 50 genes encoding different proteins and at least three genes coding for the ribosomal RNAs. As such, it shows a surprising level of conservation, both in terms of primary structure and number of components, even in highly streamlined genomes such as those of intracellular parasites, endosymbionts, and nucleomorphs arising from secondary symbiosis [27]. From the evolutionary point of view, it is unlikely that such a complex structure was present from the very early stages of cellular life. It is, then, reasonable to assume that a much simpler form of ribosome-mediated protein synthesis machinery was operative at that time, and evolved into its present stage in a step-wise fashion through a series of simpler stages. However, no such intermediate stages or simplified versions of ribosome-mediated protein synthesis have been discovered among extant organisms.

A drastic view would be to assume only few or no ribosomal proteins. In this regard, it is important to recall recent experiments carried out by several groups showing that the ribosomal RNA may be sufficient for peptide synthesis. This would mean that the ribosomal RNAs could be sufficient for a slow/basic transpeptidyl activity without the help of the ribosomal proteins. The possibility that the first ribosomes consisted of the rRNAs together with some basic peptides [28][29], or, perhaps, only by catalytic rRNA, is, in fact, reinforced by the peptide-bond-forming properties in ribozymes [30]. Further, the three-dimensional structure of the large ribosome subunit at high-resolution shows that no proteins are associated with the peptidyl-transferase catalytic site [31].

Thus, let us consider a cell that lacks the *ca*. 50 ribosomal proteins and containing only the three genes coding for the ribosomal RNAs. The corresponding 'simple-

ribosome' minimal cell would, then, be specified by *ca.* 105–112 genes (still under the assumption that all low-molecular-weight components arrive from the external medium). This is a small number when compared to the initial estimate of *ca.* 500 genes in *M. genitalium* – however, it is still a system of large biochemical complexity.

Conceivably, this cell would be capable of all that the previously considered cell could do, albeit at a much lower efficiency: in all likelihood, the rate of protein production would be slowed by several orders of magnitude. And all processes, including metabolic self-maintenance and cell reproduction, would also be less inefficient compared to present-day rates, and, perhaps, would face the risk of hydrolysis of its components – although, in a prebiotic scenario without predatory activities and without much biological competition.

**Further Reductions?** – There is no way to know for sure whether such a minimal cell would be functional. It represents, however, an interesting speculation, because it may correspond to the early stages of cell evolution. Focusing for a moment on early evolution, can we speculate about further simplifications of the genetic machinery and replication apparatus? In spite of the structural and functional similarities between the template-directed enzymatic synthesis of both RNA and DNA, double-stranded DNA cellular genomes replicate *via* a large, complex array of components in which proofreading DNA polymerases play a major role [32]. However, a number of experimental results and sequence comparisons indicate that a DNA genome with a simplified replicating enzymatic repertoire could exist [33]. Thus, the RNA-primer formation is catalyzed in mitochondria not by a primase but by the organellar RNA polymerase [34]. This suggests that a simpler set of polymerases could be functional and, in fact, may have existed during the early stages of cell evolution. Furthermore, changes in template and substrate specificity of nucleic acid polymerases can be easily achieved in the presence of $Mn^{2+}$ ions [35]. Thus, a working model of a simpler cell, in which a single polymerase could play multiple roles as a DNA polymerase, a transcriptase, and a primase, with a less-specific RNA helicase being part not only of the 'degradosome' but also of the molecular machinery involved in the opening of the DNA double-helix, is conceivable.

Similar arguments may be advanced for a simplified version of protein synthesis. For instance, EF-Tu and EF-G elongation factors are encoded by paralogous genes that resulted from a gene duplication that took place prior to the appearance of the last common ancestor of the Bacteria, Archaea, and Eucarya [36]. Thus, before the ancient separation of these three cell domains, a simpler but fundamentally similar process of protein biosynthesis involving fewer components was taking place. Pursuing this line of reasoning would correspond to speculations that make comparison with present day biochemistry more questionable. Nevertheless, the possibility of a simpler, functional ribosome-mediated system of protein synthesis includes the following *in vitro* experimental observations: *a*) initiation of translation can take place in the absence of some initiation components (*N*-formylMet-$tRNA^{fMet}$, IF-2, IF-3, and GTP) under experimentally modified conditions [37][38]; *b*) A translation system with modified cationic concentrations lacking elongation factors (EF-Tu and EF-G) achieves nonenzymatic, initiation-factor-free binding of aminoacyl-tRNAs, and ribosome-

catalyzed transpeptidation, as well as factor-free translocation reactions that result in slow but nonetheless faithful polypeptide elongation process [39–43].

All these *in vitro* data demonstrate that a relatively inefficient translational machinery may have existed without many or all of these protein factors required by modern cells. In search of further possibilities for reductions, it has been argued that the genetic code evolved from simpler, ambiguous versions involving fewer amino acids. It is possible, for instance, that Lys and Arg could be interchanged in a less-specific RNA-binding polypeptide, or that His, whose catalytic properties play a central role in the active site of many enzymes, replaced what was originally a catalytic ribozyme [44]. Thus, one may conceive, for example, that not all amino acids were present – or necessary – for the onset of the first protocells, as shown by the role of gene duplication in the evolution of aminoacyl-tRNA synthetases. It is possible, *e.g.*, that the lower number of amino acids serves to reduce the required number of genes to code for the aminoacyl-tRNA-synthetases, and also corresponds to the lower number of tRNA genes (especially when taking into account that the third base of the anticodon can be neglected in most codons). One theory holds that there might have been co-evolution between the formation of modern codons (with corresponding tRNAs/aminoacyl-tRNA synthetases) and the development of new amino acids [45]. Therefore, it is conceivable that, *e.g.*, Cys evolved from Ser at a later stage of cellular evolution, and, thus, in the early development of DNA cells, there was a limited number of synthetases and tRNAs. All these considerations decrease the number of genes by *ca.* 50 units, bringing us to a minimal cell that could be characterized by *ca.* 45–50 genes. This cell would still be able to carry out protein synthesis with most extant amino acids.

This figure is interesting in one respect: it provides a strong argument for very early involvement of compartmentalization in the origin of life and early evolution. In fact, let us make, for a moment, the hypothesis that the membrane compartment is a later development in the origin of life and early evolution (for example, appearing a good deal after proteins and nucleic acids). This hypothesis would then require the chance entrapment of all *ca.* 50 components concurrently in the same compartment at the point in time when the bilayer membrane would be closing in a spherical shell. The probability of simultaneous (or even only nearly stimultaneously) entrapment of such a large number of different components in one single small spherical compartment can be calculated as being close to zero. In force of this argument, it is much more reasonable to assume that the complexity of cellular life evolved from the inside of the compartment itself – *i.e.*, that most of the cellular macromolecular components were produced inside the early protocell itself and remained entrapped there.

**The Link with the RNA Cell.** – The staggering complexity of a minimal DNA cell nourishes the idea that the primordial living cell on Earth was a RNA cell. As already mentioned, this question has been the subject of a recent paper [19] in which the possible features and the viability of a simple RNA cell were discussed. It should be noted, however, that, in that cited paper, there is a different definition of a living cell than the one used here for the minimal DNA cell. A living RNA cell is simply a cell provided with the capability of self-replication and mutation. This corresponds to the *NASA* operational definition of life, whereby a system is said to be living when it is self-sustaining and capable of *Darwinian* evolution [46][47]. In the above-cited paper, a

self-reproducing vesicle containing self-replicating RNA has been conceived as the implementation of a simple RNA cell. In particular, it has been proposed that the simplest form of such a living cell would be one characterized by two RNA ribozymes (two RNA genes) within a self-reproducing vesicle. This is schematically represented in *Fig. 1:* one ribozyme is capable of catalyzing the synthesis of the vesicle, the other ribozyme is a RNA replicase able to self-replicate and to replicate the first ribozyme as well. The lipid precursors for the cell membrane as well as the mononucleotides for RNA synthesis are again supposed to exist in the surrounding medium and are taken up by the cell. In this way, the entire RNA cell is capable of self-replication.

The difference in complexity between the minimal two-gene RNA cell and the minimal simple-ribosome cell or further reduced DNA cell is, thus, dramatic. Of course,

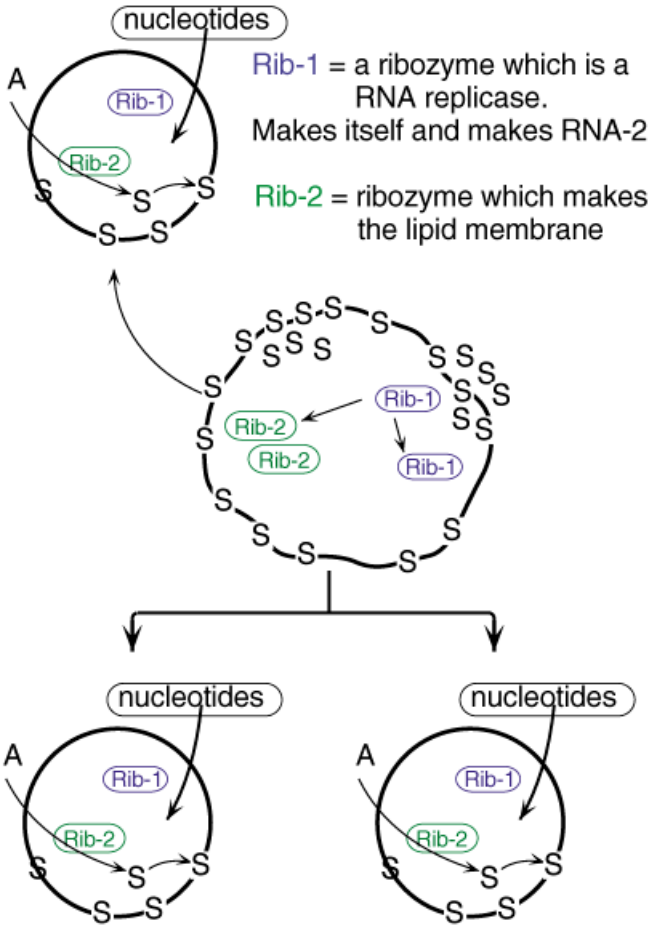


Fig. 1. *The minimal RNA cell as suggested earlier* [19] *in a different pictorial representation*. The cell contains two ribozymes (two genes provided with enzymatic activity), Rib-1 is capable of synthesizing the cell membrane by converting precursor A to surfactant S; Rib-2 is a RNA replicase capable of reproducing itself and making copies of Rib-1. All necessary low-molecular components required for the macromolecular synthesis are provided from the surrounding medium and are capable of permeating the membrane.

the minimal DNA cell is able to carry out protein synthesis, whereas the RNA cell of *Fig. 1* cannot. And, eventually, the minimal RNA cell has to develop into a protein/DNA cell. This is, in principle, possible because the ribozymes are capable of mutation and evolution. This possibility was shortly discussed in the above-mentioned paper [19] and another rendering of this hypothetical pathway is represented in *Fig. 2*.

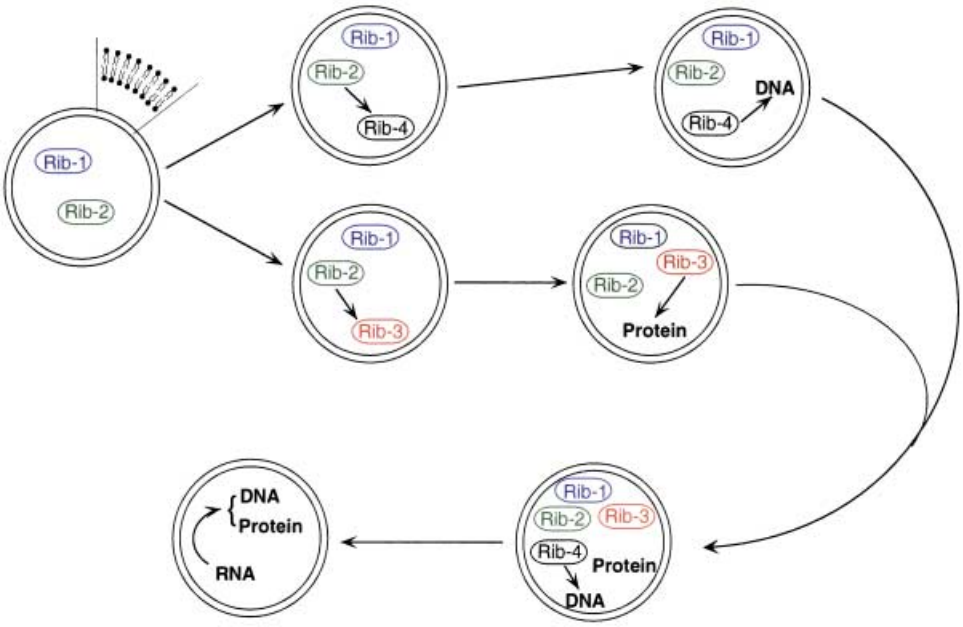


Fig. 2. *A hypothetical pathway for the transformation of a simple RNA cell into a minimal DNA/protein cell.* Rib-1 is the ribozyme that makes the membrane and is, therefore, responsible for the shell reproduction. Rib-2 is a RNA replicase able to replicate itself and Rib-1. During replication, Rib-2 is capable of evolving into novel ribozymes that make the peptide bond (Rib-3) or DNA (Rib-4). For statistical reasons, these two mutations are assumed to take place in different compartments, which then fuse with each other to yield a protein/DNA minimal cell. This mechanism is reminiscent of the double origin of life by *Dyson* [14]. Of course, one can propose a scheme in which both Rib-3 and Rib-4 are generated in the same compartment.

Thus, the link between the minimal RNA cell and the minimal DNA cells is one that is necessarily based on evolution: due to its much greater simplicity, the RNA cell is probably the way to conceptualize the very start of cell evolution (once the pivotal question 'how and where does RNA originate?' is solved). However, from a pragmatical point of view, one should consider that the two ribozymes of *Fig. 1* are still nonexistent, whereas, in the minimal DNA cell, while so much more complex, all biological tools are, in principle, presently available.

**Approaches to the Implementation of Minimal Cells.** – Having said that the required ingredients for a minimal DNA cell – unlike those for the RNA cell – are, in principle, available, the construction of a minimal DNA cell in the laboratory appears, at first sight, to be more realistic.

From the experimental point of view, one should first mention the approach used by *Venter* and co-workers: by application of transposon mutagenesis to the highly streamlined *M. genitalium* chromosome, many genes have been determined to be nonessential. These authors concluded that a minimal organism with a gene set of *ca.* 350 sequences might be able to exist [9], and that such a reduced organism might have had the ability to self-maintain and reproduce. Several years before, using a simpler but conceptually similar approach as *Venter* and co-workers, *Itaya* concluded, based on data from *B. subtilis*, that a minimal 'eubacterial-based' cell could exist with 320000 bp (about half the genome size of *M. genitalium*) [48]. On the other hand, the comparison of the completely sequenced genomes of *Haemophilus influenzae* and *M. genitalium*, species that have suffered independently the loss of many genes, led *Mushegian* and *Koonin* to suggest that the gene complement of a minimal ancestral cell might have consisted of a set of 256 sequences [49]. An additional noteworthy approach is the design of a computer simulation termed an e-cell, which 'needs' only 127 genes [50]. However, it should also be said that this e-cell is conceptually different from what we define here as minimal life: it contains the minimal gene set required to perform the most important biochemical tasks to maintain the cell (*i.e.*, protein synthesis, RNA synthesis, and energy generation by glycolysis), but is unable to self-reproduce. In addition, even if this hypothetical e-cell contains DNA that is transcribed to RNA, the entire DNA metabolism has been omitted from the theoretical construct.

The question thus becomes, how can one design an experimental approach for the implementation of minimal cells? The approach that appears as the most promising – and perhaps the only one that is reliably attempted in several biochemical laboratories – foresees the use of liposomes as model compartments for biological cells. In fact, liposomes (lipidic vesicles), with their lipid-bilayer structure, have been considered for several years the most suitable models for the shells of biological cells [13][14][43][51].

From the conceptual point of view, a schematic way by which a liposome can give rise to a cell model is represented by the chemical autopoiesis model shown in *Fig. 3*, as discussed a few years ago by *Luisi et al.* [52]. This figure represents a liposome formed by one surfactant S and hosting one reaction that transforms the reagent A into S with a velocity $v_{gen}$. Simultaneously, one membrane reaction converts S to product P, which is not able to form a membrane, with a velocity $v_{dec}$. The entire system is defined by two rate processes, and, depending on the relative values of the reaction rates, the system can be in 'homeostasis' (when the two rates are numerically equal); or it can lead to growth of the vesicles, due to accumulation of excess S in the membrane, and this can give rise to multiplication of the liposome number; or it can lead to destruction, when $v_{dec}$ is larger than $v_{gen}$. In other words, the simple liposomal system of *Fig. 3* is capable of modelling, although in the most primitive way, the various kinetic moods of a cell.

Recently, an experimental system to implement homeostasis in such a system has been presented [53], and is schematized in *Fig. 4*. It is based on two competitive reactions, one that builds the surfactants that in turn produce vesicles, and a competitive oxidative reaction that destroys the vesicles. Both reactions are taking place on the bilayer, *i.e.*, within the boundary of the system, and, therefore, the system respects also the definition of autopoiesis, the minimal living system according to *Varela* and *Maturana* [15].

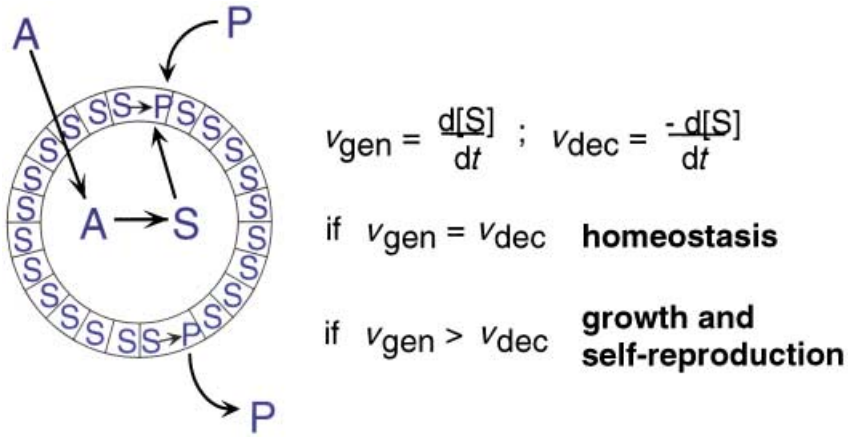


Fig. 3. *A liposome hosting two competitive reactions of shell anabolism and catabolism as a simple chemical model for a cell*

As simple as this system is, to the best of our knowledge, it is the only currently known experimentally closed chemical system that mimicks homeostatic behavior. From *Fig. 3*, as already mentioned, one can also see how a spherical surfactant-aggregate system can give rise to growth and self-reproduction. This has actually been implemented experimentally: the system is based on aggregates formed by fatty acids, such as caprylic acid or oleic acid [54–56]. As schematically illustrated in *Fig. 5*, a water-insoluble precursor of a fatty acid, such as an ester or the corresponding acid anhydride, binds to the surface of pre-existing vesicles *via* hydrophobic interactions. The ester or the anhydride is hydrolyzed on the surface of the membrane, giving rise to the free surfactant, which remains bound to the membrane, inducing vesicle growth. Eventually, together with the growth process, the system undergoes an increase of the vesicle population number, which becomes more efficient when more vesicles are present and being formed. Thus, the process is an autocatalytic one.

The mechanism of this process is not yet understood in detail. However, recently, it has been possible to prove by electron microscopy the growth and splicing of vesicles. This has been accomplished by labelling preformed POPC liposomes with ferritin, a protein having an electron-dense iron core. Then, oleate surfactant or the water-insoluble oleic anhydride was added, and the size distribution of the resulting mixed POPC/oleate vesicles was determined [20][21]. The comparison of the size distribution of the ferritin-containing vesicles before and after precursor-induced growth gives evidence of growth processes (as, after the addition, one finds larger ferritin-containing vesicles than before), and, furthermore, under certain conditions, one finds ferritin-containing vesicles that are considerably smaller than the initial ones, which could only have originated from a splicing process.

This growth and autocatalytic self-reproduction of vesicles does not involve any of the fine biochemical regulation mechanisms presently found in biological cells. Rather, it is statistically governed by energy minimization, consistent with the argument mentioned earlier that, in prebiotic times, physical forces and statistical processes were

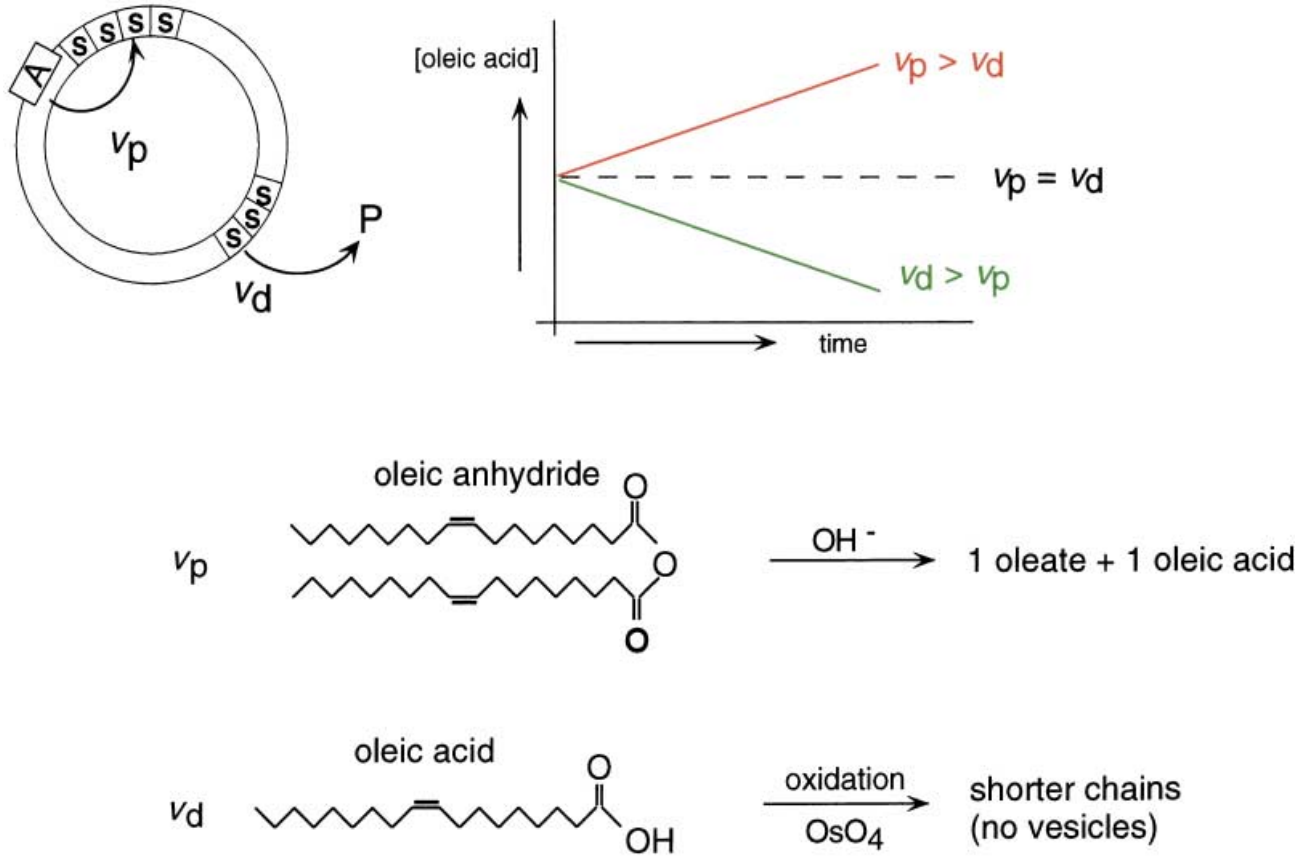


Fig. 4. *A simple chemical model of a homeostatic cellular system*. The system corresponds to the model of *Fig. 3*, and consists of oleic acid/oleate vesicles that self-reproduce upon binding the water-insoluble oleate anhydride (see *Fig. 5* for a mechanism). At the same time, the oleate molecules are partly destroyed on the membrane itself by an oxidation reaction. Depending the relative velocity of these two competitive reactions, one observes homeostasis, *i.e.*, growth and reproduction of oleate vesicles, or vesicle destruction, simulating cellular death.

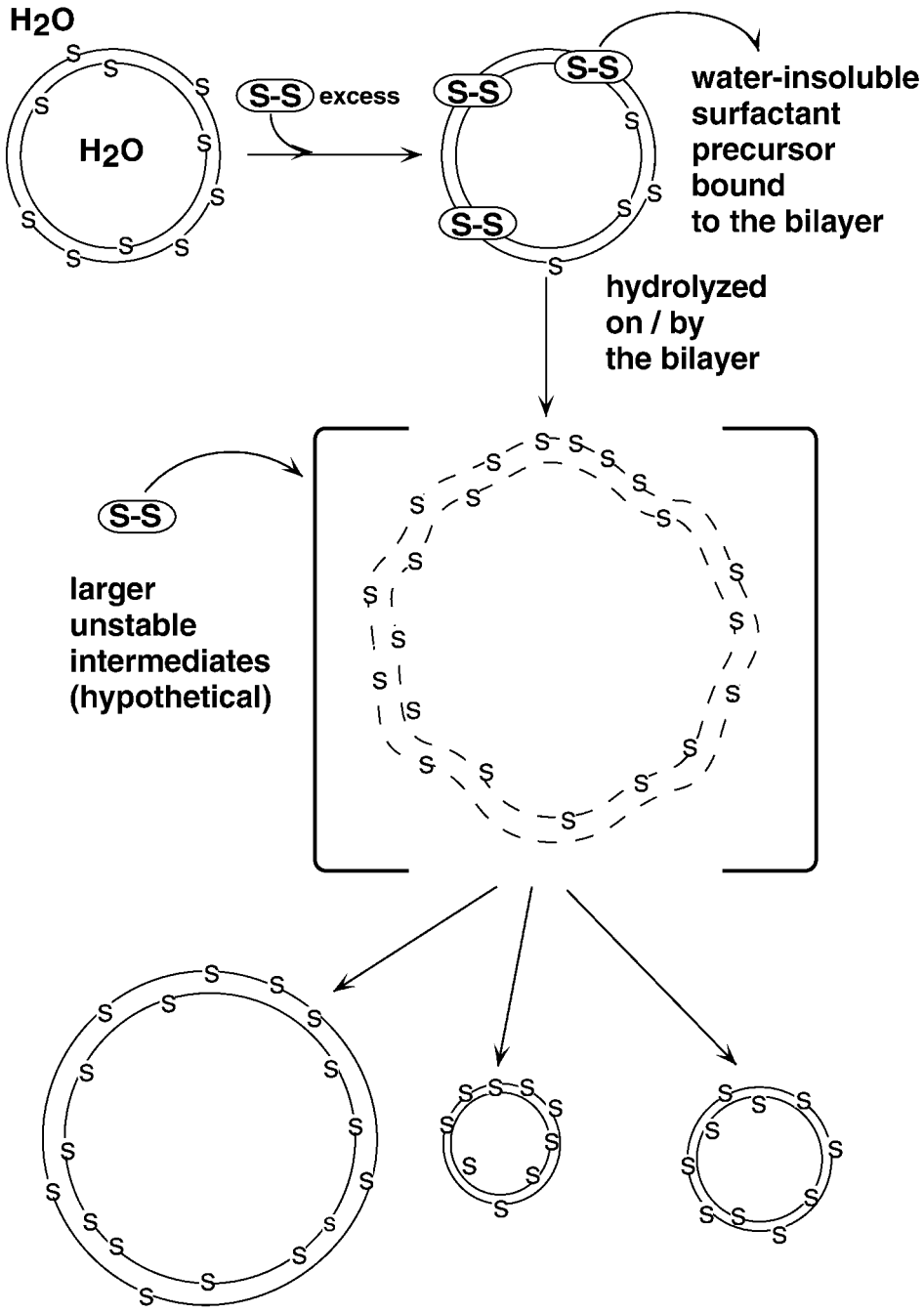


Fig. 5. *The chemical basis of the self-reproduction of micelles and vesicles.* The water-insoluble, membrane-binding precursor S–S is hydrolyzed on the membrane to the very surfactant S. This produces more amphiphilic material and leads eventually to growth and multiplication of the aggregates, which, in turn, solubilize more S–S, giving rise to an autocatalytic process.

the most likely precursors of the fine biological mechanisms developed later by evolution. These simple processes involve only the shell – the inside of the liposomes is empty. More interesting is the case in which enzymes and/or nucleic acids are present inside the liposomes.

**Liposomes and Giant Vesicles as Experimental Models for Minimal Biological Cells.** – In fact, the first step for the construction of experimental cell models with liposomes is to demonstrate the feasibility of molecular biology reactions inside the aqueous core of liposomes. A number of groups have begun experimentation in this direction. Here, some approaches should be mentioned. A couple of papers describe for example the synthesis of poly(A) by polynucleotide phosphorylase in phospholipid or oleic acid/oleate vesicles [17][57]. In a couple of experiments, the self-reproduction of vesicles was accompanied by a self-replication of nucleic acid material inside them. One case is the self-reproduction of oleic acid/oleate vesicles hosting the polynucleotide phosphorylase polymerization of ADP to poly(A) [17]. Another approach describes the self-reproduction of oleic acid/oleate vesicles hosting the enzyme Q$\beta$ replicase, an enzyme that is able to make copies of an RNA template. In first approximation, the process may proceed as illustrated in *Fig. 6*. As the enzyme is replicating its RNA template inside the vesicles (the so-called midi-variant RNA), the vesicles themselves are growing in size and number [18]. The two processes are uncoupled from each other, *i.e.*, the vesicle self-reproduction does not depend upon the RNA replication or *vice versa*. It may appear that this system is 'living' according to the definition of the RNA cell given above. However, the shortcoming of such a process is apparent from *Fig. 6:* as the number of generations increases, there is a continuous dilution of enzyme and RNA template, so that a smaller percentage of the total number of vesicles will contain both macromolecules. Eventually, no vesicle will contain both RNA and enzyme molecules, and 'life' would then stop.

Even more complex molecular-biology reactions have been successfully implemented in the inner compartment of liposomes. One example is the polymerase chain reaction [58], particularly interesting because, in this case, the liposomes containing DNA, a polymerase, and all small molecules required for PCR had to withstand more than ten temperature cycles at 55 – 95° without losing its contents. In a similar attempt, an entire translational system that was entrapped in POPC liposomes has permitted a polypeptide synthesis, although limited to the production of poly(Phe) [59]. In principle, these last two systems could also be carried out in self-reproducing vesicles. However, also in these cases there would be no coupling between internal macromolecular production and vesicle self-reproduction, and dilution would eventually take over and stop this kind of synthetic cellular life.

How can one prevent this death by dilution? One way to circumvent this difficulty in the laboratory is the use of giant vesicles (GVs). These are vesicles reaching diameters of 100 μm, and, due to their extreme size, they can be observed by conventional optical microscopy. Furthermore, it is possible to entrap biochemicals directly into their aqueous compartment by various methods. One method is based on the use of laser tweezers [60][61]; alternatively, one can use the method of microinjection as it is carried out to inject substances into eukaryotic cells [62 – 64]. One can, *e.g.*, inject a mixture of all 20 amino acids or of all possible tRNAs, a mixture

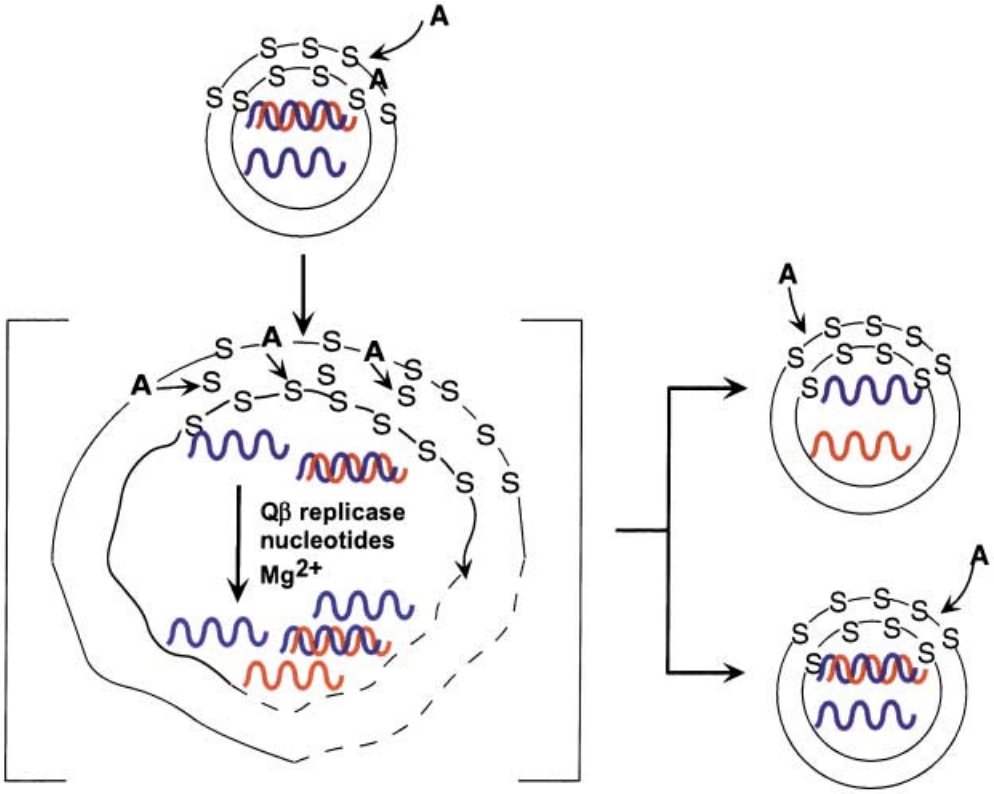


Fig. 6. *Replication of RNA in self-reproducing oleic acid/oleate vesicles* [18]. As vesicles reproduce themselves according to the mechanism shown, Q$\beta$ replicase makes copies of RNA inside the vesicles. The two processes occur simultaneously (self-reproduction of vesicles and replication of RNA) but are not coupled with each other.

of different enzymes and/or nucleotides, and even ribosomes. Generally, only a limited volume can be injected without bursting the GV (one is limited to a few picoliters) and quantification of the injected volume is not easy. By using fluorescent markers, one can ensure that the injected material is inside the $H_2O$ pool of the vesicle.

First important experiments with biological materials inside GVs were carried out by *Miyata* and *Hotani* [65], who were able to induce the polymerization of actin inside GVs, producing macromolecular filaments that deformed their GV host. It is also interesting to mention the work of *Angelova* and co-workers [66], who were able to show the incorporation of DNA in a GV *via* a process similar to endocytosis observed in eukaryotic cells. Also, *Ourisson* and co-workers are actively involved in GVs as models for cells [60].

An example of a relatively complex molecular biology reaction is given in *Fig. 7*. Here, we see the increase in fluorescence intensity inside a GV arising because of the synthesis of RNA due to the T7 RNA polymerase reaction [67] with the substrate molecules – the nucleotides – being loaded from the external medium.

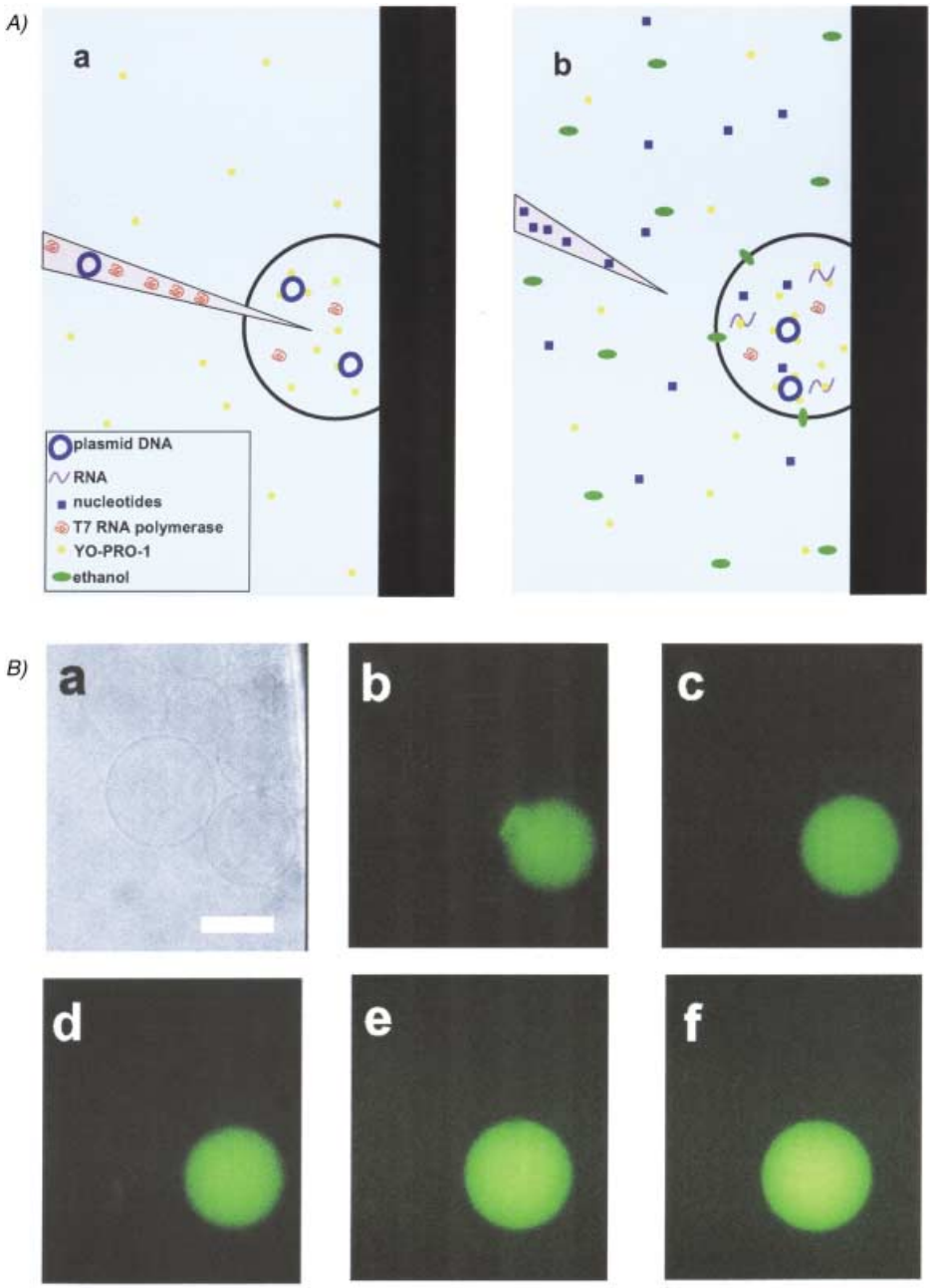


Fig. 7. *A biochemical reaction in giant vesicles (GV)*. A) *Schematic representation of the injection procedure*. In a first step (*a*), the enzyme T7 RNA polymerase and the plasmid pWMT7-EGFP (a plasmid containing the GFP gene under the control of the T7 promoter) are injected into a selected GV. Then (*b*), EtOH is added, and nucleotides are microinjected into the surrounding environment. Ethanol makes the GVs more permeable to nucleotides, thus the nucleotides can permeate the GV membrane. B) *Demonstration of the time course of the increase in fluorescence of the YO-PRO-1/nucleic acid complex due to the increased nucleic acid concentration inside the selected GV* [67]. *a*) *b*) Before addition of nucleotides; *c*) 3 min, *f*) 12 min, *e*) 22 min, *f* 32 min after the addition of nucleotides. Scale = 50 μm.

One interesting, and, now, timely, project, is the introduction of the entire translational machinery into a single GV with the subsequent synthesis of proteins, and a particularly challenging case is offered by a compartment that hosts the enzymes to synthesize the lipids (*e.g.*, lecithin) that make up the membrane itself. As the lipid is being produced inside the liposomes, the liposomes grow and eventually may divide. The idea and some very preliminary data from experiments with partially purified enzymes of such a 'self-synthesizing' liposome was presented some time ago [68]. One can now imagine a system in which these lipid-synthesizing enzymes are expressed inside the GVs, or inside a conventional liposome, thus giving rise to a real minimal cell system. Recently, *Yomo* and co-workers published, for the first time, the synthesis of proteins in conventionally prepared GVs [69], and a similar approach has been presented by our group [70].

That projects with GVs prepared by electroformation have not been realized so far is due to some fundamental problems involved in working with GVs. First, one is working with a single compartment at a time, so that the reproducibility and statistical analysis are difficult to control. Furthermore, carrying out reactions with GV makes sense only when one can directly monitor the chemical changes occurring inside, and, for that, one must rely on fluorescent reactions that can be followed by microscopy. However, since we are dealing with one single compartment and minute concentrations, significant problems with sensitivity arise. Ours and the other cited groups are presently engaged in investigations towards the solution of these drawbacks. We believe that these efforts are important, as this is the best technique for the study of many components present simultaneously in the same compartment. An interesting approach along this line has been provided in a recent article by *Ueda* and co-workers, who were able to reconstitute cell-free translation with purified components [71]. Eventually, the combination of this approach with the liposome compartment may prove extremely valuable as an alternative to 'black-box' commercial kits for protein expression.

**Concluding Remarks.** – The notion of minimal cell(s) – both the RNA cell and the DNA/protein cell discussed here – is, at this point, mostly a conceptual device providing a platform for discussion of the minimal prerequisites for cellular life and possible progress in the experimental implementation of cell models. One main line of argumentation in this paper and in the overall field is that the early cells likely adhered to this notion of minimal cell(s), namely structures that were able to display some form of primitive life without the modern cellular complexity. The relation between 'primitive life' and 'minimal cell' is indeed the main focus of research in the field. One main step towards simplicity is the assumption that physical forces, rather than fine biochemical mechanisms, were the driving forces for cell division and uptake of chemicals from the medium. Of course the assumption that all biomonomers (particularly certain essential amino acids or present-day nucleotides) were already available in the surrounding medium is open to debate.

We have emphasized here the dramatic difference in complexity between a minimal self-reproducing RNA cell and a minimal self-reproducing protein/DNA cell. Although this finding is not surprising, one can use this argument to emphasize again that the RNA cell is the most likely candidate for the beginning of cellular life; the complexity

of the DNA/protein cell, conversely, may suggest that, most likely, compartmentalization accompanied or preceded the early stages of cell origin and evolution.

The evolutionary link between the RNA cell and the DNA/protein cell is of particular importance. In *Fig. 2*, the classical view is represented, according to which ribozymes are generated by an evolutionary process and are, then, able to catalyze the synthesis of peptide bonds, which would lead to the synthesis of DNA molecules. How does one go from a cell that has a catalyst for the formation of peptide bonds, and/or the catalyst to make nucleic acids, to a full-fledged self-reproducing cell? It is clear that the synthesis of peptide chains or of nucleic acids *per se* does not help unless assisted by a self-replication mechanism to produce identical copies of polypeptide sequences (enzymes) or nucleic acid sequences. This is linked to the whole problem of the genetic code, for the onset of which no convincing and experimentally reasonable hypothesis has been yet offered.

The major components of the much simpler RNA cell in *Fig. 1* are, however, presently not available, which leads us to consider that perhaps it is currently easier to attempt to make an extremely reduced DNA/protein cell than a RNA cell. Even though as many as 50–150 macromolecular components may be necessary for a full fledged minimal cell (one that can display homeostasis, self-reproduction, and evolution), it is, in principle, not impossible to gather and transport them into a closed semipermeable membrane. If such a procedure would, indeed, lead to cellular life, this would also afford the best demonstration that life is an emergent property, a property arising from the interaction of components which are *per se* not living.

At any rate, it seems proper to end this article with the statement (see also a recent Editorial by *Philip Ball* [72]) that the enterprise of constructing minimal semisynthetic cells will be an exciting perspective of the 21st century.

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*Received December 17, 2001*