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Area review

An interpretive review of the origin of life research

DAVID PENNY
Allan Wilson Center for Molecular Ecology and Evolution, Massey University, Palmerston North, New Zealand; (e-mail: d.penny@massey.ac.nz)

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Abstract. Life appears to be a natural property of matter, but the problem of its origin only arose after early scientists refuted continuous spontaneous generation. There is no chance of life arising 'all at once', we need the standard scientific incremental explanation with large numbers of small steps, an approach used in both physical and evolutionary sciences. The necessity for considering both theoretical and experimental approaches is emphasized. After describing basic principles that are available (including the Darwin-Eigen cycle), the search for origins is considered under four main themes. These are the RNA-world hypothesis; potential intermediates between an RNA-world and a modern world *via* the evolution of protein synthesis and then of DNA; possible alternatives to an RNA-world; and finally the earliest stages from the simple prebiotic systems to RNA. The triplicase/proto-ribosome theory for the origin of the ribosome is discussed where triples of nucleotides are added to a replicating RNA, with the origin of a triplet code well-before protein synthesis begins. The length of the code is suggested to arise from the early development of a ratchet mechanism that overcomes the problem of continued processivity of an RNA-based RNA-polymerase. It is probable that there were precursor stages to RNA with simpler sugars, or just two nucleotides, but we do not yet know of any better alternatives to RNA that were likely to arise naturally. For prebiotic stages (before RNA) a flow-reactor model is suggested to solve metabolism, energy gradients, and compartmentation simultaneously – thus the intense interest in some form of flow reactor. If an autocatalytic cycle could arise in such a system we would be major steps ahead. The most likely physical conditions for the origin of life require further clarification and it is still unclear whether the origin of life is more of an entropy (information) problem (and therefore high temperatures would be detrimental), rather than a kinetic problem (where high temperatures may be advantageous).

Is the origin of life solvable scientifically?

Consider the following experiment. Take cysts (dried fertilized eggs) of the common brine shrimp (*Artemia*) – a multicellular Crustacean of considerable complexity. Place the cysts in a small capillary tube, freeze them, transfer them to liquid helium at a temperature below 2.2 °K (less than −270 °C) and leave 6 days at this almost absolute zero temperature. Then slowly warm them up to room temperature and see whether the cysts survive, hatch, eat, grow, develop (with a functioning brain and nervous system) and reproduce. They do (see Skoultchi and Horowitz 1964).

At absolute zero, although the information about the positions of atoms within chemicals is retained, virtually all prior information about electron velocity (speed and direction) and distribution in energy orbitals is lost.

Therefore, because of the brine shrimp example, life appears to be a natural consequence of a specific chemical organization of matter; in principle the origin of life is solvable. It should be possible to recreate life, but it certainly will not be easy. It should only require clutches of chemists, tapping at typewriters, designing amino acid and nucleic acid sequences to synthesize and assemble inside a membranous vesicle, along with the right combination of ions and small molecules. When done right, the new organism will start growing and reproducing – to a biologist this is *coito ergo sum*.

There were alternatives, for example, that Life (with a capital L) had properties additional to the orderings of its chemicals. It is logically possible that even when all the chemicals were arranged in the correct order, that some special combination of rotational and vibrational velocities, plus distributions of electrons in orbitals, had to be given externally; only then did this combination become self-sustaining Life. This would be a scientific version of a 'vital principle' of Life. A useful analogy (Morowitz, pers. comm. 1962) is a jellyfish that has a circular nerve around its periphery. Stimulating the nerve sends an action potential both ways around the jellyfish, which cancel each other out when they meet. But it is relatively easy to prematurely stop one action potential (a temporary lowering of nerve temperature with a piece of ice will do). The other potential just keeps rolling along; round and round, round and round the circular nerve. The basic state was there in the structure and physiology of the nerve of the jellyfish, but it needed an external stimulus to get the potential rolling. Perhaps that was what Life needed, all chemicals in the right arrangement, then a special kick-start to get Life going. However, if this were the case, compare our brine shrimp and jellyfish experiments. If jellyfish could survive to absolute zero and back, the rolling action potential would have been lost; the system would only go back to the non-living ground state before receiving the kick-start.

Thus the actual experiment of taking *Artemia* down to around absolute zero, with it still surviving, eliminates some alternative models – distinguishing life from Life. To be more cautious, the experiment does not 'prove' that life is completely defined by chemicals and their ordering; that requires actually assembling all the chemicals in the proverbial test-tube. What we conclude from the Morowitz experiment is that it is reasonable to aim to recreate life *de novo*. In contrast, there are many possible experiments that are not sensible to try, such as training a cow to jump over the moon. And in demonstrating that life appears to be a property of organized matter, we have not even given a formal definition of life – though it will be helpful later to look at the components we expect in a living system.

Early scientists find the problem

It was part of European indigenous knowledge that life continued to arise spontaneously. For example,

'*Captain Lancaster, in his voyage in 1601, narrates that on the sea sands of the Island of Sombrero, in the East Indies, he 'found a small twig growing up like a young tree, and on offering to pluck it up it shrinks down into the ground, and sinks, unless held very hard. On being plucked up, a great worm is found to be its root, and as the tree groweth in greatness, so does the worm diminish; and as soon as the worm is entirely turned into a tree it rooteth in the earth, and so becomes great. This transformation is one of the strangest wonders that I saw in all my travels: for if this tree is plucked up, while young, and the leaves and bark stripped off, it becomes a hard stone when dry, much like white coral: thus is this worm twice transformed into different natures. Of these we gathered and brought home many*.' Underlining added, (extract from Charles Darwin, Voyage of the Beagle. 1840, p. 106)

Captain Carpenter (in 1601) observed changes between 'animal, vegetable and mineral'. The indigenous view in Europe (and almost certainly in other parts of the world) was that 'kinds' or 'forms' of plants and animals continued to arise by spontaneous generation. It was accepted that there was continued interconversion between living forms, and between living and non-living matter. After all, if a caterpillar could turn into stone (a chrysalis!), which then turns into a butterfly, why couldn't a barnacle turn into a goose, or a plant into an insect? The Old Testament provided support for continued spontaneous generation

'*And God said, Let the earth bring forth grass, the herb yielding seed, and the fruit tree yielding fruit after his kind whose seed is in itself, upon the earth: and it was so*',

'*And God said, Let the earth bring forth the living creature after his kind, cattle, and creeping thing, and beast of the earth after his kind, and it was so*'. Genesis 1:11 & 24

This was interpreted as continued spontaneous generation, spontaneous generation did not occur just once thousands of years ago, it happened again, and again, and again. Indeed it was a heresy in Europe during the Middle Ages not to accept that spontaneous generation still continued, a modern creationist would have been a medieval heretic. To deny the Creator the power to make life continuously would be an affront, fully justifying being burnt at the stake (though presumably one would have to be without sin in order to light the first match). Similarly, there is no reference here to 'species', just to 'kinds' of plant or animal. The concept of a biological species did not develop until the end of 17th century, and the above translation into English authorized by King James is from the beginning of that century.

One recipe for the spontaneous generation of life is, ''*take grains of wheat and a sweaty shirt, place them under a box in a field, and leave for three weeks*''. The vapors of the shirt, together with the grains of wheat, would generate live mice – which turned out to very similar to ordinary mice! Given the caterpillar – chrysalis – butterfly example above, then couldn't human tissue make the

much simpler transformation into intestinal worms, or to liver flukes? Similarly, eggs of the barnacle goose could not be found and it was assumed that some invertebrates on the rocky shore turned into geese in the spring. The name barnacle is now associated with these marine invertebrates. In some other places it was assumed that melons turned into lambs; therefore (where meat was banned on religious grounds) eating lamb on Fridays was acceptable. In Paris, eating a particular bird as a fish was allowed during Lent, and the legend of the geese from barnacles was used to justify this. An early naturalist (John Ray, see Raven 1986) asked visitors to Paris to send him skins from the bird, and identified it as a sea-duck, the Scoter. Unfortunately (for eating meat on Friday) its reproduction was known.

It took centuries of careful work (Farley 1977, see also Oparin 1957) to move beyond European indigenous knowledge, to demonstrate that there was a continuity of like forms (species) over many generations. At the same time spontaneous generation was being questioned (and species were being considered stable), early chemists were denying that chemical elements could be transmuted (lead could not be transmuted into gold, for example). Thus there was a similar movement in scientific thought:

denying continued spontaneous generation (non-living to living);
denying transformation between chemical elements (non-living forms of matter); and
denying transmutation between living forms (species therefore having a permanence through time).

Thus we end up with the conclusion that early scientists discovered the problem of the origin of life, and that it was an important scientific advance to be able to reject continued spontaneous generation.

Statistical versus mechanistic (actualist) reasoning

Having concluded that the ultimate origin of life is a scientific problem, it is time to consider two fundamental approaches, the statistical and the mechanistic (actualist). The late Fred Hoyle (physicist, broad thinker, and science-fiction writer) only considered the statistical approach and claimed that life was just too complex to arrive all at once; therefore life had to arise elsewhere in the universe and later be transported to earth – pangenesis. To him, it was just too improbable for even a single protein of only 100 amino acids to arise all at once and in the right order. If this probabilistic approach were the only one possible, then Hoyle's calculations show it is too hard (even given the Horowitz experiment on taking *Artemia* cysts down to absolute zero) for life to arise on Earth 'all at once'.

However, no researcher accepts this probabilistic approach; consider an analogy of a simple experiment that starts with an open beaker of bright blue solution of copper sulfate left on the bench. After coming back from vacation we find that the solution has disappeared, and that the beaker contains crystals

of copper sulfate. We could sit down and calculate the probability that at one instant in time all the water molecules evaporated, and that simultaneously all the copper and sulfate ions lined up in just the right configuration for the crystals. Yes, impossible.

Instead, we expect a process of millions of intermediate steps, each incremental step following known scientific principles. We could measure the chemical activity of water in the solution and of the atmosphere, and calculate the rate of evaporation through time. We could measure the solubility of copper sulfate in water at different temperatures; study the process of crystal formation in a range of compounds, etc. Similarly, with the origin of life we need to look for intermediate steps, and the principles behind the transitions; this is standard scientific approach to understanding events in the past was pioneered by James Hutton, Charles Lyell and Charles Darwin. Basically, we aim to explain the past by, in Lyell's phrase, 'causes now in operation'. We call this the *Principle of Continuity*.

Before leaving this analogy it is important to point out that the copper sulfate example is fully reversible. If we start with the crystals, and place a large volume of water adjacent to the beaker inside a closed container, water would diffuse back to the lower chemical potential of the crystals. They would slowly take up water, dissolve, and eventually give a similar solution to the one we started with. This is full reversibility, but is stronger than we require for a scientific explanation. Reversibility in the sense of physical chemistry just requires that a process can be broken down to large numbers of microscopic steps, each of which is reversible. This is the *Principle of Microscopic Reversibility* and it is also a standard scientific approach. It is this limited use of weak reversibility, or microscopic reversibility, that is equivalent to the 'continuity' used in the previous paragraph. In the early stages of the origin of life each step will be a normal chemical one; later it will probably be a genetic mutation.

Introduction

Having now got to the beginning of this analysis of the origin of life, the preintroduction establishes the problem as appropriate to scientific study, shows how the problem arose from careful observation and experiment, and shows that no simplistic probabilistic approach will help. At this point some form of definition of life would be useful, but because that is impossible to everyone's satisfaction, it is preferable to give the components that appear necessary. These are:

An energy source (an energy gradient),
Basic biochemistry (small molecules and reactions driven by the energy gradients),
Organization (membranes, compartmentation and separation from the external environment),
Self-reproducibility (genetic inheritability, information transfer, evolvability).

Clearly, not all four can apppear simultaneously or we would be appealing to the 'statistical' (all at once) approach to the origin of life. We expect (as in any evolutionary system) a long series of intermediate forms, and therefore it is arbitrary to some extent which step we call 'living'. Here I will restrict the term 'life' to a system that shows all four of the above features, and use 'prebiotic' to the intermediate (but essential) stages. Some of the controversial claims about the 'origin of life' are really about prebiotic, intermediate stages. Fine, they may be essential stages, but they still lack some components required for a full living and evolvable system.

You could protest that these four components focus on 'life as we know it' – couldn't there be other forms of life, say, based on silicon? Computers may eventually takeover. Certainly, slight variations on existing life are easy to imagine. At the simple end of the scale, a slightly modified form of life – yeast cells coding for 21 amino acids (rather than the 20 all current life uses) have already been made (Chin et al. 2003). Such possibilities are easy to envisage, and can probably be extended step by step until there are quite a few differences from standard life. But it is still hard to imagine and experiment with completely different forms of life and so I will consider both

general principles which apply to many possible forms of living systems, and
our specific example of life, amino acids, ribonucleotides, polymerization, membranes, etc.

However, if we can solve the origin of our form of life then it will be easier to consider others.

Concurrently, I will usually give questions with a yes/no answer, simply to help direct a researcher to productive areas of research. We already have had two such questions; the life/Life alternatives, and the statistical versus mechanistic (actualist) approach. One could protest that forcing binary choices is unrealistic for such a complicated process; but the approach is for convenience. We can always subdivide a search strategy into binary choices (even if it is not the optimal search path), and we can always reformulate a question. An example we consider later is whether life is more likely to start at high temperatures at the bottom of the ocean. After rejecting a 'hot start' at high temperatures (see Moulton et al. 2000) we realize that there are at least two questions about black smokers;

the optimal temperature for the early origin of life, and
whether very high pressures are advantageous (rather than one atmosphere).

A third could be whether life could start in a dispersed aqueous phase, or whether it needs highly concentrated solutions at some stages. New questions can always be added. Thus binary choices are not limiting; we never consider all possible questions.

My approach here is a personal one in the sense that my aim is to give an analysis of critical questions, together with approaches that may allow their

solution. The coverage includes both purely theoretical studies (such as the amount of information that can be transmitted for a given error rate) as well as the better known experimental results. This combination of theoretical and empirical studies is unfortunately not common. No doubt experimentalists often think theoreticians ignore important experimental information. Similarly, theoreticians find it hard to believe that experimentalists are so slow in following up their brilliant ideas. For example, the early work of Eigen and Schuster (1977) indicated to many theoreticians that studying RNA offered the only possibility for real progress. This was strongly reinforced when Reanney (1982) showed that the Eigen/Schuster concepts explained many features of RNA viruses. However, it seemed a very long time before the experimental work on RNA systems started. It is important to increase dialogue between the two groups.

My aim here is not to cite every last reference, but to identify productive areas of research and to look for others where current assumptions may be hindering progress. There are excellent reviews of different aspects of origin of life studies, and they will give more detailed sources. The origin of the earth and the solar system is well covered in the recent book by Conway Morris (2003) and is not part of this overview. Similarly, the discovery of water (ice) on Mars (Bibring et al. 2004) opens possibilities for the future, but until we have data for the extraterrestrial origin of life, it is outside our scope.

Principles or guidelines

Physical and chemical principles, including kinetics, catalysis, thermodynamics and quantum mechanics (including energy sources and entropy) are taken for granted, as is the parsimony principle of seeking the simplest theories that are effective. Similarly, natural selection is also assumed, though some of its consequences are given in more detail below. It is sufficient to say that natural selection is the consequence of the combined effects of genetic and ecological/populational processes. Ecology includes the potential for exponential increase of numbers and competition for limited resources. Genetics gives heritable variability, some of which may increase the probability of survival and reproduction. The fundamental question, as discussed in Penny and Phillips (2004) in a different context, is whether the processes we can study in the present are sufficient to explain the origin of life. Given the above principles, the points to emphasize here are as follows.

Immediacy/current utility

There is no foresight or planning for future utility, this is basic to darwinian evolution. One of the strongest limitations of such a mechanism is that it can't

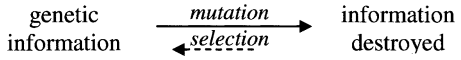
evolve anything 'because it will be useful in the future' – it has to be functional now. In theory, very slightly deleterious factors can get incorporated (fixed) into the genome, especially with small population sizes (Lynch and Conery 2003).

Continuity/microscopic reversibility

Basically this is 'no miracles'. As mentioned in the copper sulfate crystal example, we require each small step to be reversible.

Eigen limit (Information retention/loss)

Mutation loses the original sequence information; selection can favor survival and/or replication of some sequences over others. There is a balance between loss of information through mutation, and retention by selection (see Eigen 1992). In our terminology, the Eigen limit is the maximum length of sequence that can be maintained for a given error rate and strength of selection.



The higher the error-rate, the shorter the sequence length (amount of information) maintained for a given selection value. Conversely, the higher the selection the more information that is retained for a given error rate. However, in a purely chemical system there is a limitation on the strength of selection where all sequences could be copied. To a first approximation, we require less than about one mutation per replication in order to maintain the information (Eigen 1992). With more mutations than this we go into 'error catastrophe' or 'mutational meltdown' and the information is lost. A central problem is that shorter sequences can be copied faster (Biebricher and Gardiner 1997), and this erodes the information that can be retained. The consequences of the Eigen limit are particularly acute in early stages of a genetic system when error-rates are relatively high, and only short sequences can be maintained.

The Darwin–Eigen cycle

Under the right circumstances, selection can favor increased replication fidelity, which in principle allows a positive feedback cycle. Increased accuracy permits an increase in genome size, therefore additional genes can be coded, which potentially increase replication accuracy even further. We call this the Darwin–Eigen cycle (Poole et al. 1999) and Figure 1 shows a simple expression of this.

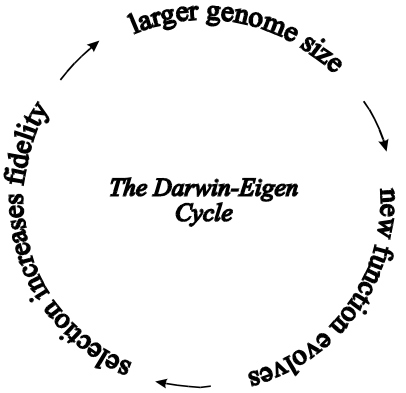


Figure 1. The Darwin–Eigen cycle. A positive feedback loop where an increase in replication accuracy allows longer sequences to be retained (that is, increases the Eigen-limit) which in turn allows more genes to be coded, allowing selection for a further increase in replication accuracy.

Universality through time

Principles were as relevant in the past as they are in the present; indeed some, such as the Eigen limit on genome size, were probably more critical in the past when error rates of replication were higher. Other problems such as keeping good combinations of genes together (avoiding random gene loss) and preventing parasitic genes entering the system are acute in early systems. It is often assumed that the advantages of recombination of genes through sexual reproduction was a new invention in eukaryotes, but linking advantageous genes together (whilst still allowing recombination) would have had major advantages as far back as the RNA-world (see later).

No reverse takeover by RNA

One principle we have used (Jeffares et al. 1998) is based on the observation that proteins are several orders of magnitude faster, and more specific in their reactions, than RNA catalysis. For example, RNA-catalyzed reactions may have turnover times of several minutes (see Table 1, Jeffares et al. 1998) whereas protein-catalyzed reactions are often 10,000 to 100,000 times faster. Thus we do not expect RNA to take over a catalytic role that proteins are already doing. This allows a direction of change for some early events (see later). Under this principle, RNA may develop new functions, including regulatory functions, amongst modern organisms; it is just that it does not takeover catalytic functions from proteins.

Autocatalytic cycles

A relatively new mathematical analysis (Hordijk and Steel 2004; Mossel and Steel, 2005) shows that autocatalytic cycles can occur with reasonable frequency in a mixture of chemicals that are potential substrates and/or catalysts. In an overly simplistic cycle, molecule A could catalyze the formation of B, B of C, and C of A. In reality many more reactions and side reactions could occur. In biochemistry the focus has been on macromolecules (especially proteins) catalyzing reactions. However, weak catalysis is a property of many small molecules and metallic ions, albeit with much lower rates. For example, we think of the break down of hydrogen peroxide (H_2O_2) to water and oxygen being carried out by the protein enzyme catalase. However, catalase has a heme molecule as a coenzyme (cofactor) that is involved in this chemical reaction, and the heme molecule by itself (without the protein) is a weak catalyst of the reaction. Again, the metal at the active center of heme, Fe^{2+}, is also a catalyst, even though even weaker than heme. Thus small molecules and ions are also catalysts. Autocatalytic cycles work in mathematical equations (Hordijk and Steel 2004) and are an important feature for future work.

These principles are all important in the search for a good explanation for the origin of life, and equally perhaps for rejecting others that rely on untestable and unlikely events. For a good explanation we require known reactions and mechanisms, a plausible series of intermediates, and ultimately testable predictions. In geology, the approach of explaining the past in terms of causes or mechanisms still available we call actualism (Camardi 1999). With those principles in mind it is time to consider some of the many steps from an early earth to living systems.

Chemical and biological starting points

It is standard to divide origin of life studies into the forwards and backwards directions.

Bottom up (chemical) approach

The forward (chemical, or bottom up) approach starts with the chemicals and conditions existing on the early earth (or elsewhere if you are of that persuasion). It studies the small molecules that would form, and how they might polymerize into larger molecules. This approach goes from the simpler to the more complex, towards a simple living system. It is more the domain of chemists and geophysicists. Topics include the following:

Conditions during the early stages of formation of a planetary system,
Detecting small organic molecules in asteroids or in gas clouds in space – towards exobiology,
Origin of small organic molecules under possible prebiotic conditions,
Formation of order (phase separation) and membranes,
Polymerization of nucleotides and/or amino acids,
A preRNA-world (which could have been quite extensive).

Top down (biological) approach

The backwards (biological, or top down) starts with life as we know it and simplifies it stepwise. What is the simplest system that could have evolved into 'life as we know it', using known mechanisms? For example, hemoglobins are not useful before high oxygen concentrations occurred in the atmosphere, so we delete this protein from our proposed early cell. This is still within our Principle of Continuity because we know from gene duplication and the subsequent divergence of function of the copies that we could recover hemoglobin from other proteins. This top-down approach is the territory of biologists and paleontologists. Subject areas for the working-backwards approach include:

Geology and the earliest fossils (though are the very earliest fossils even DNA-based organisms),
The Last Universal Common Ancestor (LUCA),
Establishing a DNA world, the origin of DNA with its takeover of information storage from RNA (except for RNA viruses),
A ribonucleoprotein (RNP) world (protein takeover of most catalysis from RNA),
An RNA-world (omitting DNA and proteins) with RNA catalysis and information storage,
RNA viruses as exemplars of the RNA-world. RNA viruses have both high rate of replication error (about a million-fold higher than DNA organisms) and consequently have short and/or fragmented genomes. Reanney (1982) initially pointed out how RNA viruses illustrated principles such as the Eigen limit. However, they will not be discussed further, it is sufficient to say that the properties of RNA viruses are fully in agreement with theoretical predictions.

Having given the forwards and backwards approaches, and for reasons that will become apparent, I will use neither – but jump straight to a potential midpoint, the proposed RNA-world. This is my Theme one, and illustrates the wide range of phenomena explained by the RNA-world model. Theme two then outlines a plausible set of stages to get from an RNA-world, via proteins, to DNA-based organisms, and thus to modern living forms. This covers the backward direction from extant organisms. Theme three considers alternatives (or variations) to RNA – how small or large is the target for chemical simu-

lations (the forwards reaction – Theme four). Without an RNA-world target it is not at all obvious how to use to choose between competing ideas on the early stages in the origin of life. But it is already a complex target.

Theme 1 – The RNA-world

Which came first – the chicken or the egg, protein or DNA?

At one time, the separation of function between DNA and proteins was a major stumbling block in the search for the simplest living system. Consider a DNA molecule. It carries information that can be replicated and passed on to descendants, but does not catalyze any metabolic reactions in the cell. It is a boring molecule from the viewpoint of catalysis. It is like a book in a library – it may contain information but it does not do anything; someone has to read the book before its information is useful.

On the other hand, proteins catalyze thousands of reactions with great speed and high precision. But proteins apparently do not directly reproduce the information in their own sequences; any change to a protein's sequence is not inherited. This left the apparent problem of 'which came first' – DNA that carried information but does not 'do' catalysis in modern cells, or proteins that carried out reactions but could not reproduce. This gave a classic, 'which came first, the chicken or the egg?' problem. Avoiding this problem has a delightfully simple solution.

The RNA-world: no protein, no DNA – no chicken, no egg.

The RNA-world hypothesis is conceptually simple: at one stage during the origin of life RNA both stored information and was the main macromolecule carrying out catalysis. In other words, in the origin of life

RNA preceded (genetically encoded) proteins, and
RNA preceded DNA.

The theory only considers the genetic (information) aspects of biology. It does not directly consider energy sources, the status of proto-metabolism, nor cellular organization. Amino acids are very likely to have been present and may have formed short peptides. However, their sequences would not have been genetically-coded in the modern sense. I will restrict the term 'protein' to cases where the amino acid sequences are heritable and (after Fox and Dose 1972) use 'protenoid' for peptides whose sequences are not inheritable (but still may have been very important). Returning to RNA, the following list gives the main roles for RNA (and its metabolic components). The first ones were discovered early, and the Introduction of Gesteland and Atkins (1993) covers these early discoveries. However, new roles for RNA keep being discovered in what is a

very active field. These functions (Jeffares et al. 1998; Meli et al. 2001; Joyce 2002) include:

RNA (like DNA) carries genetic information (as messenger RNA [mRNA] or as the genome in RNA viruses);
RNA has both catalytic and structural roles in ribosomes (rRNA) - it is both the core structure of the ribosome, and catalyses the poloymerization of amino acids into proteins (Steitz and Moore 2003);
RNA (as tRNA) translates between the mRNA code [triplets] and amino acids;
Ribonucleotides (building blocks for RNA biosynthesis) are precursors for deoxy-ribonucleotides (building blocks for DNA biosynthesis) – thus ribonucleotides probably existed first. Indeed it is a difficult reaction, requiring complex proteins, to get from ribose to deoxyribose nucleotides (Poole et al. 2001);
RNA is a primer for DNA synthesis (when a new section of DNA is synthesized, an RNA primer (which is later replaced by DNA) is required to initiate DNA synthesis – in this sense, DNA cannot be replicated without RNA);
RNA (as ribozymes, from 'ribo' and 'enzyme') has many catalytic roles, especially in processing RNA itself (Doudna and Cech 2002). There is a cascade of RNA processing effects; snRNA (small nuclear RNAs) act on snoRNA (small nucleolar RNA) which acts on rRNA (ribosomal RNA) – RNA processing a second RNA which processes a third RNA;
Most organic coenzymes [cofactors] of protein enzymes have ribonucleotide components (e.g. FAD, NAD, NADP, pterins and coenzyme A) and these coenzymes are essential for proteins carrying out the chemical reactions. For example, FAD is an abbreviation for flavin adenine dinucleotide. It has a classic dimer ribonucleotide structure; supporting the suggestion that these essential coenzymes predate protein enzymes;
RNA has many regulatory roles within cells [such as RNA interference (RNAi, Novina and Sharp 2004) and ribo-switches (Winkler et al. 2004)] where RNA molecules detect and respond to the presence of specific molecules in the cell;
The RNA-world hypothesis largely avoids the problem that amino acids exist in two mirror images (D and L) that would inhibit growth of consistent 3-D structures in pre-biological molecules. (It is likely that the L-form of ribose determines the D-form of amino acids during protein synthesis (Tamura and Schimmel 2004), though this still leaves the problem of isomers of ribose.)
Eukaryotic genes have an exon/intron structure where exons (usually) code for proteins and introns are excised and (usually) discarded. A very complex RNA/protein spliceosomal machinery exists to remove introns and the RNA-world accounts for this complexity. (It is difficult to see advantages in developing introns and the splicing apparatus if they were not already present in the earlier RNA-world; this is the introns-first theory, Poole et al. 1998. At

a minimum, introns indicate how an RNA-world could be organized even if they were lost and then later regained in eukaryotes.)

The synthesis of protein is carried out by the ribosome, which is very much an RNA machine – all the central parts that carry out the synthetic reactions are RNA (Steitz and Moore 2003). Conversely, proteins carry out DNA synthesis, including the difficult conversion of RNA precursors (ribonucleotides) to DNA precursors (deoxyribonucleotides). This reduction of ribose to deoxyribose involves a free-radical reaction (Poole et al. 2001) and is well beyond the catalytic repertoire of RNA as currently known. Thus, in an important sense, RNA makes proteins, and proteins make DNA. Although not central to the RNA-world hypothesis it is now assumed that protein preceded DNA, giving the sequence

$$\text{RNA} \rightarrow \text{protein} \rightarrow \text{DNA}.$$

It is this version that I use here. It is very important to note that there is no claim that RNA was the 'first' self-replicating molecule; just that genetically, RNA preceded both protein and DNA. Indeed, possible precursors of RNA are an important topic that comes later. Similarly, there must have been a complex cell and metabolism by the time of the RNA-world; these aspects are discussed later.

There are many functions of RNA in modern organisms, which ones might date back to an RNA-world? For this question we use the principle outlined earlier that, because protein is more effective in catalysis than RNA (in terms of both rates and specificity), we do not expect RNA to 'take back' a role that proteins were doing. Certainly, RNA functions can increase in a cell by normal evolutionary processes such as gene duplication, followed by divergence of one of the copies. Our restriction is just that RNA would not takeover a catalytic role from proteins. To identify relics from the RNA-world we (Jeffares et al. 1998) looked for functions that were central to metabolism (and therefore likely to be old), were widespread in different organisms, and were catalytic. The list (Jeffares et al. 1998) was quite impressive, and growing.

In vitro *evolution of RNA catalysis*

The simultaneous discovery (by Altman and Cech, see Gesteland and Atkins 1993) that RNA, even in the absence of proteins, catalyzed reactions was quite dramatic in that it was unexpected by the general biochemical community. Nevertheless, many theoretical biologists had favored RNA as an early functional molecule – even in the absence of direct biochemical evidence for its catalytic activity. It was not that biochemists were against RNA being important in the early origin of life; biochemists studied 'real' reactions that occurred in the present, not imaginary reactions that might have existed in the past. However, once reactions catalyzed by RNA were discovered, the way was

open to evolve ribozymes artificially through repeated rounds of synthesizing RNA (with partial randomization) and selection. This is evolution *in vitro*.

The process of *in vitro* evolution is conceptually simple.

Synthesize billions of variants of a nucleic acid sequence (this is straightforward by using mixtures of the four nucleotides for at least parts of the synthesized sequence).

Select any variants that may have even a weak function. Passing the synthesized molecules through an appropriate column is a frequent method; those molecules retained on the column can then be eluted later by a more concentrated solution).

Copy the selected RNA, often under conditions where the polymerase is 'error-prone'. This amplifies the molecules selected in the second step, but also can generate new variants differing in only a small number of mutations.

The second and third steps are repeated until RNA molecules with the desired activity are found. Calling these 'evolved ribozymes' distinguishes them from those occurring naturally. There are many variants on the basic method, but all have the cycle of starting with large numbers of variants, selection, copying, and repeating the cycle of selection and synthesis. Because of its greater chemical stability, most start with DNA, copy it to RNA, carry out selection on the RNA molecules, and then use a reverse transcriptase reaction to copy the selected RNA back to DNA for further rounds of replication and selection.

Only two applications of *in vitro* experiments are mentioned here, evolving an RNA polymerase, and demonstrating that protein coenzymes can function with ribozymes. For the first application, it is essential for any RNA-world theory that RNA can be an RNA-polymerase, and there is reasonable success in this respect (Lawrence and Bartel 2003). We will return to this reaction later under the origin of the ribosome with a suggestion how to improve the existing RNA-based RNA-polymerases. Turning to coenzymes, Jadhav and Yarus (2002) summarize a large number of evolved ribozymes that use (ribonucleotide) coenzymes (FAD, NAD, etc.), which are now only associated with protein enzymes. Overall, the results are a real success for the RNA-world theory; RNA can catalyze many chemical reactions.

Initially it was assumed that RNA was inherently better than DNA as a catalytic macromolecule – and that explained why there were no naturally occurring deoxy-ribozymes. 'It was the catalytic role of the additional hydroxyl (-OH) group on ribose that made RNA such a good catalyst', was a common argument. This was a typical *post hoc* explanation in biology; find something in nature and dream up an explanation. In this case the 'explanation' is wrong as a generalization. Artificial DNA ribozymes can be created, and are at least as effective as their RNA counterparts – and are more stable. For example, Chinnapen and Sen (2004) give an important case of a deoxy-ribozyme that, in the presence of light, repairs thymine dimers in DNA. Thus the existence of an extensive set of naturally occurring RNA (not DNA) ribozymes is important in itself and is evidence for their antiquity, relics of the RNA-world. Nowadays,

starting from modern biochemical knowledge, and given the higher stability of DNA, we might 'design' at least some ribozymes from DNA. A recent review on *in vitro* evolution of both RNA and DNA is Joyce (2004).

The RNA-world hypothesis has been an outstanding success. It started with rather vague suggestions in the 1960s and 70s that RNA must have been important early (see Gesteland and Atkins 1993, Chapter 1). This was followed by the quantitative work of Eigen and Schuster (summarized in Eigen 1992) who established some fundamental principles. In the style of Sherlock Holmes, when everything else is impossible, we have to accept the only alternative left – even if it appears unlikely. In this context, the discovery of RNA catalysis was almost necessary! In retrospect there was a conceptual problem – the early focus was on DNA as the information storage molecule and on proteins as the catalysts. RNA was considered just an intermediate. All that has changed and the slogan is now 'RNA can do [almost] anything'. But how do we get to an RNA-world, and how do we get from an RNA-world to the protein and DNA worlds?

Theme 2 – From the RNA-world to proteins and DNA

A strength of the RNA-world hypothesis is that a plausible step-by-step model can be developed, first for the origin of the genetic code, which then extends the model to the evolution of protein synthesis, and later the evolution of DNA. These steps allow the take-over of most catalysis by proteins, and most information storage by DNA. On our model, the basic driving force is the positive feedback loop of the Darwin–Eigen cycle (Figure 1). There are successive improvements in the accuracy of replication followed by increased information storage, allowing increased replication fidelity, and so on (Figure 2). The model has many intermediate steps, each following our principles of continuity, weak reversibility, and immediate utility.

From our earlier observation that 'RNA makes protein, and protein makes DNA' the reader will predict that the origin of protein synthesis must be the first major step from an RNA-world. This leads to an RNP-world (RiboNucleoProtein), a combination of RNA and protein. Proteins have a superior catalytic ability to RNA in both rate and specificity (both substrates and products). For example, RNA-catalyzed reactions may have turnover times of several minutes (see Table 1, Jeffares et al. 1998) whereas protein-catalyzed reactions are often 10,000 to 100,000 times faster (and more specific). This comparison is a little unfair in that proteins increasingly took over catalysis involving smaller molecules and so were less limited by the diffusion times of the reactants onto the macromolecular catalyst (Albery and Knowles 1976). (Neglecting solvation affects, the average rate of diffusion in solution is inversely proportional to the square root of the molecular weight.) However, this protein takeover reflects the advantage of improved catalysis when catalysis

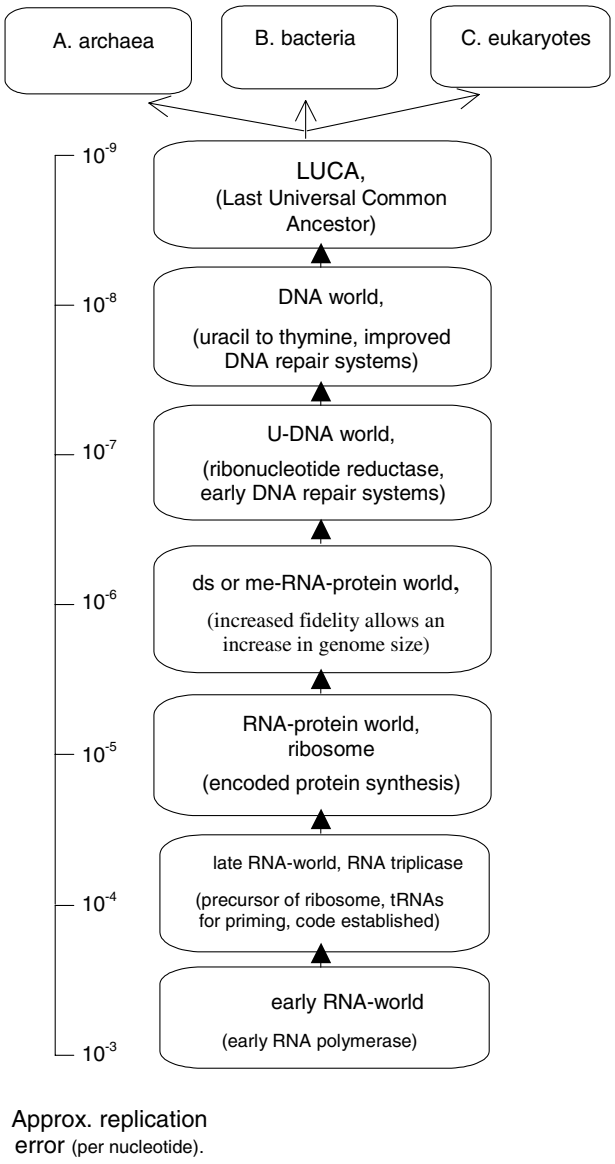


Figure 2. The Darwin–Eigen cycle in practice. A series of steps allowing a succession of increases in replication accuracy followed by an increase in the size of the genome. Every time the accuracy of replication is increased, it allows in principle in increase in the amount of information coded. This, in turn, allows for the selection of an increase in accuracy, which allows more coding, which allows selection for more information, and so on. (See the Darwin–Eigen cycle, Figure 1.)

(rather than diffusion) is limiting. Nevertheless, RNA would have retained the coding (information storage) role in an RNP-world. However, the major difficulty is getting from an RNA-world to encoded protein synthesis.

The main problem is that the origin of ribosome and of the triplet code for amino acids cannot have arisen 'for' protein synthesis – the usual explanation. The ribosome is a huge macromolecular complex and there are many steps leading up to the synthesis of the peptide bond that joins amino acids. There is no way that a ribosome could have evolved *de novo* in a single step and meet our guidelines of continuity (no miracles). It is impossible on an incremental model that a very complex structure could evolve 'for' something that does not yet exist.

A standard evolutionary explanation is that a new feature (in this case, the triplet code and the ribosome) evolved for a different purpose, and was later recruited or co-opted to a new role, in this case into protein synthesis. The classic examples are duplication of a single gene with one copy being recruited into a new function, one copy retaining the original role. A typical example is a gene for an early hemoglobin molecule being duplicated, one copy becoming α-hemoglobin, the other β-hemoglobin. Could something similar have happened with the ribosome? We must consider a prior function for a proto-ribosome, a function that allowed it to be co-opted into protein synthesis. I will discuss the origin of the ribosome in some detail to use it as an example of developing a testable evolutionary hypothesis for a very complex process.

At first sight there are two major problems; there is no obvious relic in modern cells of an RNA-based RNA-replicase, nor any obvious function for a proto-ribosome. However, there is a simple and powerful solution to both problems; namely, the proto-ribosome was initially involved in RNA replication, and later was recruited into protein synthesis (Poole et al. 1998; 1999; see also Gordon 1995). Indeed, there must have been a high-accuracy RNA polymerase in the RNA-world. Because of the Eigen-limit (around one error per replication) RNA had to be copied with a high fidelity – at least as accurate as modern protein-based RNA polymerases. Such a proto-ribosome recruited into protein synthesis could be a large RNA complex that included the following (see Table 1 and Figure 2 of Poole et al. 1998).

A total length of ribosomal RNA around 4500 bp. This is similar to current ribosomes – once proteins were available there appears little advantage in increasing the length of the RNAs.

The proto-ribosome recognizes and attaches to a single stranded RNA (ssRNA) which is being replicated (mRNA is the equivalent of this ssRNA in the modern ribosome).

tRNA-precursors that donate triplets of ribonucleotides to replicate the ssRNA. This reverses the role of the anticodon and the amino acid in modern tRNAs. Currently, tRNAs are donors of amino acids identified by the triplet (anticodon) on the tRNA – the triplet is kept, the amino acid is added to the protein. On our protoribosome model, the triplet was added to the growing RNA chain, and the amino acid on the tRNA (which aided in recognition and specificity) was retained.

A ratchet mechanism that moves the ssRNA being replicated through the proto-ribosome in steps of three nucleotides (this explains why the amino acid code is three nucleotides long, see later).

Adding ribonucleotides three at a time is expected to be more accurate in RNA polymerization than adding one at a time, I will come back to this later. Our model is that the proto-ribosome was an RNA-polymerase ribozyme that added triplets of nucleotides to the growing RNA molecule (Poole et al. 1999). We call this a 'triplicase'. To a biochemist, the triplicase has properties of both a ligase and a polymerase. A ligase because it joins (ligates) two RNA molecules; the growing chain plus the triplet. A polymerase because it repeats the operation sequentially along the new RNA strand (a ligase is usually involved in just a single reaction on a particular molecule).

Why triplets? Because it gives the length of the code, is a superficial answer. The origin of the triplet code is a similar problem to the origin of the ribosome. There is just no way under a Darwinian model that a complex code could have evolved 'for' something that did not exist – namely encoded protein synthesis. An alternative and frequent answer is evolutionarily obscene – the code is a triplet 'because' it allows 20 amino acids to be coded. This requires long-range planning and forethought! Thus the origin of the code must be broken into two questions.

Why three nucleotides?

How did our existing triplet code for amino acids arise?

In the above model, only the 'three nucleotide' question is discussed, not the origin of the code we observe today. How plausible is this model for the origin of the ribosome? Can it lead to predictions?

Could the ribosome have evolved from an ancient RNA-replicase?

When thinking in evolutionary terms we are used to considering time-scales from hundreds to millions of years. However, when treating issues of the accuracy of replication our time scale is that of chemical reactions, and events in millionths of a second are important. The issue is the extremely short time over which, for example, a free cytosine (C) ribonucleotide will H-bond with a guanine (G) in the RNA molecule being copied, to give a C≅G pairing. Protein polymerases can accurately incorporate single nucleotides. Indeed, Piccirilli et al. (1990) show that novel base pairs can be incorporated into both RNA and DNA (see later) so it appears that the information from complementary base pairing (C≅G, A = U) is sufficient for RNA and DNA replication when proteins are doing the catalysis. However, it is expected that the much longer reaction times of ribozymes (Jeffares et al. 1998, Table 1) would tend to allow dissociation of the template and lose the incoming (free) nucleotide before the reaction was complete. This is expected to lead to a higher error rate of replication (and possibly leading to a requirement for pre-tRNAs that carry the nucleotide). This conclusion is consistent with the lower accuracy of the present

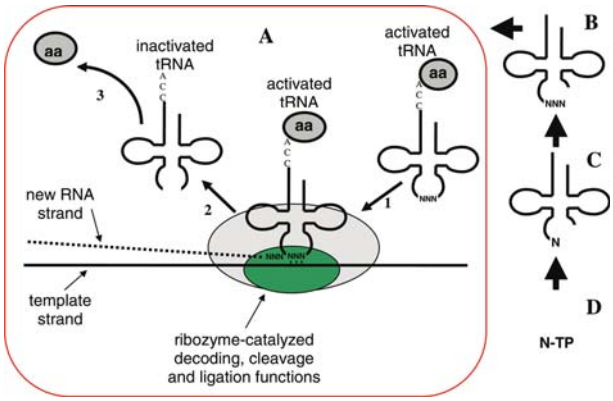


Figure 3. Model for the origin of the ribosome with an RNA triplicase that adds three nucleotides at a time to copy a RNA template. (a) is a very late stage where the triplet code is established and tRNAs are charged with amino acids and which enhances the accuracy of triplet addition to the growing RNA chain. The three main steps are shown with charged tRNAs, recognition of the RNA by binding of the anticodon, and release of the tRNA following ligation of the anticodon to the growing RNA chain (probably with release of the amino acid). Then we work backwards to earlier stages. (b) uses a pre-tRNA to add three nucleotides at a time, but without an amino acid to enhance specificity. (c) is an earlier stage with a pre-tRNA donor that enhances specificity, but adds only a single nucleotide at a time. (D) The earliest stage where nucleotides are added singly and specificity depends solely on nucleotide pairing. Clearly, the order of some events (one or three nucleotides, with or without pre-tRNA) could be reversed.

day RNA-based RNA polymerases formed by *in vitro* evolution (Lawrence and Bartel 2003).

Thus an advantage of trinucleotide over single nucleotide addition is that trinucleotides H-bond longer to the RNA template, giving our hypothetical replicase (Figure 3) more time for polymerization. For example, we expect the trinucleotide AGU to base pair longer to its complement UCT, than simply a U opposite a T. Increasing the length of an oligonucleotide increases the stability of base pairing, which can become unexpectedly stable in the interaction of two complementary triplets of tRNAs (Grosjean et al. 1976). However, the number of possible oligonucleotides also increases exponentially; there are 16 pairs of nucleotides (AA, AC, AG, etc.) but 64 possible triples (AAA, AAC, etc.) – for each additional nucleotide there are four times as many potential substrates. In principle, oligonucleotides larger than triplets are possible, but only triplets are consistent with the origin of the triplet code for protein synthesis. This is really a description of the problem, and does not explain how the length of the code (a triplet) could have arisen. This is discussed again later.

Although the discussion has been on co-opting a proto-ribosome into protein synthesis, the model for the evolution of the proto-ribosome itself is also incremental, and driven by the Darwin–Eigen cycle. The early protoribosome could evolve through a number of modules, there is no need to

invoke massive new complexes of interacting catalytic RNA arising in one step. What is now the 16S rRNA may have been the earliest component as an RNA-polymerase; the 23S rRNA (now the peptidyl transferase) a later addition that initially formed the ratchet mechanism (Poole et al. 1999). A model with amino acid tags, initially perhaps positively charged amino acids, could improve fidelity by aiding in recognition of nucleotides at the anticodon end, or by stabilising the interaction between the negatively charged RNAs. This use of amino acid tags bound to the tRNA, with trinucleotides being added to the growing RNA chain means that a relationship between codon and amino acid is already forged. The full genetic code need not have arisen at this point but a triplet code is at least partially established well before protein synthesis (Figure 3).

Experimental testing of the ribosome model, actual and potential

The first test of a model is its logical consistency and its plausibility based on known mechanisms. At this level, I think the above hypothesis for the origins of protein synthesis is the only one that even comes close to meeting these criteria – at the moment there are no other scientific models for the origin of the ribosome. There is one new experiment that supports our model, and it is critical. This is the report by Fredrick and Noller (2003) that the ribosomal RNA itself carries out the ratchet mechanism of moving the mRNA along one triplet, with concordant movement of the tRNAs – the movement does not require proteins. This really is a stunning finding; it is arguably the most important finding about the origin of the ribosome this new millennium! It supports the idea that the rRNA/ss(m)RNA/tRNA interactions are ancient, from before proteins. The RNA is the core structure of the ribosome (and presumably of the proto-ribosome). It is both the catalyst for the formation of the aminoacyl bond between two amino acids, and carryies out the major movements of tRNA and mRNA. Recent work (Hoang et al. 2004) extends the conclusion still further in that proteins close to the P-site of the ribosome (which is critical for joining amino acids) can be mutated so that the protein no longer touches the RNA; and the ribosome still functions.

The Fredrick and Noller (2003) result, though critical, was not a planned test of the model. There are good possibilities for tests in the future; the main foreseeable one is to extend (by standard RNA *in vitro* evolution methods) the ability of RNA-based RNA-polymerases. Consider the following, in a series of *in vitro* evolution experiments Lawrence and Bartel (2003) report a ribozyme RNA-polymerase. In the initial report (Ekland and Bartel 1996) the ribozyme added the first ribonucleotide fairly quickly, then the second, and finally a third much more slowly. It took forever to add any more. A similar conclusion about three nucleotides comes from the experiments by McGinness et al. (2002). They evolved a ribozyme that adds two nucleotides to a growing RNA

chain, then ligates a short RNA chain. Basically, this ribozyme is carrying out three successive reactions from a single attachment between ribozyme and ssRNA.

For both the Bartel and McGinness results, it appears that their ribozymes lack full 'processivity', they lack the ability to keep moving along the ssRNA being replicated. Our interpretation is that the replicases are only flexible enough to add three nucleotides at their active site. After three nucleotides, a ribozyme could detach and then reattach three nucleotides further along the ssRNA – a tricky operation at the best of times. Much better would be to have a ratchet mechanism that moved the ssRNA three nucleotides through the replicase. This, the 3-step ratchet mechanism, would be the origin of the RNA triplicase, the proto-ribosome, and the length of the triplet code.

The critical experiment is to aim to do just that, look for a 3-step ratchet. Start with the existing single-chain RNA polymerases from *in vitro* evolution experiments (Lawrence and Bartel 2003) and evolve, by standard *in vitro* RNA evolution techniques, a second RNA molecule that interacts with the first. Then search for variants that allow RNA synthesis to continue at an uninterrupted rate. In principle, it doesn't matter if the mRNA is moved in steps of three, just that if the moves are in steps of three then this is a prototype for a proto-ribosome (see Figure 3). Initially in such a system ribonucleotides are still added singly, but there would be later advantages for increasing replication accuracy by adding triples of nucleotides (Poole et al. 1999). Basically we are back to the Darwin–Eigen cycle, where increased accuracy allows longer nucleotide sequences to be maintained, and for RNA the relatively low replication accuracy is a major limit to information storage. An RNA-world absolutely needs a high fidelity RNA-polymerase. If the above experiment was successful – evolving a second RNA molecule that allowed continued RNA replication – it would be my candidate for the top RNA-world experiment in the next decade.

The RNA triplicase theory is attractive in that it gives a high fidelity replicase/polymerase in the RNA-world, an origin for a triplet code, and an origin for the ribosome with its RNA processing cascade. It is possible in principle to recreate such an RNA complex by *in vitro* evolution.

From DNA to Last Universal Common Ancestor (LUCA)

The origin of encoded protein synthesis appears the hardest step from RNA to modern life. By contrast, only smaller steps appear to be required to get to DNA and then to the LUCA. We (Poole et al. 2001) have discussed a series of steps: methylated-RNA; possibly a double-stranded RNA phase (but see later); ribose reduction to deoxyribonucleotides (giving DNA containing uracil, U-DNA); and the replacement of uracil (U) by thymine (T). The Darwin–Eigen cycle (Figure 1) is fundamental – more information allows more genes,

allowing increased accuracy of replication, allowing more genes, and so on. The advantage of replacing uracil by thymine is that higher accuracy replication is expected. Cytosine in DNA deaminates spontaneously to uracil. Therefore it is hard for error-correcting enzymes to distinguish when a uracil in a sequence is 'real', or whether it is a mutation that needs to be corrected back to cytosine. On a different aspect, Forterre (2001) raises the possibility that the later steps of DNA evolution may have evolved more than once. He also suggests that early viruses could have an advantage from using new nucleotides (such as T) to help avoid host defense mechanisms. Later the new nucleotides could have been integrated into the host and allowed an increase in replication fidelity. Such mechanisms are incremental in that they allow smaller intermediate steps, and it indicates additional selective forces that lead to increased complexity.

From of DNA to the LUCA there would again be many very small steps as new proteins arise (Figure 2). There are no conceptual difficulties here; they are the same processes that we observe today with gene duplication and divergence of the two copies. Similarly the maximum size of proteins increases through time, with the largest and most complex proteins being found in multicellular eukaryotes (Caetano-Anolles and Caetano-Anolles 2003). There is uncertainty over aspects of the later parts of the model. For example, Woese (2002) argues that even at the relatively late stage of the LUCA there was still extensive gene flow between organisms – 'primitive cellular evolution is basically communal'. This is a 'Garden of Eden' hypothesis (the lion lay down with the lamb) – all genes were cooperative, none were selfish.

This idealistic scenario is unlikely for at least two reasons. There are major advantages in an effective combination of genes staying together, perhaps on some form of pre-chromosome. Similarly, it is essential that any system be protected against invasion by parasitic sequences (see Boerlijst and Hogeweg 1995). At a much earlier stage, perhaps in the RNA or RNP-world, it would have been harder to hold together good combinations of genes, and mixing of genes may have been harder to prevent. It is particularly as a counter to the Woese argument that I included under the Principles section the condition that selective pressures were as relevant in the past as today. Stopping the breakup of good combinations of genes, and restricting invasions by parasites, were as important then as now. It is certainly expected that effective mechanisms would evolve early to keep good combinations of genes together, and prevent invasion by parasitic genes.

For a quick summary of Themes 1 and 2, it suffices to say that we know that RNA carries out many catalytic reactions and control functions. There is a plausible and testable model to get from the RNA-world to protein synthesis, and that the Darwin-Eigen cycle is a strong driving force for a continued increase in replication fidelity and genome size. The major problem now is the origin of the RNA-world itself; the RNA-world was probably already complex in its metabolism.

Theme 3 – are there alternatives to RNA?

The RNA-world hypothesis is an excellent mid-point for the top-down and bottom-up approaches. If the chemists could get to an RNA-world, the biologists could take over and extend along the continuum to modern life. Thus the RNA-world is an important reference point, and a target for the bottom up approach. However, RNA is unlikely to have been the first self-replicating molecule, and there are important issues with its origin in a prebiotic environment. In this section I simply ask whether there good reasons for

just four bases in RNA (adenine, uracil, cytosine and guanine);
these bases rather than other self pairing molecules;
sugars (and ribose in particular) as part of the backbone of the molecule; and
selection between optical isomers (D- and L- forms).

It is important to know how close we have to be to the target (RNA). Are there many alternatives that work well, or just a small number? If RNA is best; would some alternatives evolve easily toward it? Are there alternatives to our existing nucleotides, or to four nucleotides in RNA (is four really a sacred number in biology), and does the sugar have to be ribose?

Alternatives to A, C, G and U, and the numbers of nucleotides

Alternatives could either be completely different to nucleotides, or just alternative nucleotides. Rebek (1994) gives an account of his work with complementary self-replicating molecules, only one of which contains adenine. The other molecules are not nucleotides (one is a biphenyl molecule) but they are still self replicating in complementary pairs. An interesting aspect is that the system 'evolved' in that some molecules readily underwent self-replication and outcompeted others. Thus, in principle, molecules other than nucleotides are possible, though they are not synthesized so easily as adenine in the prebiotic world (see later). But an important principle is established; self-replication is a basic property of the appropriate molecules, a property that has been co-opted by living systems.

Why four? Given an even number of nucleotides for pairing, why not two, or six, or eight. After all, there are 20 amino acids, why not 20 nucleotides? The question may sound academic, but has flourished after Piccirilli et al. (1990) reported that an additional pair of nucleotides could be made synthetically – and could be incorporated into RNA- and DNA-like molecules. A nucleic acid with six nucleotides, not four – three pairs of nucleotides, not just two! The quantum mechanical calculations of Mac Dónaill and Brocklebank (2003) suggest that two hydrogen-donor mismatches are required for accurate discrimination between pairs of nucleotides, so this reduces the number of base pairs that are feasible. It places an upper limit because it would be difficult to have an effective pairing system with high numbers of nucleotides.

At the lower end, Reader and Joyce (2002) use standard *in vitro* evolution techniques (see earlier) and evolve a ribozyme with just two nucleotides. In this case uracil was replaced by diaminopurine to pair with thymine, giving three H-bonds between each pair of nucleotides. The resulting two-nucleotide RNA ligase ribozyme functioned, but its catalytic ability was much weaker than the original 4-base ribozyme from which it was derived. So there is a real question whether four nucleotides is a genuine optimum. Could six nucleotides be better than four, but nature did not find it before proteins 'took over' most catalysis.

Szathmáry (2003) reviews the problem, and from experiment, simulation and theoretical calculations, it seems that four nucleotides outcompetes two, six or eight. However, in simulations under some rates of mutation, six nucleotides can evolve to a new structure faster than four (Gardner et al. 2003). But this does not affect the conclusion that four nucleotides are more effective than two; it only describes the phenomenon rather than explaining the greater efficiency of four-nucleotide ribozymes.

Our suggestion (Gardner et al., in preparation) is that with only a single pair of nucleotides, each sequence can fold in an extremely large number of ways and still be close to the 3-D structure with minimum free energy (mfe). An RNA-world is unusual in that the RNA molecule is both the genotype (the actual sequence) and the phenotype (how the sequence folds in two and three dimensions). Because RNA with just two nucleotides (one pair) can fold so many ways, it appears that with a 2-letter code genotype does not specify a consistent phenotype. In contrast, with four nucleotides there are fewer potential mfe structures and therefore there is more consistency between a primary sequence of RNA, and its secondary structure. We think that this is the best explanation at present for why RNA (and therefore DNA) has more than just one pair of nucleotides, and that this idea merits further testing.

Alternative sugars

A well-recognized problem is that ribose is difficult to synthesize, and has the chirality problem of mirror images around some carbon atoms. Are there alternatives to ribose? Eschenmoser (for example 1999) has synthesized analogues of RNA with different sugars, crystallized them, and measured many physical properties. Sutherland and Whitfield (1997) summarize some of this earlier work and give the sugar analogue and the relative strength of base pairing.

Ribose (RNA and DNA)	GC > AT
Glucose – homo-DNA	GC > AA ~ GG > AT
Allopyranosyl (6C)	AA ~ GG > GC, etc.

This means that for ribose and deoxyribose (RNA and DNA) the GC pair is the strongest, then the AT pair. However, for a nucleic acid based on glucose, although GC is strongest pairing, G also pairs quite strongly with itself. Similarly, adenine (A) pairs strongly with itself, even stronger than with T. With some sugars, the relative strength of base pairings varied with the sequence, not a desirable feature for a general system of pairing. Thus for complementarity of pairing, ribose is a particularly suitable sugar. Other sugars are possible. For example, Chaput and Szostak (2003) report that a TNA (T = trehalose, a four-carbon sugar) has some elementary properties of interest and could possibly be a precursor to RNA.

Some sugars give a nucleic acid with stronger binding between the complementary strands than RNA, and these have sometimes been considered as 'more suitable'. However, it is probable that high binding energy between the strands of dsRNA is an undesirable feature – rather, the specificity of complementary pairing is more important. It is likely that in an RNA-world the positive and negative strands of RNA were kept separate – in the absence of proteins, unwinding dsRNA would be extremely difficult. In the present (DNA) world there is a complex of proteins that cut and unwind dsDNA and then rejoin it, but this would not be possible in an RNA-world. In modern cells, dsRNA does occur, but tends to be used as a signal to destroy that sequence. Good complementarity of base pairing is expected to be the main requirement.

To summarize this section, it is not necessary to decide whether the combination of ribose as the sugar and A, C, G and U (T in DNA) as the nucleotides is the optimal combination. We do not see any obviously better candidate than RNA, nor do there seem to be good alternatives for either sugars and/or nucleotides. If there were 'precursors' to RNA (which RNA eventually outcompeted) then this simplifies the situation in that a range of molecules could evolve toward RNA. In a sense the 'target size' for the bottom-up approach is larger. It is more productive at present to focus on the origin of RNA as a system that had all four of our properties for a living system, energy, biochemistry, cell organization and heritability.

Hot start/cold start?

There are two additional properties, temperature and pressure, where the properties of RNA may limit the physical conditions for the origin of life. Did life originate on earth at high temperatures (such as around hydrothermal vents deep in the oceans – black smokers), or in relatively cold conditions? Knowing the answer to this question would focus further experiments. Previously, the most popular theory was a high temperature origin ('hot-start'), and was supported by the apparent placement of the 'Last universal common ancestor' (LUCA) of all living organisms among thermophilic bacteria (living above about 80 °C). However, an improved method of building evolutionary trees (Forterre and Philippe 1999) suggests that that apparent position of the

'root of the Tree of Life' was an artifact of the earlier phylogenetic methods used. Similarly, all modern hyperthermophiles use a reverse gyrase protein to stabilize DNA at very high temperatures. But this protein appears to be a fusion production from two existing enzymes used by all organisms; it is not one of the first proteins (Forterre 2001). In popular imagination we think of life originating in dramatic fashion in the bottom of the ocean where super-heated water ($\gg 100$ °C) gushes out. Unfortunately for popular imagination, at such high temperatures,

Ribonucleotides are unstable (the nucleotides break down, Levy and Miller 1998),
RNA itself is unstable (the phosphate-sugar backbone breaks, Forterre 1995),
RNA does not fold into 2D and 3D structures (Moulton et al. 2000).

For such reasons, some form of 'cold-start' seems a more likely starting point (Bada and Lazcano 2002).

This conclusion is reinforced because, in general, the higher the temperature the harder it is to get ordered structures. Ice is highly ordered compared with liquid water, which is highly ordered with respect to steam. In thermodynamics, the decrease in order is measured as the change in entropy (ΔS) but is negative with respect to temperature ($-T\Delta S$); higher temperatures increase this negative value. With many millions of years available, the rate of a reaction is not necessarily limiting, but it is critical to find conditions where order or complexity increases. There is a fundamental issue here. Is the problem of the origin of life basically a problem of increasing order (an entropy problem), or primarily limited by rates of reactions – a chemical kinetics problem? Given also the problems of RNA at high temperatures, my guess is that the origin of life is more of an entropy problem (rather than kinetic), and that high temperatures are unhelpful.

How low a temperature? We really have little idea. At present we probably would require temperatures below 60 °C for the stability of RNA and its precursors. But if the origin of life is an entropy problem then we cannot exclude temperatures around 0 °C. There is a definite group advocating sub-zero temperatures that occur in concentrated aqueous solutions within sea ice and recent results in sea ice are encouraging (Trinks et al. 2003). The freezing of sea water, giving a concentrated solution in the unfrozen water, can (Orgel 2004b) lead to high levels of adenine from HCN (see later). These very low temperatures need exploring.

Open question: atmospheric pressure or high pressure?

However, the black smoker/hydrothermal vent suggestion raises another question. Would it be easier for early life to develop at around one atmosphere pressure – or under pressures of hundreds or thousands of atmospheres at the bottom of the ocean? There are also 'cold smokers' where chemically-rich

water comes up from the ocean floor. Alternatively, given a cold start scenario, there will be places at sea-level at one atmosphere pressure in sea-ice (see above paragraph) where there will be many highly ordered 'cells' (see later) that may be advantageous.

The question comes down to fundamental issues such as the stability of nucleotides at high pressures (including deamination of cytosine). Similarly, do two monomers (ribonucleotides) occupy a larger volume than a dimer? If so, higher pressures might then be favorable to polymerization. Is nucleotide pairing more stable at high atmospheric pressures? There is a need here for fundamental research by physical chemists. A similar question, whether sea water is the optimal medium for early stages of the origin of life was considered by Monnard et al. (2002). Their results were that sea water inhibits both membrane self-assebly and RNA synthesis. Clearly further experiments are required to search for optimal conditions.

To conclude this third section, it appears that there are no easy alternatives to RNA. In an important sense this is good news. If there are other non-RNA precursors (close to RNA, say with a different sugar such as trehalose), they are likely to have been out-competed by RNA. Thus, the RNA-world might be attainable through a variety of different routes; there need not be only a single route to an RNA-world. Such a conclusion does not imply that other forms of life were not possible, just that of the ones that might be possible in principle, the RNA-based form was easier to find.

Theme 4 – how to get to the RNA-world

At present, getting from gases and other small molecules to an RNA-world appears the hardest task for the origin of life – but probably wasn't. On current wisdom, it may have taken a mere 100 million years for RNA and DNA-based living systems to appear on earth, but another 3 billion years to get multicellular land plants and animals. Maybe getting to an RNA-world is not as hard as evolving multicellular land animals? Taking the time-to-solve-a-problem as the measure of complexity is simplistic, but is used to indicate that researchers should persist with trying to understand these early chemical phases. (The reasoning makes humans look easy, only about 5 million years from a common ancestor with chimps.)

Why does it appear so hard to get to RNA? How complex was the RNA-world? Did it have all basic metabolism and organization (including membranes)? From the discussion thus far we conclude that an intermediate stage RNA-world explains a large number of observations, and that there is a large body of experimental evidence of the catalytic abilities of RNA (ribozymes). Furthermore, it is possible to make plausible models that take us from an RNA world to the evolution of protein synthesis, to DNA synthesis, and to a last universal common ancestor (LUCA). Even better, the models lead to testable predictions. In the third section we find the number of alternatives to RNA to

be limited. But the target does not have to be just RNA, as a range of similar molecules could lead to RNA. Are we missing something obvious?

Origin of the small organic molecules used in life

In reviewing what we know of the origin of small organic molecules of living systems, we have two complementary approaches;

chemical simulations of possible prebiotic conditions (the test-tube approach), and
theoretical predictions from basic chemical properties. (What molecules are expected to occur early, and where are they likely to be synthesized?)
The results are compared to the basic biochemicals found in living systems.

In very early studies, Oparin (see 1957) tried many mixtures of chemicals in order to find what biological properties would develop in fairly simple systems, in particular gel-like microbodies (coacervates). Although some interesting physical properties of such systems were found he appeared, from our current viewpoint, to make little progress - partly because the basic genetic and biochemical problems were not understood at the time. However, the properties of coacervates may turn out to be important as part of the 'organization' of cells, we just don't have a theoretical framework to interpret them.

By far the best-known work is on chemical simulations. As is well known, in 1953 Stanley Miller (see Bada and Lazcano 2003) reported the first chemical simulations that are useful from our current understanding. He passed electric discharges through a possible primeval atmosphere (containing methane [CH_4] and ammonia [NH_3]) and found amino acids among the products! Many researchers, including Oró, Ferris, Ponnamperuma, and Orgel, followed this up; a recent review is Orgel (2004a). A startling finding was that the nucleotide adenine can be formed by the polymerization of hydrogen cyanide (HCN, one of the active chemical intermediates in the prebiotic experiments, see Sutherland and Whitfield 1997 Figure 4). This is particularly striking. Adenine, as a component of ATP is a central molecule in energy transfer reactions, is a component of coenzymes (such as FAD, NAD), and is part of both RNA and

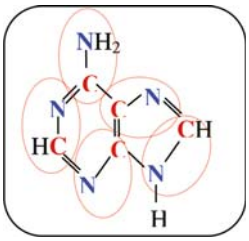


Figure 4. Adenine, as formed from five HCN molecules.

DNA. Thus basic building blocks of life occur in simple chemical simulations of prebiotic chemistry. There is no guarantee that this is the actual process by which adenine was made in prebiotic systems, but at least adenine is likely to have been present and there is a well-established chemical pathway for its synthesis (Orgel 2004b).

We all accept the importance of the chemical simulation approach, but as a theoretical biologist I also want an analysis of what small molecules are expected to occur early on the earth. Were small organic acids, amino acids, nucleotides, co-factors, and/or sugars among the first molecules in early prebiotic reactions? Were these molecules more likely to be formed in aqueous or gaseous phases, or at interfaces (including clays)? At higher or lower temperatures? At around one atmosphere pressure, or at high pressures (such as at the bottom of oceans)?

Returning to the early Miller experiment, we could consider the precise conditions of the experiments and the precise composition of the starting chemicals, and then argue about the details. Alternatively, we could look at the fundamental principles; the experiment demonstrates that known chemical reactions lead to small molecules that are of biological interest. I would argue (this week at least) that the details of the original Miller experiment on forming amino acids may not be of major interest for the early origin of life –amino acids may not have been that important so early on. On the other hand, when considering the principles, the Miller experiment is intellectually outstanding in illustrating how known chemical reactions lead to small molecules of biological relevance. But which molecules do we expect to be synthesized first?

Gas phase/aqueous phase

One model is that the small biochemical molecules formed either in reactions in the atmosphere (and then were absorbed into the ocean), or in an extraterrestrial environment and were carried to the earth during its accretionary phase (see comments in Orgel 1998). For convenience, I will include both ideas as a 'gas phase' origin, even though they assume that later stages continued in the ocean. These models focus particularly on small molecules that contain nitrogen, especially amino acids, though that may not be essential to a gas phase origin.

An alternative to the gas phase is a completely aqueous origin for the first biochemicals; in general this gives less emphasis to nitrogen-containing molecules at the earliest stages of a prebiotic system. The ideas of Wächtershauser (1992) are the best known, but I will base my analysis of the first biochemicals on that of Morowitz (1992) and Morowitz et al. (2000). I will take for granted the main chemical elements involved – carbon, nitrogen, oxygen, and phosphorus, see the discussion in Morowitz (1992, Ch. 10). The next step is to decide how to predict which molecules will occur in prebiotic systems. Morowitz et al. (2000) suggest that the most likely molecules will be:

small (no more than six carbon atoms),
water soluble, and
have low heats of combustion (therefore are relatively easy to form chemically).

The authors start with a list of over 3.5 million organic (carbon-based) compounds and select those with no more than six carbons, leaving 2790 (a thousand-fold reduction). Water solubility was measured by the relative amounts of a compound in a water/octanol mixture (a standard technique for comparing water versus lipid solubility). Other minor rules were applied including stability, the relative ease of formation, and being chemically reactive. The final list is 153 compounds – the list includes all the compounds involved in the tricarboxylic acid cycle. This cycle is shown (as the reductive TCA cycle, and as a potential flow reactor) in Figure 5, with the name of the organic acid following its number of carbon atoms).

Just like the original Miller experiment, the result is striking. The small molecules involved in metabolism in modern-day living cells are precisely the molecules that are likely to form in an early earth. This is considered a 'universal' of biology (Smith and Morowitz 2004). The conclusion only considers the earliest stages and molecules lacking nitrogen. But as the authors point out, such nitrogen-containing molecules can form from the molecules of the reductive TCA cycle (rTCA), or from related molecules no more than 1 or 2 reaction steps away. A standard chart of biochemical reactions shows many such possibilities. But to go earlier still, where do the molecules of the rTCA cycle come from. The best guess at present would be the simple 'free lunch' reaction (Martin and Russell 2003)

$$4H_2 + 2CO_2CH_3COOH + 2H_2O$$

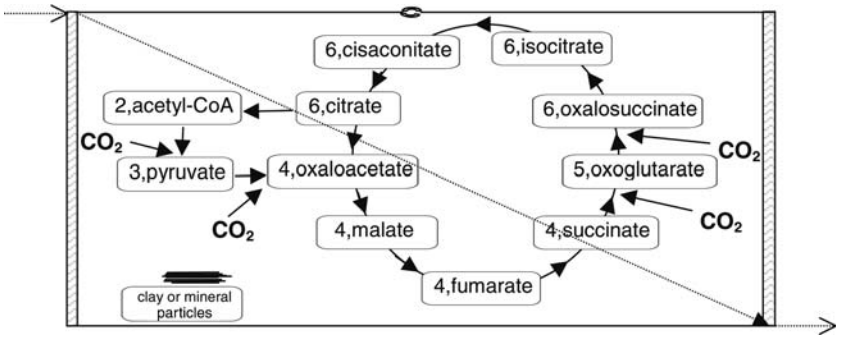


Figure 5. The Reductive Tricarboxylic Acid Cycle (rTCA) in a potential flow-reactor. There is a net synthesis of molecules, given an input of CO_2 and energy. Some form of compartmentation is indicated by membranes on the left and right, together with an energy gradient from left to right. The model combines an energy source, basic biochemical molecules, and compartmentation. Potential metallic or clay catalysts can be included in the reactor.

In a reducing atmosphere the equilibrium strongly favors acetic acid and water. Wächtershauser (1992) has the same basic cycle but adjusted for the high sulfur and iron content expected on the early earth (Anbar and Knoll 2002).

Basically, this approach leads eventually to a 'metabolism first' theory, and we will come back to it – it is the basis of much current thinking. However, it is first necessary to consider some additional features such as polymerization and organization.

Polymerization, organization and energy

In the above section, the treatment only covers possible origins of basic biochemistry, and we still need to include three other aspects of the prebiotic world - polymerization, compartmentation and energy sources. With respect to polymerization of amino acids or nucleotides, important principles are established by demonstrating the formation of short (unordered) peptides by a series of 'drying cycles'. These involved a repeated series adding a solution of amino acids to clays (particularly montmorillonite, see Ertem 2004), evaporating the solution, and heating. This cycle of adding a solution of amino acids, drying, and heating, is repeated several times. In each cycle one additional amino acid may be added to the growing peptide.

When heated dry (with amino acids absorbed onto a clay surface) any water formed during the formation of a dimer (with its peptide bond) is lost to the atmosphere, driving the reaction toward synthesis. Prior adsorption of amino acids onto the clay surface reduces entropy costs of the polymerization reaction because the amino acids are already partly ordered (on the clay surfaces) relative to their free movement in solution. In contrast to reactions on clay surfaces, in solution the high water concentration drives the polymerization reaction towards hydrolysis of peptides, and the amino acids are fully free to diffuse and rotate in solution. This establishes again that basic chemical principles are being followed in such prebiotic simulations. However, to achieve polymerization in an aqueous solution, whether of nucleotides or amino acids, is a major difficulty. Nevertheless, as outlined below, we still want a 'single compartment' answer.

'Life as we know it is cellular', is a useful slogan that introduces compartmentation and membranes. Appropriate molecules for membranes are expected to be harder to find in prebiotic systems, but suitable longer chain molecules are found even in organic material extracted from meteorites (see Deamer et al. 2002). Given the absence of proteins in the prebiotic environment, lipid bilayers are the best-known model. Such bilayer membranes form spontaneously in the right environment because of the lower free energy of having the hydrophobic ends of two lipids together. The membranes can be a layer between compartments in experimental systems, spherical membranes

separating two phases (such as surrounding gels), or spherical liposomes (with aqueous phases inside and out).

Compartmentation and membranes are certainly expected to be important. Many energy sources are used by living systems (see Martin and Russell 2003), all of which use proton gradients across membranes. Apart from a few substrate-level phosphorylation reactions, the energy currency in biology is proton gradients – they are as universal as proteins and nucleic acids. Morowitz (1981) discusses the relevance of proton gradients to the origin of life (though in a photosynthetic perspective) and concludes that a proton gradient is the only possibility. Electrons, for example, need a quite different (metallic) structure that is not found in living systems, nor is transport of large ions likely to be a solution.

Turning from the experimental to the theoretical, in addition to the observed requirement of living systems for membranes, Zintaras et al. (2002) report theoretical results where more-complex molecules can evolve in compartments. The effect comes from both increasing cooperativity (good replicators are more likely to be copying other good replicators), and also by reducing the chance of parasitism. The latter may be unrelated molecules that, for example, could be easily copied but do not reciprocate by replicating other molecules. Complementary to these theoretical results are experimental systems of authors such as Hanczyc et al. (2003) where, for example, the clay particles are included with liposomes. This is an important step to a flow reactor, But perhaps it is not sufficiently controllable to be a useful model? Thus for both theoretical and empirical reasons, and from considerations of energy use (proton gradients), it appears that membranes need to be considered very early in prebiotic stages. One suggestion for early 'protocells' has them occurring inside the small spaces of volcanic rock (Martin and Russell 2003) or other subterranean location (Trevors 2003). In general then, our conclusion is that energy sources, compartmentation and membranes, are critical for early prebiotic systems.

A new beginning?

Over the past 50 years most of the work in the prebiotic realm appears to follow the path of synthetic chemists. This is a linear model of chemical syntheses, starting with a smaller molecule, and adding moieties sequentially until the final product is reached. Why has this approach not been more successful over the last 50 years? Much has been learned about individual reactions, classes of reactions, and polymerization of amino acids on the surfaces of clays during drying cycles. But it has not lead to any major discovery such as a likely early metabolism, or how to get to an RNA-world. What are we missing?

Perhaps the linear model is not the right approach. The linear approach (including the Darwin–Eigen cycle) is a powerful model for later stages of the origin of life, after life has a genetic inheritance system. It leads from a simple RNA-world, to a complex RNA-world with an RNA-triplicase as a high

fidelity RNA polymerase reaction. This is followed by the advent of protein synthesis, perhaps methylated RNA (meRNA), to DNA, and onwards. At these later stages the linear (incremental) model is both plausible and consistent with known principles. In contrast, for the prebiotic stages of the origin of life, the linear model (with a large number of sequential chemical syntheses in the right order eventually leading to RNA) does not seem to give equivalent new and exciting discoveries.

Over the past few years an alternative to the linear scenario for the prebiotic arena is being considered; one where the three requirements of energy, metabolism, and compartmentation (including membranes) are considered together. We need to be able to consider thousands to millions of possible reactions simultaneously, just like the *in vitro* evolution experiments where routinely $>10^{13}$ variants of RNA sequences can be tested simultaneously; we need an equivalent for searching for metabolic cycles. More like combinatorial chemistry perhaps. The general idea has a long history. For example, Ninio (1982, pp. 87–89) suggested making peptides at random, separating them by 2-D chromatography, and searching for catalytic activity; he attributes the idea to L. Orgel. When the focus changed from proteins to RNA it was used successfully for evolving ribozymes.

A flow reactor is a good start to simulate the problem for metabolism, see Figure 5 for a rough idea. In this example, there is a central metabolic compartment that includes metal ions, it is bounded by membranes, and there is a strong energy gradient from left to right. Such an idealized cartoon is intended to be general, not to show particular chemicals or energy sources. However, a critical feature is a metabolic cycle, in this case the reductive tri-carboxylic acid (rTCA) cycle. Cycles are particularly interesting from the viewpoint of non-equilibrium thermodynamics (see Glansdorff and Prigogine 1971), because they lead to a high rate of energy dissipation down a steep energy gradient. A system that 'dissipates' energy faster will have cycles turning faster, incorporating additional chemicals (such as CO_2) into the system. Even at this prebiotic stage it is likely that there is selection between purely chemical systems, in the manner included in the systems studies by Rebek (1994). In this sense the system evolves, though as pointed out be different authors (e.g. Sowerby and Petersen 2002; Chen et al. 2004) it is not yet a genetic system. I call this pre-genetic evolution.

It is not intended that a flow reactor 'mimic' nature precisely; nor simulate a particular set of plausible conditions on the early earth. Rather, the intent is to examine basic principles in a controllable way, and find if there are any likely conditions where a set of self-sustaining reactions occur – given a membrane bounded structure, a steep energy gradient, and a supply of precursors. Hordijk and Steel (2004); also Mossel and Steel (2005) show that autocatalytic cycles are likely to form in a diverse chemical system. They assumed initially that each chemical could be both a reactant and a catalyst, but metal ions may be the catalysts though not otherwise participating in the cycles. Thus autocatalytic cycles are reasonable mathematically, and thermodynamically they

are advantageous. Some of the sets of conditions to try could be those described in Eschenmoser (1999) and Martin and Russell (2003).

We need to know if there are structured systems where, say, a reverse TCA cycle can arise naturally, and whether the next layers of small molecules will then appear, especially those containing nitrogen. Concepts from biomimetic chemistry (Sutherland and Whitfield 1997) are important here, the idea that pathways found in biochemistry are the most straightforward ways to more complex molecules; pathways that were found early in protometabolism. Maybe the proposed flow reactor is too simple, trying to put organization and energy gradients (plus metabolism) back together in one step. However, we need to find whether the reactions from the very earliest stages of the origin of life were autocatalytic. The conclusion here is that we need to try an organized system, with compartmentation by membranes, a steep energy gradient, and a setup that may allow cycles. This might combine our knowledge from 50 years of chemical simulations in a manner where new processes could arise.

Summary and conclusions

There is still a significant gap between what has been achieved by the top-down and bottom-up approaches, though nothing like as large as it was 30 years ago. Most researchers now consider the problem as solvable – say in another 10–20 years. This is far more optimistic than the view of 40–50 years ago when most scientists would have considered the problem inherently unsolvable, forever beyond the ability of the most powerful science then imaginable.

It is important to examine the logic behind the different conclusions. Life is a natural property of matter; its origin is a scientific problem that was, in refuting continuous spontaneous generation, discovered by science. Instead of a statistical 'all at once' answer, we are looking for a mechanistic, step-by-step approach that follows Darwin's principle of continuity (which is a version of the standard scientific principle of microscopic reversibility). In addressing the problems we need to consider both the basic theoretical work on what is possible, as well as the experimental simulations.

I have considered four main themes in order to look for a continuous set of intermediates. The RNA-world hypothesis (though qualitative) is a powerful scientific theory that is consistent with numerous phenomena, many discovered after the development of the theory. It removes the apparent chicken-and-egg problem between DNA and protein. The hardest step from an RNA-world to modern life appears to be the origin of protein synthesis, and here the triplicase/proto-ribosome theory, adding triples of nucleotides to a replicating RNA, is so far the only plausible theory. It is plausible in the sense it is both consistent with our knowledge (including selective forces) and that at least some aspects are testable. It separates the origin of the code into the questions of its length (three) and then of the specific triplet code (which is suggested to be at least partially established well before protein synthesis evolved). The

length of the code is suggested to arise from a ratchet mechanism that overcomes the problem of processivity.

Later steps to get from an RNA-world, to an RNP-world, to modern biology appear more straightforward. The Darwin-Eigen cycle is likely to be a powerful selective force in the many steps from an RNA-world to the modern biological world. However, I would like to know more of the specific situations when it should lead to an increase in complexity (total information coded). We do not know of good alternatives to an RNA-world. It is likely that there may have been precursor stages to RNA with simpler sugars, or just two nucleotides. However, we do not yet know of alternatives that were likely to arise naturally and which would have been better.

Thus the focus is still on how to get to RNA. For these prebiotic stages (before RNA) we may need to solve metabolism, energy gradients, and compartmentation simultaneously – thus the intense interest in some form of flow reactor. If an autocatalytic cycle could arise, we would be major steps ahead in our understanding. The most likely physical conditions for the origin of life require further clarification, especially in regard to temperature, pressure, chemical composition and energy sources. If its origin is an entropy (information) problem, rather than a kinetic or energy problem, then low-temperature conditions warrant much more attention. My favored analysis at present is 'metabolism, energy and organization first, metabolism makes RNA, RNA makes protein, and protein makes DNA'.

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