The Conceptual Issues

Up to now, I have tried to stay closely tied to experiments and observations. The following is not based directly on experiments. Some molecular modeling is used, and the underpinning is, of course, based on all the experimental evidence behind what has been discussed up to now. The conceptual issues spoken about earlier need to be revisited here. "Chicken-egg" questions, IC, and initiation issues will be addressed from here onward by speculation, reasoning, theory in short, not experiment. In so far as real experiments will present themselves naturally as we proceed, the reader should make note of them. It is *initiation* that is the *mysterium tremendum* at this stage. Let us set the stage.

The *primordial soup* is an energized chemical broth, vigorous with chemical ferment, driven by the Sun's UV radiation. Redox, thioester and pyrophosphate driven precursors to many contemporary biochemical processes are manifest. Thermal, wet/dry cycle niches abound on the surface of a rocky planet with a highly volcanic mantle, shallow seas of salt solutions, and iron. The UV excites electrons into excited states of oxidation. This reducing potential drives syntheses and a great variety of small molecules. Combinatorially, the numbers are low enough for multiple copies of any particular molecule to exist simultaneously with all other real molecules. Lots of amino acids of limited variety, lots of sugars, some nucleotide monophosphates. No polynucleotides and only short polypeptides generated by heat, by thioester activation, or by pyrophosphate activation are to be found. Perhaps on the shore of a shallow sea there is a tide pool zone at the upper edge of which are pools that dry out between periodic high tides. In such pools there would be diurnal cycles of UV and heat from the Sun to drive chemistry as a flow of excited electrons. Albert Szent-Gyorgyi said life was "a little electric current driven by the sunshine." (This captures the redox part. De Duve's *Thioester World* captures the thioester part, and Lipmann's *Pyrophosphate World* round out the energy couplings.) Bathing in this broth are proteinoid microspheres. The polymer formed by heating during the previous step of the cycle after the pool had dried some time between consecutive high tides. After the pool fills during the next high tide, the previously dried proteinoid is exposed to water. Microspheres self-assemble spontaneously. There is a decrease in Gibbs free energy connected to formation of the double membrane spheres. They have a chance to use the broth chemically.

Some of the polypeptide generated by the chemical factory inside the spheres can self-assemble into the membranes of the proteinoid microspheres. If these spheres divide, a process looking morphologically like cell growth is seen. What makes the growing sphere divide? Something simple may be at work here. Formation of the membrane from dissolved polymers decreases the Gibbs free energy, as must any *spontaneous* process. As the sphere's area grows so does the magnitude of the decrease in free energy. A sphere that has grown from the standard radius value, r, somewhere between half a micron and 8 microns, say, to the radius, R, is favored

thermodynamically by dividing into daughter spheres, each of radius r, provided $R = \sqrt[3]{2}r$. This value says that the volume of the sphere about to divide is 2 times the volume of a standard sphere of radius r, or said another way, volume enough for two standard sized spheres. But what about the respective surface areas? The initial surface area is $4\pi 2^{2/3} r^2$ but the final surface area is $2 \times 4\pi r^2$ which is bigger than $4\pi 2^{2/3} r^2$, because $2 > 2^{2/3}$. A change from the bigger size of sphere into two spheres of the standard size is thermodynamically favored because of the large spontaneous Gibbs free energy change, a decrease, in the formation of the needed additional membrane area. Since division could occur for any initial radius R, other *forces* are acting that thermodynamically oppose spheres being too small. These *forces* need to be identified. Surface tension (force per unit length) and osmotic pressure (force per unit area) are two possibilities.

Laplace's law for the surface tension on a vesicle is given by

$$P_{inside} - P_{outside} = 2\,\frac{Tension}{r}$$

For permeable membranes, the pressure difference across the membrane is the result of osmotic pressure caused by the trapped polymers. Otherwise the inner and outer pressures balance. Using van't Hoff's formula for the osmotic pressure yields

$$P_{osmotic} = \frac{N}{V} k_B T = 2\,\frac{Tension}{r}$$

In which k_B is Boltzmann's constant, T is the temperature, N is the number of *polymer* molecules, irrespective of size, and $Tension$ is the vesicle surface tension. For the moment one can think of just the inner membrane of the two. The generation of polymer is an energy driven process because activation energy is required. As generation proceeds, the N increases and if all else is left equal then the tension must increase. The volume expands because of increased osmotic pressure. The radius, r, grows. However, the volume, V, also depends on r.

$$V = \frac{4}{3}\pi r^3$$

Thus, for a growing r and N, the tension is given by

$$Tension = N\frac{3k_B T}{8\pi r^2}$$

If a fluctuation reduces the radius, the tension rises and opposes the reduction. If the radius were to be decreased enough the high tension would lyse the sphere. In lipid vesicles the tension at which vesicle lysis begins is about or above 3 mN/M (I prefer dyne/cm.). For T = 300 K, and for a *one micron* radius, one gets

$$Tension = N \times 0.1194 \ldots \times 4 \times 10^{-6}\ ergs/cm^2$$

$$Tension = N \times 4.776 \ldots \times 10^{-7}\ ergs/cm^2$$

This means that 10^7 polymer strands would create enough osmotic pressure and membrane tension to rupture lipid vesicles. The microspheres may be stronger, supporting a greater tension before rupture. 10 times stronger? 100 times stronger?

The polypeptides that are synthesized from activated amino acids, either by thioesters or by pyrophosphate, or by other means, afford the first opportunity to understand what *selection* can do. Polypeptides will form in this environment because of the presence of activation free energy. Even though the average length is small, there are plenty of hexamers, say, and multiple copies of all possibilities are feasible. Among these possibilities may exist catalysts for *activation* and for *polymerization*. The thermally generated hexamers (and longer polymers) have catalytic abilities and so should the oligomers made from chemically activated monomers. An *activator* catalyst is today some sort of *kinase* and the *polymerization* catalyst is today a *polymerase*. With such naturally occurring catalysts, these microspheres would grow faster and divide more frequently. The generalized broth is shared by all spheres. The spheres with these two catalyst are in greater numbers than those without. Since these polypeptides are in multiple copies, division can share them as well. However, unless new ones a produced spontaneously the division process eventually spreads the copies too thin. It is minimalist to assume that these special catalyst are uniformly available inside all spheres. Remember, each sphere can try out all sequences of hexamers, heptamers, etc. in multiple copies. Can anything really special happen in this scenario since all spheres potentially contain the same possibilities? Why don't they have genetics yet? How do polynucleotides of great length come into being, and why?

Some of the growth in N can be incorporated into the membrane by self-assembly. This increases the radius (and volume) of the spheres. The osmotic pressure must also grow. The rate of polymer production must cover membrane enlargement and polymer density, i.e. osmotic pressure. If the density stays constant during radius growth, the tension must also grow to compensate (see above). Division, lowers the tension but requires added membrane (if the volume divides by 2, the surface area divides by $2^{2/3} < 2$). More membrane must self-assemble during the division than is present at division initiation. Nevertheless, Gibbs free energy decreases during this phase. The possibility exists that hexamers and heptamers are too short for self-assembly. Perhaps lengths three times bigger are needed instead, lengths such as 18-20 units. If a polypeptide *ligase* existed that joined hexamers with hexamers, as though hexamers were the new monomers, then longer polymer could occur. These hexamers are still activated at the carboxyl end because the amino end grows as activated carboxyl groups react with amino groups. Thus the activated carboxyl group of one hexamer attacks the amino group of the other. This polypeptide ligase activity is intrinsic to the natural products found inside a microsphere as

a short polypeptide. If it had alternating plus minus charged residues, it could facilitate linkage of the negatively charged carboxyl phosphate and positive amino group. The expected number of linked units is Napier's e. That makes polypeptides 18-20 units long. These should surely self-assemble into membranes as do thermal proteinoid polypeptides of comparable size.

The possibility of short polypeptide formation insides the spheres leads to a proposal about the origin of chirality. Suppose that within the natural products of short polypeptides we have been imagining, there is a proto-polymerase sequence that catalyzes polymerization of activated monomers. Suppose that it happens to favor *L with L* amino acid linkages. The internally grown polypeptides of the spheres would become chiral. The absolute chirality is arbitrary and frozen in today. Chiral polymerization might produce a faster mechanism for faster divisions. With polypeptides, it is difficult to imagine a chiral advantage to pure L or pure D instead of a racemic mixture. One expects all varieties to work well as catalysts and structural elements. A manifest advantage does attend polynucleotide synthesis however. By making pure chiral polynucleotides, replication becomes possible on purely stereo chemical grounds (the racemic polymers snag when processed either for replication or for transcription). A natural hypothesis is that chirality evolved simultaneously with the evolution of polynucleotides, and locked in when polynucleotide replication locked in. The L amino acid D ribose coupling is only relative, not absolute, DL could work just as efficiently. What about LL or DD?

It has already been stated that polynucleotide production introduces added difficulties. Interesting progress has been made by experimenters on several fronts. Hydrogen cyanide, despite its positive free energy of formation, is available and adenine, for example can be thought about as $(HCN)_5$ and has been generated in the laboratory (J. Oro). The formamide reaction, with phosphate added, is an even richer system for generating the bases needed for polynucleotides (R. Saladino, E. Di Mauro and co-workers). The formose reaction is a way to generate sugars including ribose. This reaction is based on the conceptual idea that sugars are "polymers" of fomaldehyde, i.e. $(CH_2O)_n$. However, it is said that ribose is not very stable in such reactions. It may help to add phosphate to the reaction in the hope that a ribose-5-phosphate would be produced *and* is more stable, just as is the case in the Calvin cycle. In the Calvin cycle ATP and reducing potential are used to generate sugars. The end products of the cycle that pile up if activation energy, ATP and reduction, isn't available are the three pentose-5-phosphates, and ribose-5-P is one of them [*energy metabolism*]. To the extent that the formose reaction plus phosphate mimics the precursor in evolution to the Calvin cycle, we can expect ribose phosphate to now be available. How the bases are attached (it is another dehydration linkage) and which bases make up the available set are open questions. Contemporary nucleotides (bases already attached) are made in metabolism by a series of steps that are construction steps for building the base on site on the ribose-5-P. The simple view of using ready made bases, must explain how the bases are added and include at least ACGU. How big was the set of bases that includes ACGU ? Work in Nick Hud's laboratory (Heather Bean) has shown that glyoxylate is another candidate

for linking together nucleo**s**ides. Their model has several attractive features. However, glyoxylate is relatively high in Gibbs free energy and is easily hydrolyzed

$$C_2HO_3^- + 3H_2O = 2HCO_3^- + 2H_2 + H^+ + 142.3\ kcal/mol$$

Nevertheless, glyoxylate may occur in higher concentrations as part of Art Weber's triose model (Origin of Life and Evolution of the Biosphere (2007) 37:105–111).

The ideas expressed above regarding: 1) compartmentalization and division, 2) polymerization and polymer trapping inside compartments, 3) numbers of different sequences of different lengths for small numbers of different types of monomer, 4) natural selection from what is available !, 5) short polymers predominate until ligase activity is selected, 6) microsphere self-assembly from proteinoids and from polymers made from activated monomers, 7) selection of catalytic activities that promote sphere growth and division, 8) the emergence of chiral purity; these are just some of the conceptual issues addressed here. For each of these ideas there is already promising experimental evidence.

The stage is now set for the emergence of polynucleotides and their selection as information polymers. In the orthodox RNA World point of view, polynucleotides, RNA's, are selected for their *catalytic* activities. I have argued above that this is not the natural emergent property of polynucleotides. Rather catalytic precursors already occur robustly: in proteinoids, in polypeptides made from activated monomers, and, in proto-coenzymes that carry the pyrophosphate world signature. What is still lacking in this model is *genetics*, and that is where polynucleotides play a special role. It is also tantalizing that chiral purity may have emerged with polynucleotides as a necessity for **replication** and **transcription**, functions that are needed for *genetics*.

Experiments and observations about real molecules are the focus. We leave these material methods and do some conceptualizing instead. This activity is tempered by the constraints of reality as suggested by the experiments and observations. How far can we go into the *molecular logic*, nay, *molecular algebra*. We can do a molecular algebra and build models of what might have happened on the Earth. If this works, we have a way to understand the 20 unique aaRS's that couple cognate amino acids and nucleotide triplets.

My choice to state that the microsphere is the simplest unit that can be seen as living, like a cell, in the emerging structure built by the primitive Earth geochemistry has antecedents. I was influenced by the writing of Francois Jacob in his *The Logic of Life: A History of Heredity* (Pantheon Books, New York, 1973) in which he introduced the **Integron**. He concluded that the cell was the least living integron, not RNA. The RNA World took place well into the history of geochemical developments. Why does RNA coding for polypeptides evolve? Or, *What is it good*

for? It *is good for* directing production of polypeptide sequences in a coded way. What selects for the structures and the mechanisms? Certain short polypeptide sequences are in fact catalysts. They may represent a small percentage of what is available. If these sequences were much more available in the microsphere interior distribution of sequences then growth and division would be faster and the population of these types would flourish. However, the catalytic ability of polypeptides is universal among spheres. No genetic advantage can be established using these molecules. Thus, in a natural way RNA's would become coders for polypeptides. But do we really need, from the beginning, ribosomes, tRNA's, mRNA's and aaRS's? How does something molecular get started that can in fact evolve into this rather complex, multifaceted mechanism? Direct synthesis of polypeptides from RNA's activated at their ribose 2' hydroxyls by activated amino acids is imagined to occur with a conformational change in the RNA helix, driven by the attachment of amino acids (my book: *Energy and the Evolution of Life*, W.H. Freeman and Sons, New York, 1988). The primitive mechanism uses just one type of RNA, a proto-messenger RNA, or proto-mRNA. The tRNA's and the aaRS's must come into play later and together. Thus, so must the ribosome and its components. The primitive mRNA serves as a messenger because it directs polypeptide synthesis, and as a gene because it can be replicated.

What is the origin of the coding of codon triplets with amino acids. Strands of RNA rich in G and C replicate and code for polypeptides rich in arginine by direct affinity between arginine and the codon CGN (where N means *any base*). A hexamer of RNA only codes for 2 amino acids if the three base, one codon, rule is adhered to molecularly from the start. An amino acid hexamer requires an RNA at least 18 units long. This is a trimer of hexamers, and is available if a polynucleotide proto-ligase exists to connect the polynucleotide hexamers. As we saw, trimers (of hexamer/heptamer.. units) are the primary product, but there are also longer products with 4, 5,.. trimeric units. Poly-arginine is a candidate for a proto-ligase because it would be attracted to polynucleotides through an electrostatic interaction between the positive charge of the arginine residue and the negative charge of the polynucleotide back bone. This is a short range interaction with a Debye length under 10 angstroms. Nevertheless, when short polymers are properly aligned this strong interaction is very favorable thermodynamically, and such *oriented proximity* is the basis of catalysis. The RNA is of order 20 units long (trimeric in a hexamer, heptamer,… mix). It can replicate, at least produce a complementary copy, if there is a good polymerase (then the complement of the complement is a replica). Maybe polyarginine can facilitate monomer attachment, i.e. polymerization, by helping a different alignment (ligasing joins hexamer while polymerasing joins an activated monomer to a growing chain). Then a certain CGN rich sequence of RNA, 20 units long, could code for its replicase, and for the ligase needed for its synthesis from shorter segments, both in one molecule, a poly-arginine perhaps 5 units long. Or perhaps 2 arginines, 2 others and 2 arginines, in that order. This structure is a linker of negatively charged structures. In the case of arginine, there has been demonstrated using SELEX technology (Mike Yarus) a direct attractive interaction between the amino acid

and the codon. The code has a physico-chemical origin in this construction. The details will be explained in the next section.

The strands of RNA are trapped inside the spheres. If they replicate fast enough, the population of replicas within a sphere can be maintained during growth and division of the spheres. The *genotype* spreads. A gene coding for a polypeptide formation catalyst, a proto-polymerase for polypeptides, would also lead to increased populations of its genotype. The entire progression of genetic acquisitions is of great interest to imagine and unravel.