HOW SMALL CAN A MICROORGANISM BE?

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Abstract

Much of the volume of a bacterial cell is filled with machinery (ribosomes) that converts information in the genetic biopolymer (DNA) into information in the catalytic biopolymer (protein). This places a limit on the size of a two-biopolymer living system that all but certainly excludes cells as small as (for example) the structures observed in the Allan Hills meteorite derived from Mars. Life that uses a single biopolymer to play *both* genetic and catalytic roles could conceivably fit within a smaller cell, however. No biopolymer has yet been found that can play both roles, and the chemical demands for genetics and catalysis are frequently contradictory. A catalytic biopolymer should have many building blocks; a genetic biopolymer should have few. A catalytic biopolymer should fold easily; a genetic biopolymer should not. A catalytic biopolymer must change its physical properties rapidly with few changes in its sequence; a genetic biopolymer must be COSMIC-LOPER (Capable Of Searching Mutation-space Independent of Concern over Loss of Properties Essential for Replication), with physical properties largely unchanged by changes in sequence. This article reviews the chemical plausibility of a single biopolymer that might make an effective compromise between these competing demands, and therefore permit life within very small cells.

Two-biopolymer Life-forms and One-biopolymer Life-forms

In terms of its macromolecular chemistry, life on Earth is a "two-biopolymer" system. Nucleic acid is the genetic biopolymer, storing information within an organism, passing it to its descendants, and suffering the mutation that makes evolution possible. Nucleic acids also direct the biosynthesis of the second biopolymer, proteins. Proteins generate most of the selectable traits, from structure to motion to catalysis. The two-biopolymer strategy evidently works well. It has lasted on Earth for billions of years, adapting to a remarkable range of environments, surviving formidable efforts by the cosmos to extinguish it, and generating intelligence capable of exploring beyond Earth.

The terrestrial version of two-biopolymer life contains a well-recognized paradox, however, one relating to its origins. It is difficult enough to envision a non-biological mechanism that would allow either proteins or nucleic acids to emerge spontaneously from non-living precursors. But it seems astronomically improbable that *both* biopolymers arose simultaneously *and* spontaneously, and even more improbable that both arose spontaneously, simultaneously, and as an encoder-encoded pair.

Accordingly, "single-biopolymer" models have been proposed for life that may have preceded the two-biopolymer system that we know on contemporary Earth (Joyce et al., 1987). Such models postulate that a single biopolymer can perform both the catalytic and genetic roles and undergo the Darwinian evolution that defines life (Joyce, 1994). RNA was proposed some time ago as an example of such a biopolymer (Rich, 1962; Woese, 1967; Orgel, 1968; Crick, 1968). This proposal became more credible after Cech, Altman, and their coworkers (Cech et al., 1981; Zaug and Cech, 1986; Guerrier-Takada et al., 1983) showed that RNA performs catalytic functions in contemporary organisms. The notion of an "RNA world," an episode in natural history when RNA served both genetic and catalytic roles, is now part of the culture of molecular biology (Watson et al., 1987).

Single-biopolymer Systems and Extraterrestrial Life

"Single-biopolymer" models for Darwinian chemistry have relevance to the search for extraterrestrial life. For example, some biologists have argued that the microstructures identified by McKay et al. (1996) in the Allan Hills meteorite, which are 20 to 100 nanometers across, are too small to be the remnants of living cells (Kerr, 1997). The argument is that the ribosome is 25 nm across, ribosomes are a requirement for life, and placing ribosomes (ca. four ribosomes across the short dimension of the "cell") in the cell would exclude virtually every other biomolecule.

This view is narrowly formulated. Ribosomes are a requirement for a two-biopolymer life-form, such as those known on contemporary Earth. If a single-biopolymer (such as RNA) can serve both genetic and catalytic functions, ribosomes are not required. A smaller cell may be sufficient to hold a single-biopolymer life-form.

How much smaller might the cells of a single-biopolymer life-form be (excluding parasitic cells)? Translation places demands upon the volume of a typical two-biopolymer cell. If we do not consider water, approximately half of the material inside an *E. coli* cell is ribosomes, tRNA, and mRNA (Lewin, 1985). Thus, a single-biopolymer cell can be half the size of a two-biopolymer cell simply by discarding the translation material. Of the remaining half of the dry weight of the intracellular contents of *E. coli*, aminoacyl tRNA synthetases, proteins that form transcription complexes, and proteins catalyzing amino acid biosynthesis are a major contributor. Together, biomolecules required to support translation comprise more than half of the soluble proteins that form the "core metabolism" encoded by the protogenome (Benner et al., 1993), the organism at the hypothetical threefold point joining Archaea, Eucarya, and Eukaryota in the universal tree of life.

Models can be built for a minimal metabolism that might be used by a single-biopolymer life-form. If that biopolymer is RNA, Figure 1 offers an autotrophic metabolism that involves fixation of carbon dioxide (4 catalysts, by analogy with the reductive tricarboxylic acid cycle), carbohydrate biosynthesis (5 catalysts exploiting cyanide-based couplings and aldol reactions), triphosphate generation (6 catalysts), nitrogen metabolism (3 catalysts), and nucleotide biosynthesis (28 catalysts, adopted directly from contemporary pathways). Ignoring the thermodynamics of this pathway (which are expected to be favorable under reducing conditions; see McCollom and Shock, 1997), this model sustains a single-biopolymer life-form with ca. 50 biocatalysts. Although additional macromolecules would undoubtedly be useful to biosynthesize membrane components, transport metal ions and cofactors, and participate in gene regulation, a plausible model for minimal single-biopolymer autotrophic life could almost certainly be limited to fewer than 100 macromolecules, less than 10% of the number found in a typical autotrophic two-biopolymer genome. If different types of catalysts can have the same size, a single-biopolymer life-form might fit within a cell having 5% the volume of a contemporary terrestrial bacterium. This implies that the microstructures in the martian meteorite might *not* be too small to be fossils of a single-biopolymer form of life. Conversely, if the meteorite structures are indeed fossils, then they almost certainly are fossils of an organism that used only a single biopolymer.

Does a Single Biopolymer Exist That Is Capable of Genetics *and* Catalysis?

This discussion suggests that the answer to the title question depends on the answer to the question: Does a single biopolymer exist that can robustly do both genetics and catalysis. In this discussion, we focus on RNA as the most highly regarded candidate for the single biopolymer.

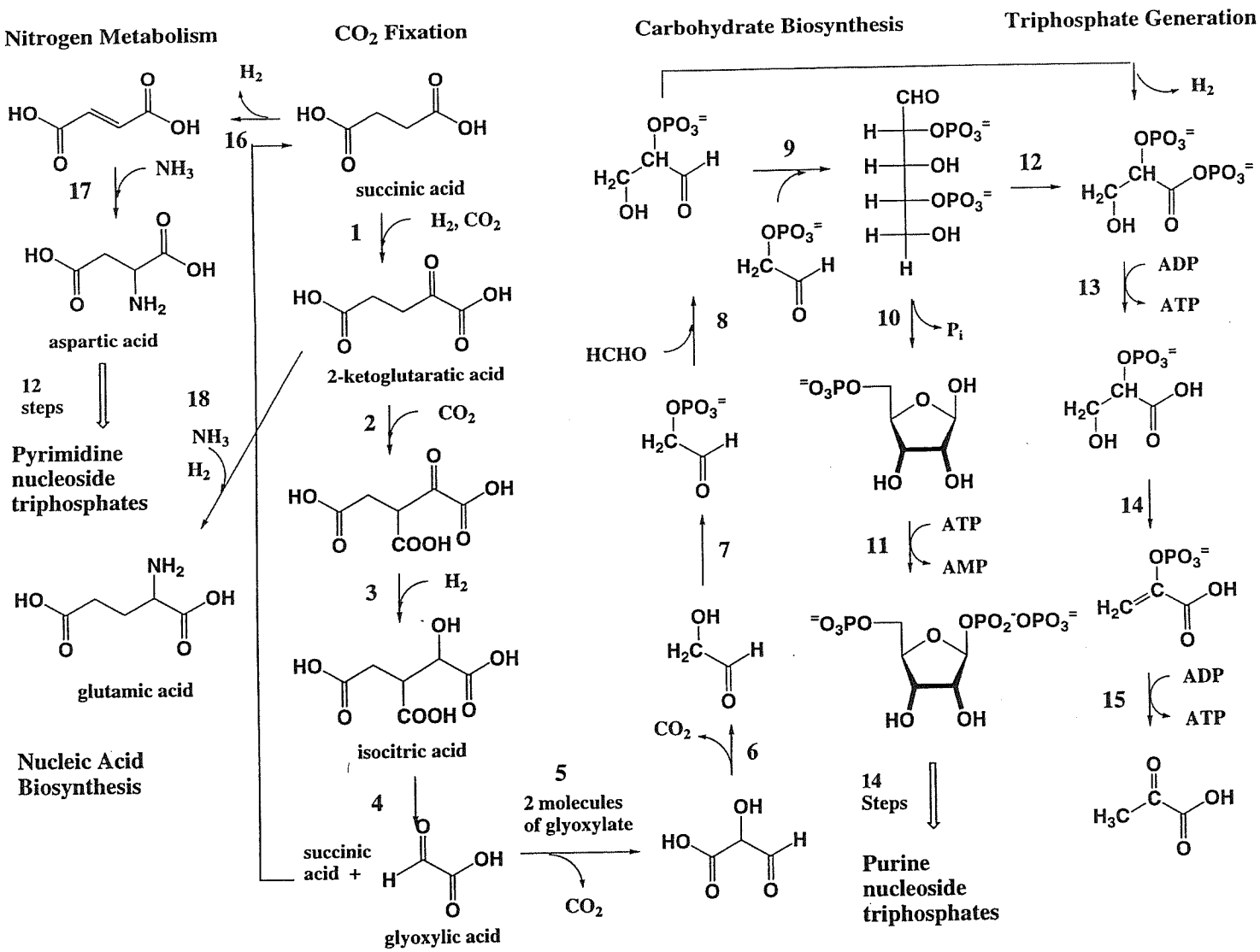

Figure 1. A hypothetical metabolism for a single-biopolymer autotrophic life-form based on RNA. Each reaction has a precedent in known chemistry, biological or non-biological. The driving force for the overall synthesis is not defined. The overall process would be exergonic under reducing conditions (see McCollum and Shock, 1997).

The Requirements for Genetics

A NASA workshop defined life as "a self-sustaining chemical system capable of undergoing Darwinian evolution" (Joyce, 1994). The genetic component of this definition is contained within the concept of Darwinian evolution. It includes not only the ability to be reproduced, but also the ability to survive mutation in a way that can create a change in phenotype that is selectable.

As discussed elsewhere (Benner and Switzer, 1998), many molecular systems can be reproduced and can form structures, catalysis, or other lifelike phenotypes. The most substantial challenge facing those attempting to develop a system that models life is to identify a biopolymer that can undergo mutation in a non-destructive way. Specifically, to support Darwinian evolution, a biopolymer must be able to search "mutation-space" independent of concern that it will lose properties essential for replication. If a substantial fraction of the mutations possible within a genetic information system cause a biopolymer to precipitate, unfold, or otherwise no longer be recognizable by the catalyst responsible for replication, then the biopolymer cannot evolve. We designate polymers that have this property as COSMIC-LOPER biopolymers (Capable of Searching Mutation-space Independent of Concern over Loss Of Properties Essential for Replication).

DNA and RNA are COSMIC-LOPER biopolymers. A mutant of a DNA sequence is as likely to dissolve in water, pair via Watson-Crick rules, template complementary strands, and be a substrate for DNA polymerases as its parent. The COSMIC-LOPER behavior is not absolute. If an RNA sequence wanders into a G-rich region of sequence space, it may become insoluble, or otherwise incapable of acting as a template. But these regions are exceptions.

Because of the familiarity of the "rule-based" molecular recognition properties displayed by DNA and RNA, the uniqueness of nucleic acids with respect to their COSMIC-LOPER behavior is often overlooked. In fact, very few classes of organic molecules can suffer changes in structures without significant changes in their physical properties. Perhaps the best example is proteins. The physical properties of proteins (including their solubility) can change dramatically upon point mutation within the mutation space allowed by the 20 standard amino acids. Again, there are many examples of this phenomenon in Nature (for example, hemoglobin in sickled cells). Designed peptides provide other examples. For example, altering their structure of a peptide designed to catalyze the decarboxylation of oxaloacetate by a single acetyl group changed substantially their level of aggregation, while altering their internal sequence at a single residue changes substantially their helicity (Johnsson et al., 1990, 1993). If solubility and/or helicity are essential to the replicatability of a peptide template, a large range of plausible mutation would destroy it. Protein is not COSMIC-LOPER, and is not expected to serve well as a genetic biopolymer, despite its acknowledged virtues as a catalytic biopolymer.

Starting in the 1980s, various groups altered the structure of nucleic acids to learn what structural features enable the rule-based molecular recognition properties (for a review, see Benner et al., 1998). The polyanionic nature of the oligonucleotide backbone appeared to be an important component of the COSMIC-LOPER behavior of nucleic acids; modifications of that backbone to remove the repeating charges created a biopolymer that no longer displayed rule-based molecular recognition (Richert et al., 1996).

Further, work expanding the number of letters in the genetic alphabet uncovered an intriguing relationship between the number of building blocks in a biopolymer and the fidelity of its synthesis. A genetic polymer should be replicated with a high (if not perfect) degree of fidelity. From both theory and experiment (Szathmary, 1992; Lutz et al., 1996), one expects higher fidelity with smaller genetic alphabets than large genetic alphabets.

The Requirements for Catalysis

Binding and catalysis (which may be viewed as binding to a transition state) require that the biopolymer present a series of specific interacting groups to the substrate. Here, diversity is advantageous. A case can be made that the 20 amino acid side chains found in natural proteinogenic amino acids provide a good sampling of the diversity that is available, in that it includes cationic groups, anionic groups, hydrophilic neutral groups, hydrophobic aliphatic groups, aromatic groups, and heterocycles, general acids and general bases, and nucleophilic groups. It has deficiencies. The standard 20 amino acids underrepresent heterocycles (compared, for example, with the U.S. Pharmacopoeia), it lacks a range of redox active side chains, and it is missing an electrophilic reactivity. But much of the diversity required for catalysis is present in standard proteins.

Catalysts must also surround a transition state, delivering contacting interactions from all sides. This, in turn, requires folding. Via a backbone with an equal number of hydrogen bond donors and acceptors, peptides fold well. Indeed, the feature most characteristic of proteins is that they precipitate (Benner, 1988b). Precipitation is folding, arising when the peptide prefers to interact with other peptides than with solvent. DNA and RNA in contrast, have a backbone of repeating negative charges. In the absence of cofactor (most commonly, divalent metal ion), there is no backbone-backbone interaction that supports the folding of an oligonucleotide (Richert et al., 1996).

The Contradicting Chemical Features Required for a Biopolymer That Does Both

This discussion makes evident that catalysis on one hand and genetics on the other place competing and contradictory demands on molecular structure. This implies in turn that it is difficult to find a single biopolymer that does both, suggesting that single-biopolymer life-forms might be less robust than two-biopolymer life-forms and that the small cells that single-biopolymer life enables might be scarcer in the universe than large cells. Let us review three specific contradictions:

1. A biopolymer specialized to be a catalyst must have many building blocks, so that it can display a rich versatility of chemical functionality required for catalysis. A biopolymer specialized for genetics must have few building blocks, as a way of ensuring faithful replication.
2. A biopolymer specialized to be a catalyst must fold easily so that it can form an active site. A biopolymer specialized for genetics should not fold easily, so that it can serve as a template (Richert et al., 1996).
3. A biopolymer specialized for catalysis must be able to change its physical properties rapidly with few changes in its sequence, enabling it to explore "function space" during divergent evolution. A biopolymer specialized for genetics must have physical properties largely unchanged even after substantial change in sequence (the COSMIC-LOPER property).

At the very least, a single-biopolymer attempting to support Darwinian evolution must reflect some sort of structural compromise between these goals. No fundamental principle guarantees that a polymeric system will make this compromise in a satisfactory way, however. The demands for functional diversity, folding, and rapid search of function space might be so stringent, and the demands for few building blocks, templating ability, and COSMIC-LOPER ability so stringent, that *no* biopolymer structure achieves a suitable compromise. Even if one exists, it may perform genetics and/or catalysis with poor robustness. Single-biopolymer life would then be fragile and easily extinguished. Life would be scarce in the universe because most of the initial forms would be driven to extinction before they

could leap to a two-biopolymer structure. Conversely, if many-polymeric systems exist that make an acceptable compromise between the demands of catalysis and the demands of information storage, life would have emerged rapidly via single-biopolymer forms and be abundant in the universe in diverse forms.

Theoretical Evidence for a Robust Single-biopolymer System

Well before experiments were brought to bear on this problem, a theoretical argument was available that suggested that a single-biopolymer life-form might be possible. It began with three stipulations: (1) that life on Earth did not arise via divine intervention, (2) that spontaneous generation of a two-biopolymer system is not possible, but (3) that spontaneous generation of one biopolymer is possible. From the (obvious) fact that life exists on Earth, it can be concluded that a single biopolymer must have existed that performs both genetics and catalysis; this is the only way to explain the origin of life on Earth.

This proposal in one of various forms was made in the 1960s (Rich, 1962; Woese, 1967; Orgel, 1968; Crick, 1968). The extent to which the proposal begs questions was ameliorated by a rational analysis of contemporary biochemistry that began in the 1970s, when Usher and McHale (1976), White (1976), and Visser and Kellogg (1978) suggested that elements of contemporary metabolism (in particular, the structure of cofactors) might be viewed as vestiges of an "RNA world" (Gilbert, 1986). The emerging field of genomics was then used to generate internally consistent reconstructions for the ancient single-biopolymer life-forms. These reconstructions concluded from the abundance of its vestiges in modern metabolism that the RNA world was metabolically complex (Benner, 1988a; Benner et al., 1989; Benner et al., 1993). In modern metabolism, RNA fragments play roles for which they are *not* intrinsically suited. This suggests that these fragments originated during a time in natural history where RNA was the only available biopolymer, rather than by convergent evolution or recruitment in an environment where chemically better-suited biomolecules could be encoded. If the RNA world developed the RNA cofactors, ATP, coenzyme A, S-adenosylmethionine, and NADH, it follows that the RNA world needed these, presumably for phosphorylations, Claisen condensations, methyl transfers, and oxidation-reduction reactions (respectively).

These models imply that the RNA-based single-biopolymer life upon which all terrestrial life is founded had a complicated metabolism. This, in turn, implies that RNA *can* catalyze a wide variety of chemical reactions. This may be taken as indirect support for the existence of single-biopolymer life-forms, and from there, the possibility of very small cells.

The Experimental Evidence

These types of arguments, together with the discovery of RNA catalysis, made hopes high when Szostak (1988), Joyce (1989a,b), Gold (Irvine et al., 1991), and their coworkers introduced "in vitro selection" as a combinatorial tool to identify RNA molecules that catalyze specific reactions. If RNA was indeed as effective a catalyst as the reconstruction of the RNA world would imply, in vitro selection should rapidly generate the ultimate goal, an RNA (or DNA) molecule that catalyzes the template-directed polymerization of RNA (or DNA), a molecular system able to undergo Darwinian evolution. If selection procedures were appropriately designed, they should also produce RNA catalysts for almost any other reaction as well.

In contrast with these hopes (and only by this contrast), in vitro selection has been disappointing. RNA has proven to be an intrinsically poor matrix for obtaining catalysis, especially when compared

with proteins. For example, to have a 50% chance of obtaining a single RNA molecule capable of catalyzing a template-directed ligation reaction by a modest (by protein standards) factor of 10,000, Bartel and Szostak (1993) estimated that one must sift through 2×10^{13} random RNA sequences 220 nucleotides in length. To obtain a catalyst with a factor of 10 greater catalytic power, one must increase the size of the library being searched by a factor of 1,000. This is poor catalysis, at least by comparison with proteins.

Although many laboratories have tried, only a few have managed to extend the scope of RNA catalysis beyond the phosphate transesterification reactions in which it was originally observed. For example, attempts to obtain an RNA catalyst for a Diels-Alder reaction using in vitro selection failed (Morris et al., 1994); the same reaction is readily catalyzed by protein antibodies (Gouverneur et al., 1993). Attempts to obtain RNA that catalyzes amide synthesis have succeeded, but with difficulty (Zhang and Cech, 1997; Wiegand et al., 1997). The fact that such successes came only after many attempts is indicative of a relatively poor catalytic potential in oligonucleotides.

The comparison with peptides is instructive. For example, short (14 amino acids) peptides accelerate the rate-determining step for the amine-catalyzed decarboxylation of oxaloacetate by more than three orders of magnitude (Johnsson et al., 1993), not far below the acceleration observed for the first-generation ligases observed in the Bartel-Szostak selection beginning with 10^{13} random RNA sequences. Further, the peptide is less than 10% the size of the RNA motif. Combinatorial experiments starting from this design (Perezpaya et al., 1996; Baltzer, 1998) suggested that perhaps only 10^7 random sequences must be searched to get a similar catalytic effectiveness as is observed in a library of 10^{13} RNA molecules. This suggests that peptides are intrinsically a millionfold fitter as catalysts than RNA.

The comparison is imperfect, of course, as it involves different reactions and different design strategies. This imperfection characterizes most of the comparisons that can be made at present. Not surprisingly, ribozymes are most frequently sought for reactions where oligonucleotides are most likely to be effective catalysts (for example, where oligonucleotides themselves are substrates), while peptide catalysts are most frequently sought for reactions suited for peptide catalysts (for example, those that make use of functional groups found on amino acid side chains). This makes the comparison non-quantitative, but useful nevertheless as an estimate of how well oligonucleotides and oligopeptides respectively perform when challenged by their favorite target reactions.

Biopolymers That Are Not (Exactly) RNA Or DNA

The failure of in vitro selection experiments with RNA to rapidly generate self-replicating systems challenges the notion that life emerged in a fashion directly analogous to the way in which in vitro selections are presently being done in the laboratory. This, in turn, means that these experiments failed to provide positive evidence that a single-biopolymer system exists, which in turn implies that we cannot confidently invoke a single-biopolymer life-form when we wish to argue that a very small structure (for example, on Mars) is a vestige of a primitive cell.

These experiments provided a direction, however. The apparent superiority of proteins as catalysts compared with RNA reflects (at the very least) the availability to proteins of a wider range of building blocks and catalytic functionality than in RNA. RNA lacks the imidazole, thiol, amino, carboxylate, and hydrophobic aromatic and aliphatic groups that feature so prominently in protein-based enzymes. RNA has only hydroxyl groups, polar aromatic groups, and phosphate groups. An uncounted number of studies with natural enzymes and their models has illustrated the use of this functionality by protein catalysts (Dugas, 1989).

Several groups are now seeking to add functionality to RNA and DNA. RNA might gain functionality

using cofactors, much as contemporary proteins gain the functionality that they lack through vitamins. In a sense, this was already done in in vitro selection experiments, which nearly universally use the divalent magnesium cation, essentially as a cofactor. More recently, Breaker and his coworkers have expanded the approach to include organic molecules as second ligands in riboenzymes (Tang and Breaker, 1997).

A second solution was to append functionality to the standard nucleotides (Tarasow et al., 1997). Prompting this suggestion was the observation that contemporary tRNA and rRNA contain much of the functionality found in proteins but lacking in contemporary *encoded* RNA, including amino, carboxylate, and aliphatic hydrophobic groups (Limbach et al., 1994). These functional groups are introduced by post-transcriptional modification of encoded RNA. Some of these might even be placed by parsimony in the protogenome (Benner et al., 1989).

A third way to expand the functional diversity of nucleic acids is to increase the number of nucleotides in the nucleic acid alphabet. This can be done by using the non-standard hydrogen-bonding patterns permitted by the geometry of the Watson-Crick base pair (Switzer et al., 1989; Piccirilli et al., 1990). Additional letters in the genetic alphabet could carry a richer diversity of functionality. Indeed, one might imagine a new type of biopolymer, one carrying functionalization like proteins but able to be copied like nucleic acids (Kodra and Benner, 1997).

Conclusions

Each approach outlined above to increase the catalytic power of RNA as a single-biopolymer is only beginning to be explored. The title question will be answered only as this work proceeds. We believe that some of the most exciting results in chemistry in the next decade will come from efforts attempting to resolve the contradictions between catalysis and genetics in single-biopolymer systems in a way that will generate a biopolymer capable of both genetics and catalysis.

This question has implications for planetary exploration. The experiments with nucleic acid analogs has suggested as a hypothesis that a universal chemical characteristic of genetic biopolymers in water is a repeating charge, either an anion or a cation. This repeating charge may be both necessary and sufficient for COSMIC-LOPER behavior (Richert et al., 1996; Benner and Switzer, 1998). A repeating charge is a convenient biomarker for non-terrean genetic molecules. Future planetary probes might well search for such molecules.

Further, a single-biopolymer system should sustain work on Earth to learn how metabolic pathways might have emerged. In vitro selection permits would permit sequential selection for catalysts for individual metabolic steps (as shown in Figure 1). This would provide an experimental approach to identify the minimal cell, may generate new biomarkers, and could assist in the search for life on other planets.

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