TEMPLATE-DIRECTED CHEMISTRY AND THE ORIGINS OF THE RNA WORLD

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Abstract. Prompted by the growing number of reports about reactions catalyzed by ribozymes, this paper summarizes mechanistic and kinetic aspects of template-directed (TD) chemistry important for the synthesis of a diverse population of polynucleotides and analogues possibly up to 100 units long. Assuming that this chemistry takes place in a microenvironment conducive to life under the constant influx of mM concentrations of activated monomeric building blocks, the proposed scenario represents a working hypothesis for the prebiotic synthesis of the RNA world.

1. How Did the RNA World Emerge?

Recent advances in biotechnology have made routine the synthesis, analysis and manipulation of nucleic acids. Exploiting this technology, Cech, Altman and coworkers established the ability of certain RNAs to catalyze reactions at the phosphodiester linkage with 'enzymatic' specificity (Cech, 1986a,b; Been and Cech, 1988; Gurrier-Takada *et al.*, 1983). More recent work brought up the possibility that ribosomal RNA may catalyze the formation of the peptidic bond (Noller *et al.*, 1992; Piccirilli *et al.*, 1992). Using *in vitro* genetics (Szostak, 1992), Szostak's and Joyce's group demonstrated the potential of RNAs to evolve in a Darwinian sense (Beaudry and Joyce, 1992; Lehman and Joyce, 1993; Green and Szostak, 1992). These results make the RNA world hypothesis (Gilbert, 1986) more appealing every day.

Nevertheless, the prebiotic synthesis of the RNA world has been seriously questioned (Shapiro, 1988; Joyce, 1989; Joyce and Orgel, 1993). Two major difficulties have been identified: Firstly, the synthesis of the ribonucleotides requires a series of steps that all suffer from low yields and low regiospecificity and secondly, problems inherent in the non-enzymatic, template-directed (TD) synthesis of RNAs make it doubtful that substantial amounts of long RNA oligomers could be made using this type of chemistry (for reviews on TD reactions see Orgel, 1986; Joyce, 1987; Orgel, 1992).

The present paper addresses the latter problem, i.e. the synthesis of a large and diverse population of RNAs from activated ribomononucleotides. To this end I will review kinetic and mechanistic aspects of TD chemistry that are relevant to this problem, and then offer a scenario for the synthesis of long informational biopolymers.

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2. Metal Ion and Mineral Catalyzed Oligomerization of Nucleotides

It is clear that TD reactions require the presence of a preformed oligo- or polynucleotide which can act as a template. Hence the condensation of monomers facilitated by other catalysts besides a template is an important first step in the creation of the RNA world. A recent review (Ferris, 1993) enumerates related studies and concludes that small amounts of oligomers at least up to twenty units long could have been formed on mineral surfaces and/or by means of metal ion catalysis.

3. Pitfalls in TD Reactions

Experiments performed by Orgel's and Schwartz's groups support the idea that a variety of short oligomers of nucleic acids and nucleic acid analogs (Visscher and Schwartz, 1988; Visscher *et al.*, 1989; Tohidi and Orgel, 1989; Harada and Orgel, 1990) enhance the synthesis of a family of oligomers that can be regarded, in a broad sense, complementary to the template used. In special cases templates can be as short as a tetramer (Zielinski and Orgel, 1987). However, copying of a random sequence RNA molecule is still not feasible, especially if it contains a stretch of purine bases (Wu and Orgel, 1992c). An additional problem is that in the ribonucleotides the 2′-OH is intrinsically more nucleophilic than the 3′-OH (Lohrman and Orgel, 1978; Sawai, 1988) and in most experiments an abundance of the unnatural 2′-5′ linkange is obtained. In view of the discussion to follow, these two problems may not be as daunting as they seem on first inspection. A third, more serious, problem relates to the enantiomeric inhibition of oligomer synthesis observed in two cases so far: with DL racemic 2-MeImpG in the oligoguanylate synthesis (Joyce *et al.*, 1984) and with Rp and Sp diastereomers of adenosine-5′-thiophosphobenzimidazolide in the UO_2^{2+}-catalyzed oligothioadenylate synthesis (Shimazu *et al.*, 1993). It is unknown how general this inhibition will turn out to be in other systems, but it is quite clear that no satisfactory answer has been given yet to the origin of the optical purity of the biomolecules (Bonner, 1991).

4. Intricacies of TD Chemistry

Although the inadequacy of TD chemistry to produce self-replicating RNA-like systems has been widely discussed (Joyce and Orgel, 1993), it is my contention that its possible contribution to prebiotic evolution has been underestimated. Let me briefly review what has been learned about TD chemistry, so that its potential can be evaluated.

4.1. Cooperativity

A template can be visualized as a string replete with binding sites. The binding sites can be filled by complementary monomers. The affinity of a monomer to an isolated site on the template is determined by its hydrogen-bonding capability

(Watson-Crick base-pairing). The association of a monomer with a site immediately adjacent to one that is already occupied is stronger than with an isolated site because the binding is enhanced by stacking (see more on base stacking later). This means that the association of monomers with the template is cooperative (Davies and Davidson, 1971; Hill, 1985) and monomers will associate preferentially as stacks rather than bind to isolated sites on the template. This is even true at rather low template and monomer concentrations. The complex between the segment of the template and the stack of monomers bound to it usually becomes part of a double helix. The thermodynamics of homopolymer-monomer interactions have commonly been treated by postulating two association constants, q and Q (Gukovskaya *et al.*, 1980). q refers to the association of a monomer on an isolated site and Q refers to the association of a monomer adjacent to another monomer; the difference between the corresponding energies, E_Q–E_q, is the stacking energy between the two monomers. Q is presumably independent of the length of the stack. The larger the ratio, Q/q, the stronger the cooperativity, and hence the higher the preference for longer stacks. The degree of association is enhanced by specific salt effects, higher ionic strength, lower temperature (Davies and Davidson, 1971; Gukovskaya *et al.*, 1980) and possibly the presence of metal ions and other molecules that could stabilize the duplex. The number and the values of the association constants necessary to describe heteropolymer/monomers interaction can be estimated based on the nearest-neighbor approximation.

It should be noted that even though the above thermodynamic treatment of polymer-monomer interactions is valid, the actual mechanism of association of monomers with the template may be more complex. This is because nucleotides have a tendency to form stacks even in solution (Ts'o, 1974). For example, we have evidence that at room temperature, at ionic strength of 1M with NaCl and at concentrations in the order of 5–10 mM, 5′GMP, 5′AMP and 5′CMP self-associate; 5′UMP does not (Kanavarioti *et al.*, 1992). Base-stacking may be more pronounced with the phosphoimidazolide derivatives than with the free nucleotides because at physiological pH the former are predominantly zwitterionic, whereas the nucleotides carry two negative charges that destabilize intermolecular interactions (Howard *et al.*, 1966). Hence it is likely that not only single monomer molecules but entire monomer stacks become associated with the template in one step (Huang and Ts'o, 1966).

It is predicted and shown experimentally that polynucleotide-mononucleotide complexation is independent of the concentration of the template* but requires a 'critical' concentration of monomer – in most cases a few mM – above which most of the additional monomer accumulates on the template until the template is practically 100% saturated (Davies and Davidson, 1971; Hill, 1985). The implication with respect to a prebiotic microenvironment is that an informational biopolymer,

* True statement only if the polymer is infinitely long or of such length that end-effects can be neglected. With oligomers acting as templates complexation would depend both on the length and the concentration of the oligomer.

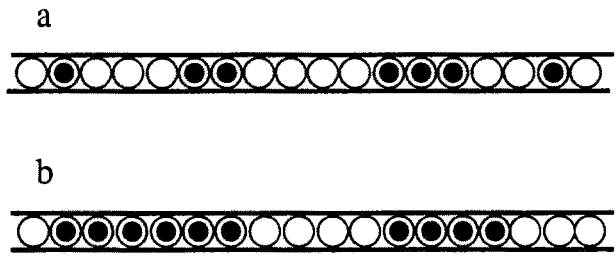

Fig. 1. Polymer/monomer interactions: Template represented as string of open circles; associated monomer as filled circles. Schematic illustration of systems (a) with low template site occupancy and (b) high template site occupancy (see text).

no matter how little is present, will be complexed by the complementary monomeric units as long as their concentration exceeds the critical value. Unfortunately thermodynamic studies on polymer/monomers systems relevant to oligomerization reactions are scarce (Kanavarioti and Hurley) but will hopefully be forthcoming in the near future. A desirable objective of such studies will not only be to evaluate the cooperativity of specific systems but to search for conditions or potential stabilizers to enhance this cooperativity. Stable complex formation, although not a sufficient requirement for oligomer synthesis, should be considered a prerequisite condition when testing for a template effect.

4.2. PARAMETERS THAT INFLUENCE THE DEGREE OF POLYMERIZATION

In the discussion to follow I presume that complex formation is established much faster than any chemical step. An important factor then that affects the degree of polymerization is occupancy of the template by monomers. Low template occupancy could be the result of low hydrogen bonding affinity of the monomer for the template (q), and/or low cooperativity (Q/q) and/or low monomer concentration (Figure 1a). Conversely, high template occupancy is the result of a high q, and/or a high Q/q, and/or high monomer concentration (Figure 1b). For obvious reasons elongation of a preformed oligomer will be faster on a more saturated (Figure 2a) than on a less saturated template (Figure 2b). But there are additional parameters that influence the degree of polymerization.

Let us base our discussion on a specific reaction that has been extensively studied. The reaction is the poly(C)-directed oligomerization of guanosine 5′-phosphate-2-methylimidazolide (2-MeImpG) shown in equation 1 where M stands for the monomer 2-MeImpG and G_2, G_3... G_i... are oligoguanylates of length 2, 3, etc. (Inoue and Orgel, 1982; Kanavarioti *et al.*, 1993). For simplicity, poly(C) and Mg^{2+} that are known to catalyze each step are not shown in eq 1. As implied by eq 1 elongation steps are believed to be stepwise, that is the tetramer is formed from the trimer, the pentamer from the tetramer and so on.

$$M \xrightarrow{k_2[M]} G_2 \xrightarrow{k_3[M]} G_3 \xrightarrow{k_4[M]} G_4 \longrightarrow\longrightarrow G_{i-1} \xrightarrow{k_i[M]} G_i \longrightarrow\longrightarrow \xrightarrow{k_n[M]} G_n \qquad (1)$$

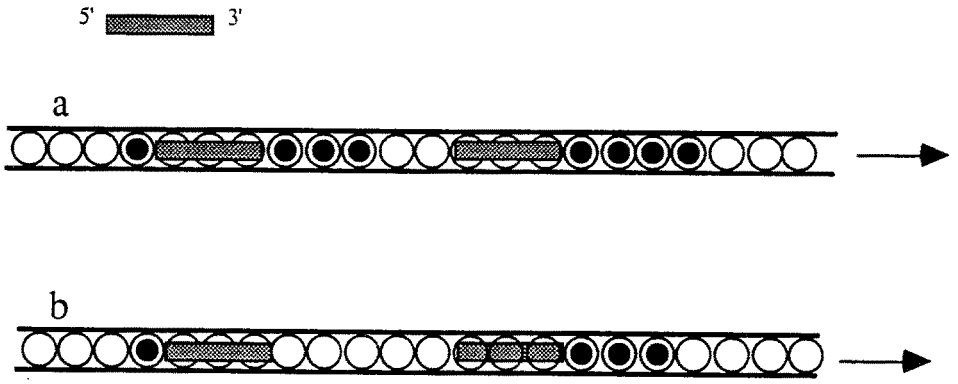

Fig. 2. The elongation process. Rectangles represent oligomers which can only elongate at their 3′ end (right). Filled circles represent associated monomers. (a) High template site occupancy. Both oligomers have associated monomers at their 3′-end. (b) Low template site occupancy. Two oligomers shown, but only one is reactive at any given time because there is not enough monomer.

Envision a poly(C) template that is 100 units long and completely saturated with monomers. The oligoguanylate products are expected to span a range from the dimer up to the 100-mer. An important question is where does the maximum of this distribution lie? The mean oligomer length depends, among other parameters, on the competition between dimerization and elongation, based on the following reasoning: Dimerization is the only reaction that can happen immediately following association. Once a small number of dimers is present, dimerization competes with formation of the trimer and with the other elongation steps for the consumption of the monomer. Competition between elongation and dimerization can be understood as follows: If elongation is only as fast as dimerization, few oligomers longer than the dimer are expected. This is because all monomers have a neighbor and statistically all can react to form a dimer, but very few monomers will be associated at the reactive end of a dimer, since dimers are just beginning to form. On the other hand, the faster the elongation as compared to dimerization, the longer the mean length of the products will be at the onset of the reaction (Kanavarioti and Bernasconi, 1990). If the systems are left to equilibrate, ligation, i.e. the reaction of two oligomers, will become important and, in principle, both systems (fast or slow elongation) should lead to the formation of a single product, i.e. the 100-unit long complementary polynucleotide. This will of course only occur in 'ideal systems' where the activated monomer is not subject to deactivation (hydrolysis) on the time scale of the oligomerization. In 'real systems' oligomers deactivated at the 5′-end as well as excess of deactivated monomer molecules adsorbed on the template will impede elongation and ligation and therefore the synthesis of the perfectly complementary product will not be realized. Experiments where the reaction is rekindled, i.e. furnished with new activated monomer, result in longer products (Joyce, 1984) and clearly show the detrimental effect of monomer hydrolysis even

if the latter is seemingly slow (Kanavarioti *et al.*, 1989). Hence deactivation of both the monomer and oligomers is another major parameter in determining oligomer length. In conclusion, for a TD reaction to produce long oligomeric products it is necessary that the relevant association constants favor stable and long helical complexes between template and monomers (the optimal case being a saturated template) and that the competition between hydrolysis, dimerization and elongation favors the latter. This conclusion is valid even when activated monomer is produced at a constant flux.

4.3. The effect of neighboring molecules

Although surprising at first, there is compelling evidence that, for optimal reactivity, TD processes require additional template-bound monomers besides the one(s) involved in the reaction. It was shown for many different combinations of nucleobases that elongation becomes increasingly more efficient in the presence of up to two additional neighboring monomers (Wu and Orgel, 1992a,b,c and Figure 3a). It is even more surprising that this phenomenon also applies in the case of a G-oligomer elongating with a G-monomer (Wu and Orgel, 1992a; Kanavarioti *et al.*, 1993), because this combination exhibits the strongest base-stacking and the strongest hydrogen-bonding to the template and hence would be expected to require catalysis the least. If the need for next-neighbors is not due to general acid-base catalysis (Wu and Orgel, 1992a), then it is plausible that the presence of associated neighbors stabilizes the transition state comprised of the template, oligomer, stacked monomers and one or more Mg^{2+} ions by enforcing a more rigid configuration between the reactants (Kanavarioti *et al.*, 1993). In a way, elongation becomes more akin to ligation with the advantage that in a ligation (reaction of two oligomers) the reacting groups are to some extent forced into the conformation of the product whereas in an elongation (reaction of an oligomer with a monomer) the monomer has additional degrees of freedom.

Another surprising recent finding is that the template-assisted formation of a G-dimer occurs within a stack of six or more G-monomers (Kanavarioti *et al.*, 1993 and Figure 3b). This does not mean that dimerization in a stack of two units is impossible, only that it is kinetically much less favored. Considering that dimerization has to compete with monomer deactivation, the presence of longer stacks where bond formation is efficient becomes rather critical in determining whether or not dimers and other oligomers will be produced. If, in general, dimerization among nucleotides is favorable only within a long stack, this requirement could easily explain why in some cases oligomerizations are inefficient. For example, the inability to detect oligouridylates in the poly(A)/mono(U) system (Stribling and Miller, 1991) is consistent with the negligible base stacking exhibited by uracil derivatives.

Two interesting implications result from the postulated mechanisms of dimerization and elongation. Firstly, in the presence of small amounts of reactants, elongation (oligomer+3monomers) is entropically more favored than dimerization

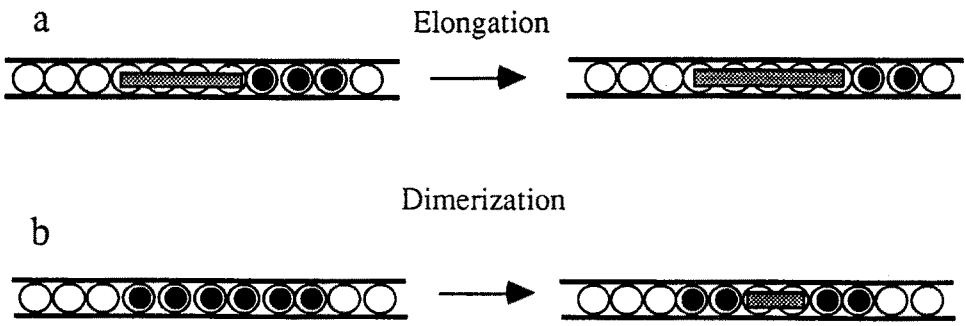

Fig. 3. Elongation versus dimerization. The template is represented as a string of open circles and the template-bound monomers as filled circles. Rectangles represent oligomers of length equal to the number of occupied sites. (a) Elongation of a template-bound tetramer to form the pentamer is facilitated by the presence of at least two additional next-neighboring monomers. (b) Formation of the dimer is favored within a long stack of monomers, six shown here (see text).

(6 or more monomers) because of the large number of monomers required by the latter. Furthermore, in one case where the intrinsic reactivity within an optimal complex was measured, we found that elongation is 80 times faster than dimerization (Kanavarioti *et al.*, 1993). Here intrinsic reactivity acts in addition to the entropic effect to ensure that monomer will be preferentially consumed to form longer polymers instead of dimers. The second implication is that a mechanism with a stringent requirement for additional monomers and with a double helical complex extending beyond the reactive site may have a useful 'screening' function in a prebiotic milieu. It ensures that molecules, e.g. nucleotides and others, that do not fit well into the dublex, will be less prone to be incorporated into the product. In a mechanism without the above requirement any electrophile that to some degree is able to associate with the 3′end of the monomer (dimerization) or oligomer (elongation) may lead to product indiscriminately.

4.4. KINETICALLY DETERMINED REACTIONS

The effect of competing processes in the polymerization reactions is not very intuitive. Rate determinations of individual steps in the poly(C)/2-MeImpG polymerization, in conjuction with computer simulation, allowed an assessment of the effect of changes in the elongation rate constant, k_n, ($k_n = k_4 = k_5 = k_6 =$ etc. up to k_{14} see Eq 1 and Kanavarioti *et al.*, 1993) on the yield of the oligomers. Such changes affect the yields of all oligomers but the effect is amplified in every elongation step and therefore the longest oligomers are the most strongly impacted. For example, a 4-fold reduction of k_n leads to a 1000-fold reduction in the yield of the 14-mer! This result implies that a set of conditions or a catalyst that enhances k_n by a small factor may have a dramatic effect on the yield and length of the oligoguanylate products. With a heteropolymeric template, it is plausible that k_n

depends on the specific nucleophile/electrophile combination* and it is likely that each combination may require its own catalyst. Hence, with a heteropolymer as the template, more than one catalyst may be necessary in order to attain a product distribution similar to the one anticipated with a homopolymer.

In most prebiotic syntheses yields are low. On the other hand, there is a strong belief that evolution and the origin of life required large amounts of materials. However, there are situations where less may be better. Computer simulations approximating the competing processes in the poly(C).$(pG)_n$.2-MeImpG system, where $(pG)_n$is a preformed oligomer, led to the following general conclusions: The fraction of oligomer that has reacted as well as the product distribution of the various oligomer lengths do not depend on the absolute concentrations of the reactants, i.e. oligomer and monomer (M), but only on their ratio, ([M]/[$(pG)_n$]) (Kanavarioti and Bernasconi, 1990). This is true as long as dimerization and hydrolysis are not responsible for consuming most of the monomer; mM amounts of monomer are sufficient.

It is instructive to review some specific cases of this computer simulation (Kanavarioti and Bernasconi, 1990) in order to illustrate the effect of the ratio [M]/[$(pG)_n$] on efficiency and product distribution: (i) With n = 7 and [$(pG)_7$] = [2-MeImpG] at any concentration in the range 1.7 to 170 mM, synthesis will result in partial consumption of the oligomer and elongation up to the 11-mer, i.e. addition of four units only. This is because when the amounts of monomer and oligomer are comparable, a relatively large fraction of monomer is consumed to elongate the oligomer by just one unit. (ii) In contrast to the case above when, for example, [2-MeImpG] = 170 mM and [$(pG)_7$] = 1.7 mM, oligomers longer than the 35-mer are predicted with a distribution of products that exhibits the highest relative concentration at the 28-mer. (iii) With a concentration of monomer that is barely enough to make elongation compete with hydrolysis, i.e. [2-MeImpG] = 1.7 mM and with [$(pG)_7$] $\leq$ 1.7 mM, oligomers up to the 11-mer are formed which, interestingly enough, is a similar distribution as the one obtained with concentrations of materials in the range 1.7 to 170mM (see (i)). The conclusion drawn by comparing the above cases is that higher concentrations of starting materials do not necessarily lead to longer products or better efficiencies. Longer products and higher efficiencies are obtained when the monomer is in excess of the oligomer.

4.5. Ligation of Oligonucleotides

Ligation, i.e., the formation of a phosphodiester bond between two oligomers, seems to be the most obvious and direct pathway for obtaining long polymers, simply because in just one step the length of the starting material can be greatly

* There is experimental evidence that elogation rates depend on the combination of the reacting bases (Wu and Orgel, 1992a,b,c). However, since these determinations are not supplemented by thermodynamic studies, it is not clear how much of the slowing down of the rate is due to intrinsic reactivity and how much is due to poor complexation.

increased, e.g. self-ligation will double the length of the reacting oligomer. TD ligation, in contrast to elongation, is unaffected by problems associated with the inability of some monomers to cooperatively bind onto the template. Even medium size oligomers, like 4-8 nucleotides long, are expected to form stable double helices with an appropriate template. Although ligation of two oligomers in solution will greatly depend on their concentration and become improbable in a dilute medium, the template serves as an anchor for both molecules and due to proximity facilitates their reaction. Earlier, we came to the conclusion that mM concentrations of monomers may be sufficient for elongation reactions. It seems that μM-nM of oligomers should be sufficient for TD ligation.

Two types of non-enzymatic TD ligation are formally possible and have been documented by experiments. On the one hand, ligation of two oligouridylates on a poly(A) template was reported as the first example of a template-directed reaction (Naylor and Gilham, 1966). More recent work indicates that oligomers condense in the presence of a template, even when mismatches are incorporated in the sequence 2 nucleotides away from the reaction site (Harada and Orgel, 1993). The presence of additional catalysts, besides the template, can further increase the speed and the yield as observed in the reaction of oligoguanylates in the presence of hydroxylapatite (Acevedo and Orgel, 1986), as well as in the impressive 7 million-fold acceleration of a TD ligation by *in vitro* selected ribozymes (Bartel and Szostak, 1993).

On the other hand, ligation is possible between two pyrimidine oligomers that find themselves head-to-tail, based-paired in a Hoogsteen sense with the purine strand of a double helix (Beal and Dervan, 1992). In one example, the condensation reaction between the 3′OH of the first oligomer and the 5′-phosphate group of the second was performed in the presence of simple condensing agents such as BrCN, imidazole and $NiCl_2$(Luebke and Dervan, 1989). Other non-enzymatic ligations using triple strand recognition have been reported (Luebke and Dervan, 1991 and 1992). Oligomers to be condensed in such arrangement need to be relatively long – at least 11 nucleotides long – and fully complementary to the template. This is because the complexation of the third strand decreases substantially with the length and with the number of mismatched pairs (Collocci *et al.*, 1993). As compared with the ligation in the Watson-Crick sense, ligation within a triple-stranded complex will necessarily be less common in a pool of random sequence oligomers, but more faithful.

Ligation reactions using a double helix as the template is an interesting alternative for accomplishing non-enzymatic self-replication of polypyrimidines using oligomers as building blocks instead of monomers. This can be envisioned as follows: In analogy to an earlier proposal (Kanavarioti, 1992), a polypyrimidine/polypurine double helix, YR, could serve as a template to synthesize a third strand, possibly by ligation of the appropriate fragments. The new strand, a polypyrimidine Y′, fits in the major groove and is hydrogen-bonded to the purine of the Watson-Crick duplex via Hoogsteen base-pairing. Under certain conditions, i.e.

pH, temperature, ionic strength etc., disproportionation of the triple-stranded complex, YR • Y′, to the duplex and the single polypyrimidine strand would be favored. Under those conditions, free Y′ could direct the synthesis of its complementary polypurine, R′. Y′ and R′ are expected to be associated in a Watson-Crick type dublex, Y′R′. The latter could direct the synthesis of a new strand, all polypyrimidine, complementary to R′ in a Hoogsteen sense. Assuming that no mismatches occured in any of the above steps, the new strand should be an identical replica of Y, that is the polypyrimidine of the duplex that initiated these cycles.

In conclusion, TD chemistry incorporates a repertoire of reactions, such as dimerization, elongation and ligation within a double or a triple helix. It is the kind of chemistry that lends itself to information transfer, primitive screening, a preference for synthesizing long vs short polymers and accomplishes all this under conditions such that [template] $\ll$ [oligomer] $\ll$ [monomer]. Next, I would like to speculate on what can be done with such chemistry, but only after defining the stage in the origin of life that may be relevant to this chemistry.

5. Fidelity in the Replication of RNA

Fidelity of self-replication is a prerequisite for information transfer and the creation of progeny. The high fidelity is instrumental for retaining integrity and perpetuating the self. Undoubtedly, a very low error threshold for self-replication and translation is required in order to perpetuate the efficient and finely tuned extant systems. A somewhat less fidele system of replication applies to many viruses, which for that reason, propagate not as a strictly defined molecule, but as a coherent, self-sustaining group, so called quasispecies (Eigen, 1993). For similar reasons, satisfactory fidelity is required if the RNA that needs to be replicated participates in a series of coupled reactions that are responsible, for example, for the synthesis of activated β-D-ribomononucleotides or for the synthesis of certain catalytic peptides that transform UV-energy into chemically activated compounds. Hence fidelity becomes an issue only when the RNA is constituent of a cell or of a protocell (presumed here to be the first anscestor), or at least participant in a set of coupled reactions or hypercycles (Eigen and Schuster, 1977).

6. TD Chemistry before the Hypercycles

Enzymatic, TD chemistry is responsible for information transfer and Darwinian evolution both in nature and in the test-tube (see *in vitro* genetics, Szostak, 1992). On the other hand, thirty years of experimentation suggests that non-enzymatic TD chemistry results, at best, in a set of molecules that are similar in composition and length to the complementary of the template. The latter chemistry could have operated at an early stage in prebiotic chemistry not as the tool for directed evolution, but as a mechanism for enhancing the population of RNAs on prebiotic Earth. Since the stability of the duplexes depends on the number of mismatches,

the imperfect complementarity would allow for the coexistence of a considerable number of single strands together with double helices. The availability of single strands is advantageous in establishing a true turnover of the templates acting as catalysts. Template turnover is probably the most important requirement for the creation of a large population of polymers and could come about by physical means, e.g. the everyday cycling of the temperature from day to night, or by the chemical properties of the participating molecules, e.g. a more favorable association in the ternary reacting complex between 'template' and 'monomers' than in the binary product complex (Von Kiedrowski and Terfort, 1992). I propose that TD chemistry could have been instrumental in creating a relatively large pool of RNA and RNA-like materials. Supposedly, it is these materials together with other chemicals from the prebiotic environment that organized themselves in the form of hypercycles.

It also seems probable that in the beginning there may have been a disproportionately large amount of polynucleotide-*analogues* as compared to *ribo*polynucleotides (for examples of acyclic sugar derivatives see Visscher and Schwartz, 1988; for cyclic sugar derivatives see Eschenmoser, 1992). It is also plausible that these analogues and their mixtures adhered to some extent to the rules of TD chemistry, discussed earlier, and evolved by forming longer polymers. How a 'take-over' by the ribocomponents occurred may become clearer once the properties of these analogues have been determined and their crossinteractions with DNA or RNA are established (Eschenmoser, 1992).

7. 'Protozymes'

Thirty years ago it was thought that nucleic acids could replicate with relative ease. This view has not been borne out by experiments. Recently the emphasis has been placed on discovering one self-replicating RNA (Doudna *et al.*, 1993). However, it is questionable whether or not such RNA was in the path of evolution or just a sidestep from it. Some of the reasons that shed doubt on the involvement of a self-replicating RNA in the prebiotic chemistry that led to the origin of life are as follows: (i) Based on theoretical and experimental reasoning such a molecule is predicted to be quite rare (Joyce and Orgel, 1993). (ii) The only chemical system with the capability to evolve (Eigen and Schuster, 1977) is one that constitutes a hypercycle. A hypercycle consists by definition of more than one molecule, hence a self-replicating RNA can not represent an evolving system. Simply said, what good would it do to have in a pool of trillions one self-replicating RNA that uses up all available mono- or oligonucleotides to make more copies of itself? This RNA would only hinder evolution. However, the value of finding such RNA, besides being interesting in its own right, may be that it would help to assess the availability of such type of ribozymes within a random pool of RNAs and provide clues about the structure associated with the self-replicating function.

Current evidence suggests that non-enzymatic replication, if it ever existed, was not only aided by metal ions and minerals but by more sophisticated catalysts,

possibly RNA molecules and/or peptides (the case for peptides has been described in detail by Lahav (Lahav, 1993)). I propose that within a population of diverse and long peptides, polynucleotides and analogues, a few molecules, henceforth called 'protozymes', will turn out to have some catalytic activity. I chose the term 'protozyme' in order to underline that these molecules are envisioned as the first (proto) catalysts of organic nature and because 'zyme' makes no commitment whether or not the molecule was a peptide or an RNA or even an RNA-like molecule. These protozymes are thought of as short and of minimal catalytic activity at the onset of the TD processes. At a later stage, when the pool of organic material consisted of long peptides and polynucleotides, 'protozymes' may have been correspondingly longer and more efficient. The catalytic action of these molecules could have been to improve the efficiency of TD syntheses, to facilitate the synthesis of the mononucleotides from their constituents, to assist the condensation of short peptides to longer ones, etc.

This idea of the existence of 'protozymes' is not unreasonable in light of findings suggesting that very short RNAs and peptides exhibit catalytic ability: For example, UUU trinucleotide catalyzes stereospecific phosphodiester bond cleavage A-G in AGGG tetranucleotide (Kazakov and Altman, 1992). Trivaline catalyzes the oligomerization of 5′-pdGTT to 6-, 9- and 12-unit long oligomers (Streltsov *et al.*, 1992) and 5-hydroxycytidine was found to catalyze a simple redox reaction (Yanagawa, 1990). Current research supports the idea that ribozymes as well as some of the enzymes catalyzing phosphoryl transfer reactions do it by properly placing two metal ions at the transition state while using very different functional groups (Pyle, 1993; Yarus, 1993; Steitz and Steitz, 1993). It is conceivable that such function could be accomplished by a variety of polymers, i.e. peptides, polynucleotides, as well as analogues thereof. It should be realized that *in the absence of a satisfactory replication mechanism these catalysts ('protozymes') would come and go.* However, as long as the availability of mononucleotides and activating agents is well ahead of polymer hydrolysis, 'protozymes' would appear again. Due to statistical reasons their catalytic potential would be a function of the size and diversity of the pool of polymers. The appeal of this proposal is that it may be possible to test it experimentally.

8. From Mononucleotides to the RNA World and to the Hypercycles

Assuming that a method of synthesis for ribonucleotides or nucleotide analogues was available and that building blocks were furnished to prebiotic Earth at a constant flux, three major stages in oligonucleotide synthesis can be conjectured (Figure 4):

(i) Metal ion as well as mineral-catalyzed oligonucleotide synthesis that resulted in short RNA type oligomers, maybe up to 10 bases long, with no specific or predetermined sequence. A small number of these oligomers could have ligated in

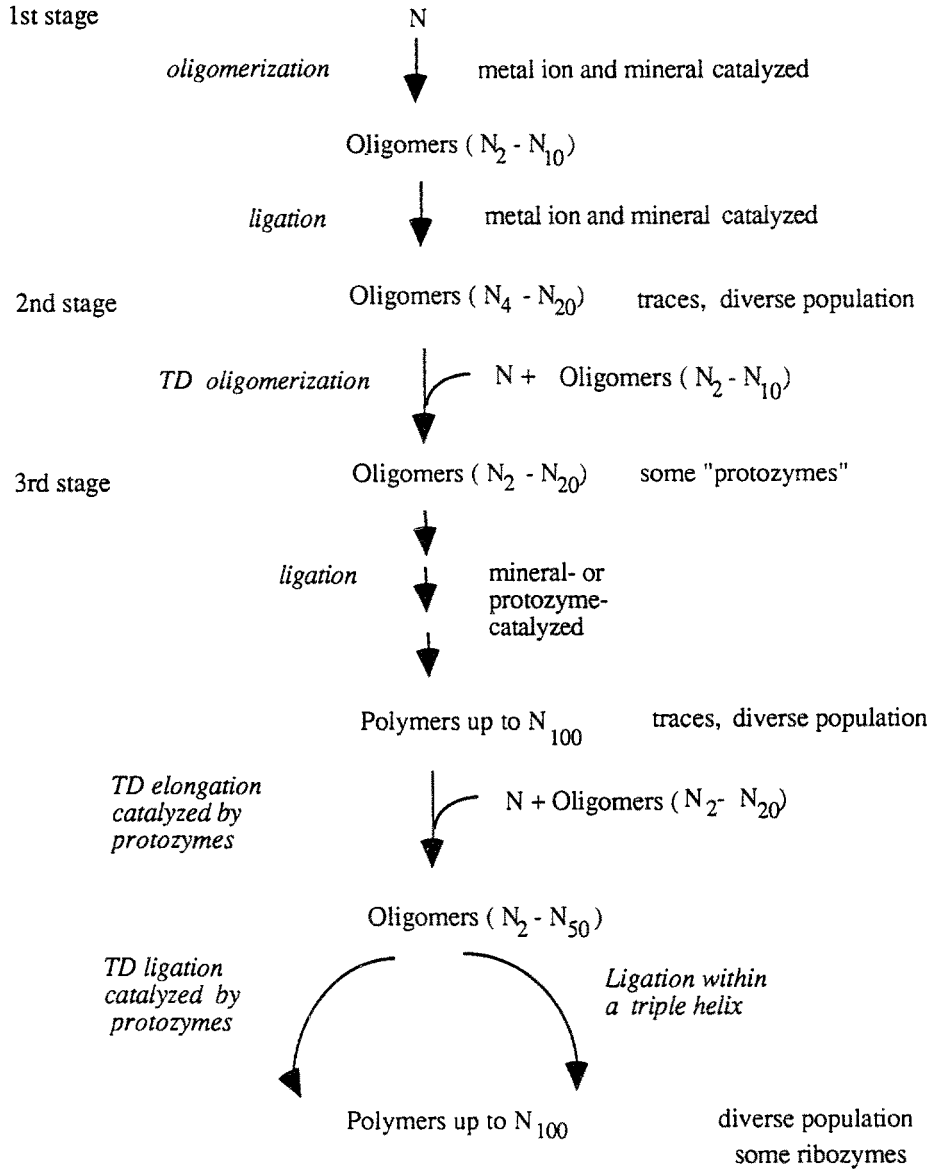

Fig. 4. Scenario for the prebiotic synthesis of an 'RNA world'.

the presence of appropriate metal ions to form traces of oligomers up to 20 bases long.

(ii) These oligomers, once formed, served as templates for template-assisted oligonucleotide synthesis and TD elongation of the shorter ones. With the prerequisite of true turnover these templates were responsible for enhancing the population of the 20-mers much beyond the range of traces. It is conceivable that among these oligomers a few could act catalytically ('protozymes') in facilitating processes

such as syntheses of the ribonucleotides, polymer/monomer complexation, dimerization, elongation, ligation and transesterification. Transesterification may have been necessary for the synthesis of a diverse population that incorporates all four bases, before the advent of efficient polynucleotide synthesizing systems. Hence the appearance of a 'protozyme' to facilitate transesterification may have been an important step in evolution.

(iii) In the presence of activating agents, and possibly helped by minerals or other catalysts, a small amount of these oligomers condensed to form traces of polymers up to say 100 bases long. In turn, these polymers could act as templates and direct the elongation of the existing oligomers to form products possibly up to 100 bases long. This could have been accomplished with the help of a polymerase-like 'protozyme' or peptides (Lahav, 1993) or combination of materials not discovered yet. Longer oligomers could also now be formed by template-assisted ligation of two units within a triple-stranded helix. Among this diverse and relatively large population of oligomers up to 100 bases long a few may turn out to be of similar function as some of the ribozymes discovered in the last decade.

The gist of the scenario presented above (Figure 4) is that with the help of the 'protozymes' and other materials in the prebiotic milieu the RNA population steadily increased in absolute number, variety and length of the polymers. *With diversification, better 'protozymes' appeared, not by selection but by chance.* As long as the availability of activated monomers was well ahead of polymer hydrolysis, the system as a whole evolved randomly towards more complex structures, *until* some of the existing materials organized themselves in the form of a hypercycle where evolution in the Darwinian sense could take place.

9. Conclusions

Until recently TD chemistry has been looked upon with suspicion, mainly because the existence of a polynucleotide to act as template was considered prebiotically implausible, and because there is no random RNA sequence, short or long, that will replicate itself non-enzymatically. A better understanding of the principles underlying TD chemistry allows us to envision a form of supramolecular chemistry, with capabilities of information transfer and chemical selection through kinetically controlled processes, which can be seen as the evolutionary link between monomers and polymers.

Exploiting this type of chemistry, a scenario is proposed that, assisted by 'protozymes' formed endogeneously and by chance, leads gradually to a population of long and diverse polymers. In this framework, the origin of a protocell may not have been the result of a robust reaction that some of us are looking for. On the contrary, evolution may have exploited very many reactions and may have occurred in so many stages that one would not dare enumerate. Nevertheless, little by little, catalysts and informational molecules as well as other important constituents of

the protocell were coevolving, independently and slowly in the beginning, more efficiently and intertwined at a later stage.

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