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"Prebiotic Evolution of RNA"

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Publications

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2) G. Costanzo, R. Saladino C. Crestini, F. Ciciriello, E. Di Mauro. 2007. Nucleoside phosphorylation by phosphate minerals. The Journal Of Biological Chemistry 282, 16729-16735.

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Introduction

EMERGENCE AND THE ORIGIN OF LIFE

Scientists assume that life arose on the blasted, primitive Earth from the most basic of raw materials: air, water, and rocks. Life emerged nearly 4 billion years ago by natural processes completely in accord with the laws of chemistry and physics, yet details of that trasforming origin event pose mysteries as deep as any that faces science. How did nonliving chemicals become alive?

It is possible, of course, that life arose through an improbable sequence of many chemical reactions. If so, then living worlds will be rare in the universe.

Alternatively, the universe may be organized in such a way that life emerges as an inevitable consequence of chemistry, given appropriate enviroments and sufficient time. This scenario allows fruitful systematic scientific study. If life is likely to arise whenever and wherever appropriate condition occur, then scientists can hope to study life's origins in the laboratory through experiments that simulate those conducive conditions. Not surprisingly, most origin-of-life investigators favor the view that life is a cosmic imperative and that it is only a matter of time before we figure out how it happened (Hazen, 2005).

A central theme of life's origins is the concept of emergence. What do we mean when we say that something emerges? In common usage, a shadowy figure emerges from the dark, a submarine emerges from the sea, a plot emerges in a novel. But emergence has come to have different meaning in scientific terminology. Researchers are increasingly beginning to use emergence to describe processes by which more complex system arise from simpler systems, often in unpredictable fashion.

This use of the word "emergence" is in a sense the opposite of reductionism, the view that any phenomenon can be explained by understanding the parts of that system. Because reductionism has been such powerful tool in sciences, some scientists shy away from the concept of emergence, thinking it to be slightly weird. But there can be little doubt that the word itself is useful in referring to

some of the most remarkable phenomena we observe in both nature and in the laboratory. Such emergent phenomena, phenomena that exhibt self-organization, are common in our everyday experience.

The laws of chemistry and physics permit certain kinds of molecules to self-assemble into aggregates that have surprising structure and properties. Sometimes the process is spontaneous, as in the formation of vescicles, but in other instances an imput energy is required to drive self-assembly. If we did not know by observation that soap molecules can self-assemble, we could not have predicted that vescicles would suddenly appear if we simply increased the concentration of soap molecules in solution. And even though we know that vescicles form, there is still no equation that can predict exactly what concentration of soap is required to form them.

The origin of life is the most extraordinary example of an emergent phenomenon, and the process by which life began must involve the same kinds of intramolecular forces and self-assembly processes that cause soap to form membranous vescicles. The origin of life must also have in some way incorporated the reactions and products that occur when energy flows through a molecular system and drives it toward even more complex system with emergent properties. Life on Earth arose through a sequence of many such emergent phenomena. Imagine that we could somehow travel back in time to prebiotic Earth, some 4 billion years ago. It is very hot-hotter than the hottest desert today. Asteroid-sized objects bombard the surface. Comet crash through the atmosphere, no oxigen yet, just a mixture of carbon dioxide and nitrogen, and add more organic molecules to a globe-spanning ocean. What did happen in 100 million years that led to the origin of life? This is a fundamental question of biology, and the answer will surely change the way we think about ourselves as well as our place in the universe, because if life could begin on Earth, it could begin by similar processes on Earth-like planets circling other stars throughout the universe (Deamer, 2005).

If you want to enunciate a law that characterizes emergent systems, then the first step is to examine everyday samples. Scientists have already identified key

aspect of the problem. Many familiar natural systems lie to equilibrium, they are very stable and unchanging, and thus they do not display emergen behavior. Away from equilibrium, dramatically different behaviors occur. Rapidly boiling water, for example, dispays complex, turbolent convection. Water flowing downhill in the gravitational gradient of a river valley interacts with sediments to produce the emergent landform patterns of braided streams, meandering rivers, sandbars, and deltas. These patterns arise as energetic water moves. Emergent system seem to share this common characteristic: they arise away from equilibrium, when energy flows through a collection of many interacting particles. Such systems of agents tend spontaneously to become more ordered and to display new, often surprising behaviors. And as patterns arise, energy is dissipated more efficiently, in accord with the second law of termodynamics. Ultimately, the resulting behavior appears to be much more than the sum of the parts. All emergent systems display the rather subjective characteristic of "complexity", a property that thus far lacks a precise quantitative definition. Such complexity is the hallmark of every emergent system. What scientists hope to find, therefore, is an equation that relates the properties of a system on the one hand (its temperature or pressure, for example, expressed in numbers), to the resultant complexity of the system (also expressed as a number) on the other. Such an equation would in fact be the missing "law of emergence". Of all known emergent phenomena, none is more dramatic than life, so studies of simpler emergence can provide a conceptual basis, a jumping-off point, for origin-of-life research. The beginning and end points of life's emergence on Earth seem resonably well established. At the beginning, more than 4 billion years ago, life's simplest molecolar building bloks emerged inexorably through facile chemical reactions in numerous prebiotic enviroments, from deep space to deep crust. A half-century of compelling synthesis research has amplified Stanley Miller's breakthrough experiments. Potential biomolecules must have littered the ancient Earth. The end point of life's chemical origin was the emergence of the simple, encapsulated precursors to modern microbial life.

WHAT IS LIFE?

Some old definitions of life, which are relevant for the arguments presented here, will be given without further comments: one interesting and often forgotten definition goes back to M. Perret in the early fifties (Perret, 1952). This definition was later taken up by J. D. Bernal (1965).
It reads: *'Life is a potentially self perpetuating system of linked organic reactions, catalyzed stepwise and almost isothermally by complex and specific organic catalysts which are themselves produced by the system'.*
I find particularly fascinating the definition of life given 1894 by Engels (yes! Friedrich Engels of Karl Marx' memory) and particularly what he says about life and chemistry (Engels, 1894).
His definition reads: *'Life is the existence form of proteic structures, and this existence form consists essentially in the constant self-renewal of the chemical components of these structures'* (Engels knew about Haeckel's work, who in turn knew about Rolle's ideas – none of them at that time had a clear notion of what proteins really were (Luisi, 1998).
At first sight, the problem of defining life should be easier for us in the field of origin of life by the selfimposed restriction to deal only with *minimal life*, i.e., the simplest possible form of life. This restriction makes it possible to ignore all those very beautiful and very difficult properties of human life (intelligence, consciousness, ethics, etc). In order to have a general prospective of the situation, let us consider Figure 1. This represents the view which we all widely accept (although not demonstrated yet).
Accordingly, the living originates from the inanimate matter throughout a continuous process of increase of molecular complexity and organization – a process loosely referred to as 'molecular evolution'. The first one to propose this idea in a written form was Darwin himself – you remember his little warm pond full of salts and other good ingredients – which later on should become famous as prebiotic soup.

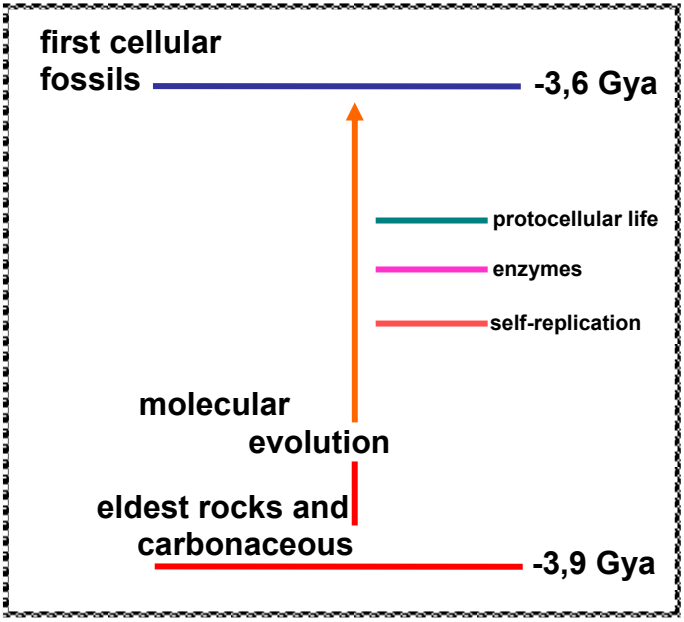


Figure 1. Oparin' scenario of transition to life, with the two signposts we can experimentally relay upon, the dating of the eldest carbonaceous rocks, and the dating of the first fossil cells; and the three waystations to the transition to life along the pathway of molecular evolution, selfreplication, enzymes and precellular life. The assumption here is that a macromolecular system with the property of selfreplication is more probable – and therefore may have occurred prior to – a full fledged enzymatic system (ribozymic or proteinaceous) (Luisi, 1998).

But Darwin didn't think too much about origin of life; some of the contemporary scientists who popularized his view, however, did it for him, most notably Ernst Häckel, who stressed that there is no difference in qualities between the inanimate and the animate world ('Anorgane und Organismen') and therefore there is a natural and continuous flux from the one to the other (Häckel, 1866); and Friedrich Rolle, who most clearly advocated that life derives from the inanimate (Rolle, 1863)*. But in the chemical literature not so much is known about Rolle and Haeckel or Lamarck. The first name of note for us is Alexander Ivanovic Oparin, who in 1928 wrote his famous book 'The origin of life' (Oparin, 1928), the one in fact who pushed the idea into modern science. In fact, I will refer to Figure 1 as 'Oparin' scenario*, as he is certainly the main responsible for our time. Implied with this is not the particular chemical scenario of Oparin' (for example the methane atmosphere) but the general concept of transition to life as a process which goes spontaneously from the inanimate matter to the first living cells. According to this view, transition to life is a *continuum*, a process which moves between the two signposts ca. 4.5–4.6 billions years ago (origin of Earth) and 3.6–3.7 billions years ago (first cellular fossils). Actually, as the beginning of the transition to life one should take the age of the eldest rocks, about 3.9 billions years ago, when the Earth had reached a certain stage of geological equilibrium. Concerning the final signpost, one may argue that at this time life was already full fledged, since these fossils look remarkably similar to modern cyanobacteria.

* A citation from this 1863 work is worth mentioning: 'The hypothesis of an originary arising of life from the inanimate matter ... can at least offer the advantage to explain natural things by natural pathways, thus avoiding to invoke miracles, which are actually in contradiction with the foundations of science'.

* A. I. Oparin himself gave a description of life based on six properties: (1) capability of exchange of materials with the surrounding medium; (2) capability of growth; (3) capability of population growth (multiplication); (4) capability of selfreproduction; (5) capability of movement; (6) of being excited.
He also added however some additional properties, such as the existence of a membrane (a cardinal principle for him); and the interdependency with the milieu (Oparin, 1961).

Then, the beginning of life forms must have started earlier. In conclusion, then, as diagrammatically represented in Figure 1, the transition to life should encompass a period of only a few million years or even shorter (Lazcano and Miller, 1993). In view of this arbitrariness on where to put the marker, is any definition equally good? Surely not, as one definition may be more meaningful than another, depending on what you want to do with it. In fact, the following criteria appear important: a definition of life should permit one to discriminate between the living and the nonliving in an operationally simple way and it should not be too restrictive (i.e., the discrimination criterion should be applicable over a large area and should be capable of including life as it is as well as hypothetical previous forms). All forms of life we know about should be covered by such a definition. Once decided upon, the definition should also help to design experiments on the production of minimal life in the laboratory, consistent with the definition. It should help space explorers in the attribution of the term 'life' to novel biological forms. Finally of course it should be logically selfconsistent. The criterium of discrimination between living and nonliving should clarify 'difficult' cases, such as a virus, which generally is not included in the list of the living; a dead cell, which has still a lot of nucleic acids; and a growing crystal; ... and others. (Luisi, 1998).Perhaps the simplest definition of life is the one presently used by the Exobiology Program within the National Aeronautics and Space Agency as a general working definition of life. It is one of the very few which one finds in a written form and perhaps for this reason it enjoys considerable popularity – it is for example often cited in origin of life meetings. This working definition goes asfollows (Joyce, 1994): *'Life is a self-sustained chemical system capable of undergoing Darwinian evolution'*. This definition was used before by Horowitz and Miller (1962). Implicit in this definition is the fact that the system is driven by an external source of energy– as nobody, not even in the field of the origin of life! – intends to violate the second principle of thermodynamics. In other words, the self-sustaining proceeds at the expenses of transformation of nutrient/energy provided by the environment.

THE CHEMISTRY OF THE ORIGIN OF LIFE

It was more than 50 years ago, when a paper by a young graduate student from the University of Chicago was published in the journal *Science*. It described a simple apparatus in which a mixture of gases was subjected to a spark discharge and the products were condensed into an aqueous solution. With his work, Stanley Miller, at that time in the laboratory of Harold Urey, presented experimental proof that it is possible to synthesize biologically relevant organic compounds such as amino acids from simple non-biological compounds – gases such as methane, ammonia, hydrogen and water – under conditions that could have occurred on the primitive Earth. This experiment is considered to be the starting point of experimental exobiology, an interdisciplinary field of research that investigates the origin of life on the Earth and its possible occurrence elsewhere in the universe. There are three competing theories for the chemical evolution of life. First are *gene-first* theories that suggest that the origin of life be intrinsically tied to the appearance of the ability to pass on acquired catalytic abilities. Second are *membrane-first* theories that speculate that that the appearance of encapsulating lipid bilayers made the origin of energy harvesting membranes possible. Both these theories have in common that they require an initial set of organic compounds to be present on the early Earth (heterotrophic origin)(Bada, and Lazcano, 2002). The third theory is a *metabolism-first* hypothesis, which speculates that life began with mutually catalytic chemical reactions that could also occur at elevated temperatures, and that is often associated with hydrothermal systems. In this theory, carbon (in the form of atmospheric CO_2) is fixed through chemical reactions and transformed into organic compounds (chemoautotrophic origin).

DATING THE PREBIOTIC EARTH

We focus here on the period just before the emergence of life on Earth. The prebiotic Earth is the place where the last steps of chemical evolution led to the

first biological processes. This concept becomes of course very fragile if one considers the possibility of panspermia (Napier, 2004). It is usually assumed that the emergence of the terrestrial life occurred on Earth, panspermia being considered as a more exotic hypothesis. The assumption of a terrestrial origin, however, is not clearly supported by scientific arguments. In order not to eliminate any possibility, let us consider that the prebiotic Earth was the place where the conditions allowed life to evolve, whether its origin was endogenous or extraterrestrial.

THE ORIGIN OF WATER

Scenarios assuming a continuous and local accretion of the Earth did not provide a consistent theory for the origin of water. The correlation between the water content and the orbital distance of asteroids (assuming that their present orbital distance does not differ too much from where they formed) suggests that material formed at 1 AU was dry (Morbidelli et al., 2000). This implies a late veneer of chondritic or cometary material to feed the water and volatile reservoir of the Earth. Several authors proposed the comets as a late source of water (Chyba, 1987; Delsemme, 2000) but both deuterium constraints and dynamical models limit the contribution of comets to less than 15% of the terrestrial water content (Morbidelli et al., 2000). Abundances of noble gases and metals may provide an even more drastic limit on the cometary contribution (Dauphas and Marty, 2002), though the abundance of these elements in comets is still derived from laboratory studies and models. A chondritic origin of the water fits the D/H ratio of Earth's oceans but would require the delivery of a "super veneer" of about 1% of the terrestrial mass, which contradicts recent re-estimates of the bombardment. The existence of very old zircons also requires enough liquid water on Earth to form continental crust as early as -4.4 Gyr (Wilde et al., 2001). Finally, one would have to explain why the late delivery was inefficient on Mars. Indeed the upper limit on the initial relative water and volatile contents are much lower than on Earth and are difficult to explain only by scaling the impact rate or by escape

processes (Lammer et al., 2004). The relatively small initial Martian water reservoir can be understood in this context: at the orbital distance of Mars, planetary formation is less efficient because of the influence of Jupiter; and Mars is probably a remaining dry embryo (or the result of a very small number of dry embryos) formed locally and on which water was only brought by the bombardment (Lunine et al., 2003). The accretion of the Earth being fed by distant formation regions, a late delivery of water is no longer necessary. Such delivery certainly contributed somewhat, and can even be required to explain some anomalies of siderophiles or noble gas content of the Earth, but the bulk of the terrestrial water can be present from the ”beginning”, that is before -4.45 Gyr.

GIANT IMPACTS AND ATMOSPHERIC EROSION

In a collision similar to the Moon-forming impact (i.e., a collision betweeen a proto-Earth and a Mars-sized planet)(Genda and Abe, 2003), less than 30% of the atmosphere of both bodies is lost to space. Therefore, giant impacts can result in a net delivery of gases to the growing proto-Earth (in the case of volatile-rich impactors) and are unlikely to reset to zero the atmospheric content, because:

- 30% is the extreme upper limit for the escaping fraction estimated by these authors,

- impacts with Mars-sized bodies are among the largest ones experienced by proto-planets, according to accretion models (Chambers, 2001),

- a significant fraction of the volatiles can be kept in the mantle (especially CO_2 and H_2O, but also N_2 depending on the oxidation state reached by the mantle at that stage. This is demonstrated by the survival of near-solar isotopic ratios of neon in the deep mantle.

INSIGHTS INTO THE COMPOSITION OF THE PREBIOTIC ATMOSPHERE

The bulk of the terrestrial water was likely brought to the Earth by one or several wet embryos, formed beyond 2-3 AU. The formation of these embryos from km-sized objects takes typically 10˙000 yrs. In order for the embryos to radiate their internal energy ($> 3GM^2/5R$) during this period of time, the mean cooling rate must exceed (by orders of magnitude) the $\sim 300 Wm^{-2}$ runaway greenhouse threshold. This runaway produces a "magma ocean" phase, during which a dense steam atmosphere equilibrates with a molten rocky surface (Sleep and Zahnle, 2001). For embryos with masses between 0.01 and 0.1 M_{Earth}, this phase lasts 0.5 to 4 Myr, which is shorter or comparable to the typical lifetime for protoplanetary nebulae (Lyo et al., 2003; Armitage et al., 2003). This has interesting consequences: first, embryos may capture directly a solar-composition atmosphere from the nebula, while the opacity of the disk provides a protection against the solar radiation, which is able to induce gravitational escape of the atmosphere. Second, during the magma-ocean phase, a nebular component is incorporated into the embryo through the molten surface, which is consistent with the isotopic and elemental abundances of noble gases in the terrestrial mantle and crust (Dauphas, 2003).

SURVIVAL OF REDUCED ATMOSPHERES AND EARLY OXIDATION OF THE EARTH

During the accretion of embryos by the growing Earth (before -4.4 Gyr), such magma-ocean phases must have occurred (at least once the water abundance reached a certain value). But in this case, the disk no longer exists and the atmosphere is submitted to the strong XUV radiation from the young Sun. If the core segregation is still not complete (until at least 30 Myr from Hf-W dating) water reacts with iron in the mantle, releasing large amounts of molecular hydrogen in addition of the captured nebular atmosphere (Zahnle, 1998). The lifetime of such an atmosphere is extremely short due to the hydrodynamic

escape driven by the XUV heating: a mass of hydrogen equivalent to the mass of the present Earth's atmosphere is lost in less than 1 Myr during the first 150 Myr. Therefore, the existence of a primitive H_2-dominated atmosphere depends on the production rate of H_2 by the oxidation of iron. In an atmosphere where H_2 is not the main compound, the escape rate of hydrogen can still be of the same order (Hunten et al., 1989). Indeed, during the first 100 Myr, the XUV solar flux would produce exospheric temperatures above the critical temperature at which H atoms have a mean Kinetic energy above their gravitational energy (~7500 K for an Earth mass). Gravitational escape from an initial nebular atmosphere is consistent with the observed fractionation of residual atmospheric xenon and neon. During these epochs, the Ly-α irradiation was also a major source of H through the photodissociation of H_2O or CH_4. The Ly-α emission of the young Sun was able to photolyse several meters of precipitable water every Myr. The result of this production of rapidly escaping hydrogen is a fast oxidation of the atmosphere and the upper mantle. This oxidation started as early as the incorporation of water into the Earth (before -4.4 Gyr from zircons and numerical accretion models).

This fast oxidation is consistent with the early degassing of N_2 (before -4.3 Gyr, Tolstikhin and Marty, 1998) inferred from its isotopic distribution. Because such degassing is incompatible with reducing conditions (Libourel et al., 2003), the upper mantle should have reached its present oxidation state as early as 4.3 Gyr ago. It is likely that the fast and early oxidation of the upper mantle was made possible by the chemical action of water combined with the activity of the young Sun. If the bulk of water would have been delivered later, when the Sun was less active and the loss of atmospheric hydrogen less efficient, the fate of the Earth might have been different. The fact that atmospheric neon and xenon are highly fractionated; while nitrogen seems not to be, suggests that these noble gases were submitted even earlier to an intense fractionation through atmospheric hydrodynamic escape. This also requires a very reducing mantle able to keep nitrogen in the silicates and thus an incomplete core segregation when the bulk of noble gases was lost. The relatively short period of time during which nitrogen

was kept in the silicates may then be of high importance by protecting nitrogen from gravitational escape. A secondary nitrogen component may have been brought later by comets (Owen and Bar-Nun, 2001), although this contribution is constrained to low values by D/H constraints. A limited chondritic delivery is also possible. The partial presure of N_2 in the prebiotic atmosphere was probably similar to its present one. Indeed, the upper mantle contains a reservoir of N_2 smaller than 15% of the atmospheric N_2 (Zhang and Zindler, 1993).
Therefore, the partial pressure could have been slightly higher but probably not lower. The recycling of atmospheric N_2 into the mantle is indeed likely to be more efficient nowadays because of the biological fixation of nitrogen (Marty and Dauphas, 2003a, 2003b). The partial pressure of CO_2 is a subject of debate and is discussed in the next section. Immediately after giant impacts producing a runaway greenhouse, the partial pressure of CO_2 (and H_2O) was only limited by its reservoir and its solubility in magma. Out of these extreme conditions, it seems reasonable to say that it was in the range of 10-200 mbars, despite a huge reservoir in the crust and upper mantle respectively equivalent to 60 and 150 bars (Zhang and Zindler, 1993). The composition in other gases is much more speculative: CO is often pointed as a major prebiotic gas because of its formation during impact and its abundance in the protosolar nebula; however, no quantitative study about the sources and sinks of CO are available. Moderate levels of CH_4 are plausible (see next section). Nitrogen-bearing prebiotic compounds like HCN and NO can be produced by lightning and impacts at a rate depending critically on the level of CO_2 and CH_4 (Navarro-Gonzalez et al., 2001; Commeyras et al., 2004a). Oxygen (O_2) was kept to very low levels by the release of reduced volcanic gases despite its production by CO_2 photolysis and H_2O photolysis associated with H escape (Selsis et al., 2002).

THE TEMPERATURE OF THE EARLY EARTH

As describe above, the formation of the Earth implies huge impacts by planetary embryos with masses between 1% and 10% of Earth's mass. Post-impact conditions induced a magma ocean surrounded by an atmosphere of several hundred bar of H_2O and CO_2. During the cooling and condensation of this atmosphere, a hot ocean (230°C) can be found in equilibrium with a 300 bar atmosphere. The thermal evolution between this extreme phase and the present conditions is still very obscure. Knauth and Lowe (1978) used the isotopes of oxygen ($\delta^{18}O$) in cherts to infer the temperature of the early ocean. However, the high temperatures they found (80-100 °C, 3.5 Gyr ago) are now interpreted as the temperature in hydrothermal systems (Pinti et al., 2001), which is not related to the mean ocean temperature. The coldest times in Earths history are the Snowball Earth events characterized by an ice cover down to the equator. At the beginning of these events, the runaway ice-albedo feedback makes the global mean temperature drop to −50°C for a few tens of thousands of years (Schrag et al., 2002). This temperature drop is followed by a period of a few million years during which the mean temperature is around −10°C.

The most ancient known snowball events occurred near the beginning of the Proterozoic (2.45 - 2.22 Gyr ago), and another one may have occurred 2.7-2.8 Gyr ago (Crowley, 1983). These ancient low-latitude glaciations may be a consequence of the first release of biological oxygen (Selsis, 2002). The absence of other major glacial event suggests a mean surface temperature well above the freezing point of water and thus an efficient greenhouse warming. However, after the emergence of life, the climate can be strongly influenced by biological activity. In particular, methanogenesis is known as a very primitive metabolism, able to sustain a warm climate even under low solar irradiation (Pavlov et al., 2000). Therefore, high temperatures recorded in the Proterozoic or even the Archaean should not be extrapolated to prebiotic periods, for which our knowledge of the thermal conditions still rely on climate models.

CLIMATE REGULATION BY THE CARBONATE-SILICATE CYCLE

Walker et al. (1981) proposed the attractive hypothesis that the level of atmospheric CO_2 is regulated in such way that the temperature is always above and close to the freezing point of water, except for geologically brief periods. If, for any reason, the greenhouse warming becomes unable to maintain such a temperature, surface water freezes making ineffective the removal of atmospheric CO_2 into carbonates. In the absence of a CO_2 sink, the release of volcanic gases restores the level of CO_2. Thanks to this feedback mechanism, a global glaciation is not an irreversible trap. Assuming such regulation throughout Earth's history, and a constant albedo, the minimum level of CO_2 can be expressed as a function of time from the evolution of the luminosity (Kasting and Grinspoon, 1991). Paleosols of 3 Gyr age seem to indicate that the level of CO_2 was below the freezing limit (Rye et al., 1995), implying another greenhouse gas. The nature of these geological samples and how they should be interpreted in terms of CO_2 level is highly debated but the problem can be solved anyway by the production of methane through the primitive biosphere (Pavlov et al., 2000), without questioning the role of the carbonate-silicate cycle in abiotic conditions.
Recently, however, Sleep and Zahnle (2001) developed a detailed model for CO_2 cycling on the early Earth. They find CO_2 levels lower than the warming limit, except for brief post-impact periods. For these authors, other efficient sinks for atmospheric CO_2 (mainly the basalt carbonatization) make a dense CO_2 atmosphere unstable. They conclude that life certainly emerged on a frozen planet. The model is however sensitive to several parameters affected by large uncertainties, like the impact rate and the quantitative coupling of the different carbon reservoirs.

ABIOTIC METHANE

The existence of a globally-reduced prebiotic atmosphere has been generally abandoned during the last decades, but there is still room for methane as a minor

(but maybe precious) atmospheric gas. Indeed, a small production of methane (small compared to the biogenic one) exists in present hydrothermal systems. This production is due to the reaction of hot water with iron associated to a production of H_2, and then, CH_4 in the presence of CO_2. To contribute to the surface warming, the abiotic production of CH_4 had to be orders of magnitude higher than today, especially considering the intense Ly-α emission from the early Sun which drives the photo-oxidation of CH_4 into CO_2 and H_2O. An extended and efficient source of hydrothermal methane is however supported by the geological context on the prebiotic Earth (Shock et al., 2000).
The production of methane after large iron-rich asteroid impacts has recently been studied (Sekine et al., 2003), mostly for its implications on HCN production and prebiotic chemistry. The effect on the climate is negligible, considering the other consequences of the large impacts considered. Impacts by comets, which contain a significant fraction of methane, can also sporadically deliver CH_4 (Kress and McKay, 2004). Nonetheless, only a continuous production of this gas can sustain a sufficient level in the atmosphere because of the short photochemical lifetime of this molecule.

ORIGIN OF ORGANIC MOLECULES ON THE EARLY EARTH

There are three principal theories for the origin of prebiotic organic compounds on the early Earth (and perhaps other planets): The atmospheric/oceanic synthesis via Miller-Urey-type synthesis using electric discharges, UV light or other high-energy radiation as energy sources. The second possibility is mineral catalyzed synthesis in and around hydrothermal vents, perhaps via Fischer-Tropsch-type (FTT) synthesis. And the third theory states extraterrestrial synthesis, either in the interstellar medium or inside asteroids and comets, followed by delivery to planetary surfaces. At present, since very little data are available regarding the atmospheric, oceanic or geological conditions on the early Earth, it is impossible to determine conclusively which of these sources

was the most significant one, and probably all three sources contributed to the inventory (Ehrenfreund et al., 2002).

Although the Miller-Urey (MU)-Experiment is considered the starting point of experimental exobiology, (Miller 1953) its relevance to the origin of organic compounds on the early Earth has been somewhat diminished. The main reason for this was the changing picture about the composition of the Earth's early atmosphere. While Miller, based on Urey's ideas, used a mixture of reduced gases in his experiment, contemporary geochemical models tend to favor a more oxidized composition of mainly CO_2, CO and N_2, with minor amounts of reduced gases such as ammonia and methane (Kasting et al., 2001). If the MU experiment is carried out under these conditions, the yield on amino acids is drastically reduced, and the only amino acid detected in significant amounts is glycine (Schlesinger and Miller, 1983). However, there is the possibility that reduced conditions could have been present in localized environments such as volcanic plumes, where discharges may have driven prebiotic synthesis.

The investigations of the synthetic mechanisms leading to the formation of the compounds found in the MU-experiment has contributed to the understanding of how these molecules could be formed abiotically (Miller, 1957; 1984).

An alternative pathway for the formation of prebiotic organic compounds on the early Earth can be identified as part of the "metabolist" theory proposed by Wächtershäuser and coworkers (Wächtershäuser, 1992; 2000). Central to this thesis is the role of iron sulfide as both catalyst and a source of energy (in the form of reductive power) for life attempting to thrive using oxidized, mantle-derived volatiles (such as CO_2, CO, H_2S, N_2 etc.). It has been proposed that carbon fixation in such a "Iron-Sulfur-World" would be accomplished via the reductive citrate cycle (RCC), in which CO_2 would be the source of carbon and reduced carbon compounds such as citric acid would be the major products. Although individual steps of the RCC have been shown experimentally to be viable under geochemical conditions (Huber et al., 1997; Cody et al., 2001) the overall concept of the emergence of self-organizing biochemical cycles has been criticized (Orgel, 2000). In addition, this theory is generally placed in a

hydrothermal environment at temperatures of 100°C or higher and high pressure (which has been thought to be consistent with the apparent ancient phylogeny of hyperthermophiles, see below). Under these conditions, organic compounds, and particularly biologically relevant polymers such as RNA, are decomposed very rapidly (Miller and Bada, 1988; Miller and Lazcano, 1995; Levy and Miller, 1998). In contrast to this 'hot' origin of life on the Earth, experiments based on the stability of prebiotic organic molecules have provided additional evidence that a 'moderate' or even 'cold' origin of life should be favored (Bada, et al., 1994). With respect to RNA folding, it was shown that high temperatures strongly reduce the stability of three-dimensional, and therefore functional, structures of RNA molecules (Moulton et al., 2000).

HCN polymerization is another compelling process for the formation of organic compounds under prebiotic conditions (Ferris et al., 1984). Concentrated solutions of HCN produce nucleic acid bases and amino acids, whereas in dilute solutions hydrolysis becomes dominant. For example, one of the biological nucleobases, adenine, can be synthesized from 10 to 15 M ammonium cyanide solution at temperatures between 27 and 100 °C (Oró, 1960; Oró et al., 1961), while guanine is formed at yields 10 to 40 times lower than adenine (Levy et al., 1999). Steady state concentrations of HCN in the primitive ocean were estimated to be maximal 2×10^{-6} M (at 0°C) (Miyakawa et al., 2003). These concentrations are too low for HCN to polymerize. Because HCN is more volatile than water, evaporation in lagoons or in drying beaches can not occur if the pH is lower than the pKa of HCN (9.2 at 25°C) (Miyakawa et al., 2003). It is suggested that eutectic freezing may be a potential mechanism to concentrate HCN in an aqueous solution, for example in a lake or an ocean. The eutectic temperature of an HCN-water mixture is –21°C, at which point the mixture contains 78 weight-% HCN, which is concentrated enough to polymerize (Miyakawa et al., 2002). In an experiment that demonstrated the possible formation of nucleic acid bases from HCN, a wide variety of these compounds were identified as products of a dilute frozen ammonium cyanide solution that had been held at –78°C for 27 years (Levy et al., 1999; Miyakawa et al., 2002). Formamide (Ferris et al., 1981)

is easly formed by hydrolisis of HCN and is amoung the most abundant products obtained from pyrolysis and tetramethylammoniumhydroxide thermochemolysis of HCN-polymers. Calculation of the steady state concentration of formamide, in the primitive ocean by Miller and co-workers, showed that is always lower than that of HCN under similar conditions. Formamide has a boiling point of 210°C with limited azeotropic effects and, at difference from HCN, it can be easly concentrated by simple water evaporation in lagoon and on drying beaches (ECT Kirk-Othmer Encyclopedia of Chemical tecnology, 1978). In most anhydrous conditions the hydrolysis of formamide is a minor side-process, thus favouring this compound as a prebiotic precursor for the syntheses of nucleic acids bases at moderate temperatures. The one-step synthesis of purine by heating neat formamide at 160°C was first reported by Yamada and co-workers (Yamada and Okamoto, 1975). When the reaction was performed under similar experimental conditions in the presence of doubly enriched HCN, adenine was obtained as the main product along with a low amount of purine (Ferris et al., 1978). In a similar way, ^{13}C NMR studies of adenine prepared either from doubly enriched HCN or formamide showed that the adenine ring was formed from three molecules of HCN and two molecules of formamide (Yamada et al., 1978). It is well known that formamide decomposes at 180-190°C to give a mixture of ammonia, carbon monoxide, water and HCN at a rate of about 0,5%/min (ECT Kirk-Othmer Encyclopedia of Chemical tecnology, 1978). Titanium dioxide is a well known photocalyst used for a variety of organic trasformations including the photodegradation of formamide to formaldehyde (Friesen et al., 1999). Because formaldehyde is the main prebiotic precursor of sugars, the contemporany presence of formaldehyde and formamide in the reaction mixture is expected to generate the optimal conditions for the sinthesis of nucleic acid bases and nucleoside derivates.

Purine and pyrimidine acyclonucleosides are able to pair with natural nucleic acids bases and were indicated as possible precursors for the synthesis of primordial nucleic acids molecules, such as a peptide nucleic acid derivates (Schwartz and Orgel, 1985). Because of the lack of efficient prebiotic procedures

for the condensation of preformed nucleid acid bases with sugars (Sanchez and Orgel, 1970), the possibility of building the sugar moiety directly on the formylates bases opens interesting scenarios for a novel prebiotic synthesis of nucleosides on the primitive Earth. Since Bernal's analysis of the physical basis of life (Bernal, 1951), several prebiotic scenarios invoking clays have been proposed. Accordingly, these minerals have been used as microreactors to concentrate organic reagents, as templates for polymerizations (Cairns-Smith, 1992) and protective environments for lowstability biomolecules (Franchi et al., 1999; Franchi and Gallori, 2005). The third source for abiotic organic compounds is extraterrestrial delivery by asteroids, comets, meteorites and interplanetary dust particles (IDPs). Results from space missions (McDonnell, 1991), space and ground-based observations (Molster et al., 1999), and laboratory analyses of IDPs indicated the presence of olivines, that are sizeable components of cosmic dust. Principal sites of cosmic dust formation are envelopes of giant stars that belong to the asymptotic branch (AGB) and supernovae (SN) (Gehrz, 1989; Jones, 1997). Once formed, grains are injected in the interstellar medium (ISM) and collected during planetary system formation as building blocks of large bodies (Botta and Bada, 2002). "Fluffy" grains of amorphous olivines were synthesized in the laboratory as CDAs with the aim of reflecting the chemical composition and morphology of silicate dust expected in different astronomical environments and tested (Brucato et al., 2006); and the detection of purines and pyrimidines in meteorites (of which Murchison is only one instance) (Botta and Bada, 2002) provides a clear example of extraterrestrial syntheses and poses the question of their origin. Given that large amounts of organic molecules were deposited on the primitive Earth by asteroids, comets, meteorites, and IDPs (Chyba and Sagan, 1992; Botta, 2004).

Figure 2: Image of a piece of the Murchison meteorite that fell in Australia on September 28, 1969 (Kvenvolden et al., 1970).

Amino acids have been identified in a class of meteorites called carbonaceous chondrites, in the early 1970s (Kvenvolden et al., 1970; Botta and Bada, 2002). Since then, more than 70 different amino acids were identified in the Murchison meteorite at a total concentration of approximately 60 parts per million (ppm) (Cronin and Chang, 1993). Nucleobases were also identified in the Murchison, Murray and Orgueil meteorites at a level of several hundred parts per billion (ppb), and based on the low terrestrial contamination, were assumed to be indigenous (Stoks and Schwartz, 1979; 1981). Only guanine could be identified in a set of Antarctic meteorites (Shimoyama et al., 1990). Finally, sugar-related compounds were also detected in Murchison and Murray (Cooper et al., 2001).

Although it is difficult to estimate the flux of extraterrestrial carbon to the early Earth from the current data (Cohen et al., 2000; Lazcano and Miller, 1994) (Love and Brownlee, 1993), it was estimated that between 109 (at 4.4 Gyr) and $5 \cdot 10^7$ kg (at 3.5 Gyr) of organic carbon could have been delivered to the planet per year in the first billion years of its existence, mostly in the form of IDPs (Chyba and Sagan, 1992). IDPs, called micrometeorites when they enter the atmosphere, have been found to provide the major source of extraterrestrial organic carbon, mainly due to their high flux rate (under the assumption of a similar organic composition as carbonaceous chondrites). Complex aromatic molecules have been identified in IDPs (Clemett et al., 1993).

However, one problem associated with the delivery of organic compounds by these micrometeorites is that, depending on their mass and size, they can suffer full-depth heating to temperatures (~200°C to 1200°C for IDPs with a diameter of ~100 μm) during atmospheric entry deceleration (Glavin, 2001). This means that organic compounds present inside the IDP that are thermally unstable at these temperatures, such as amino acids, would be decomposed during this flash-heating event. It has been suggested that sublimation could be a possible mechanism by which volatile organic compounds could escape and survive atmospheric entry heating by vaporizing off the surface of IDPs and even from larger meteorites, before they are melted and destroyed (Glavin and Bada, 2001). However, although most pure amino acids sublime with high recoveries at temperatures around 500°C, only glycine could be detected after sublimation from Murchison meteorite powder at temperatures above 150ºC, providing the only evidence that amino acids could sublime from micrometeorites and survive atmospheric entry heating (Glavin, 2001). None of the nucleobases present in the meteorite powder sublimed at a temperature of 450ºC. Although it is not fully understood why most of the amino acids, purines and pyrimidines do not sublime from Murchison, experimental evidence suggests that divalent cations such as Ca^{2+} and Mg^{2+}, and/or the presence of kerogen-type organic polymers in Murchison may inhibit the sublimation of these compounds (Glavin, 2001). Obviously organic compounds survive entry and impact in small to medium

sized (few tens of meters diameter) meteorites, but the organic carbon fluxes from these objects are estimated to be about five orders of magnitude lower than for the IDPs. Theoretical studies about impact processes on the Earth suggested that most organic compounds contained in a big impactor such as an asteroid or comet would be destroyed by the high temperatures produced in these collisions (Chyba et al., 1990). In contrast, extraterrestrial amino acids were detected in the sedimentary layers that were formed when the bolide that formed the Chicxulub crater impacted the Earth 65 Myr ago (Zhao and Bada, 1989). Experimental studies of impact events in the laboratory can be extended to planetary scales of hundreds of kilometers by computer simulations. New high-resolution hydrocode modeling simulations of asteroid and comet impacts, which trace the impactor's thermodynamic evolution, coupled with experimental data for amino acid pyrolysis in the solid phase suggest that amino acids would survive the shock heating of large (kilometer-radius) cometary impacts at the percent level (Pierazzo and Chyba 1999). Exogenous delivery by asteroids, comets and IDPs appears to have been an important mechanism for seeding the terrestrial planets with complex organic compounds. These compounds probably triggered the increase in molecular complexity that may have been necessary for the origin of life to occur.

THE RNA WORLD

It is generally believed that there was a time in the early history of life on Earth when RNA served as both the genetic material and the agent of catalytic fuction.

Gerald Joyce, 1991

Most origin experts dismiss the idea of a purely metabolic life-form in favor of a genetic first scenario. In order to reproduce, event the simplest known cell must pass volumes of information from one generation to the next, and the only known way to store and copy that much information is with genetic molecule similar to

DNA or RNA. No one has thought more deeply about genetics and the origins of life than Leslie Orgel at the Salk Institute for Biological Studies in San Diego. His “Evolution of genetic apparatus” has guided generations of researchers, and he continues to exert a tremendous influence on origin theory and experiment. Orgel states that the central dilemma in understanding a genetic origin of life is the identification of a stable, self-replicating genetic molecule, a polymer that simultaneously carries the information to make copies of itself and also catalyzes that replication. Accordingly, he catologs four broad approaches to the problem of jump-starting such genetic organism. One possibility is the emergence of a self-replicating peptide of the kind made by Reza Ghadiri’s group at Scripps, or perhaps a protenoid as championed by Sidney Fox. The idea that proteins emerged first and then “invented” DNA holds some appeal, because amino acids, the constituents of protein, are thought to have been available in the prebiotic enviroment.

The second of Orgel’s possibilities, the simultaneous evolution of protein and DNA, seems even less likely, because it requires the emergence of not one but two improbable macromolecules.

Graham Cairn-Smith’s Clay World scenario provides an intriguing third option, with genetic-like sequences of elements replicating and acting as templates for organic assembly.

The fourth and favored genetics-origin model of Orgel and many followers is based on a nucleic-acid molecule such as RNA, a single-stranded polymer, that acts both as a carrier of information and as a catalyst that promotes self-replication.

Orgel proposed this model, long before any experimental evidence supported such a notion: “I must confess to a strong, longstanding bias in favor this explanation”, he remarked : “It is, a the very least, the model that can be studied most easly in the laboratory”. How to choose? When evaluating various origin-of-life models, scientists aren’t restricted to chemical experimental alone. The metabolism-first models of Wächtershäuser, de Duve, and others are equally

influenced by top-down** studies of molecular phylogeny, which point to deeply embedded, primordial biochemical pathways.

It is argued that the evolution of the genetic apparatus must have required the abiotic formation of macromulecules capable of residue-by-residue replication. This suggests that polynucleotides were presented even in the most primitive ancestors of contemporany organism.

Leslie Orgel, 1968

Few events have electrified the origin-of-life community as much as the early 1980s discovery of RNA ribozyme—strands of RNA that not only carry genetic information, but also act as a catalysts. Sidney Altman of Yale and Thomas Cech of the university of Colorado indipendently demostarted that a particular segment of RNA can accelerate key biochemical reactions. This startling finding, which won Altman and Cech the Nobel Prize in 1989, ispired a new vision of life's origin.

Many scientists adopt the "top-down**" approach: they scrutinize all manner of unambiguous living and fossil organism to identify the most primitive entities that are, or were, alive. This strategy is limeted, however, because all known life-forms, whether living or fossil, are based on biochemically sophisticated cells containing DNA and proteins. By contrast, a small army of investigators pursues the so-called "**bottom-up**" approach. The device laboratory experiments to mimic the emergent chemistry of ancient Earth enviroments. Eventually, the bottom-up goal is to create a living chemical system in the laboratory from scratch—an effort that might clarify the transition from nonlife to life.

Modern life relies on two complexity interrelated molecules: DNA which carried information, and proteins, which perform chemical functions.
This interdipendence leads to a kind of chicken-and-egg dilemma: Proteins make and maintain DNA, but DNA carries the instructions to make proteins. Which came first? RNA, it turns out, has the potential to do both jobs. The RNA World theory quickly emerged following the discovery of ribozyme. It champions the central role of genetic material in the dual tasks of catalyst and information transfer. Over the years, "RNA World" has come to mean different things to different people, but three precepts are common to all versions of the theory:

1. Once upon a time, RNA rather than DNA stored genetic information
2. Ancient RNA replication followed the same rules as a modern DNA replication by matching pairs of bases
3. Ancient RNA played the same catalytic roles as modern enzymes.

In this scenario, the first life-form was simply a self-replicating strand of RNA, perhaps enclosed in a protective lipid membrane. According to most versions of this hypothesis, modern metabolism emerged later, as a means to make RNA replication more efficent. Recently Orgel and co-workers (Paul et al., 2006) have demonstrated that, an RNA ligase ribozyme was converted to a corresponding deoxyribozyme through in vitro evolution. The ribozyme was prepared as a DNA molecule of the same sequence, and had no detectable activity. A population of randomized variants of this DNA was constructed and evolved to perform RNA ligation at a rate similar to that of the starting ribozyme. When the deoxyribozyme was prepared as an RNA molecule of the same sequence, it had no detectable activity. Thus, the evolutionary transition from an RNA to a DNA enzyme represents a switch, rather than a broadening, of the chemical basis for catalytic function. This transfer of both information and function is relevant to the transition between two different genetic systems based on nucleic acid-like molecules, as postulated to have occurred during the early history of life on Earth.

RNA's probable antiquity is underscored by growing list of other biochemical studies. In modern cells, RNA nucleotides play key structural roles in a variety of essential biological catalyst called coenzymes. Preliminary studies, not yet confirmed, discovered "riboswitches", remarkable segments of RNA that change shape when they bind to specific molecules in the cell. These chemical sensors then regulate the cell's chemistry by turning genes on and off. The inevitable conclusion: RNA is a very ancient molecule that seems to "do it all".

MY STARTING POINT

Life is a sturdy phenomenon and its initial steps *bona fide* originated from robust chemical frames based on firm thermodynamic ground. These assumptions on the simplicity and the necessity of the pre-biogenic processes are mitigated by the consideration that the genetic mechanisms onto which relies life-as-we-know-it today are combinatorially elaborated. In passing from the initial self-organization of chemical information to the potentially infinite complexity of interplaying genotypes and phenotypes that we experience today, evolution did necessarily play the key role. In my work I have focused on two aspects of the problem: the definition of a plausible chemical frame into which the first spontaneous syntheses could have taken place; the evolutionarily relevant selective properties and constrains that the first informational polymers had to deal with. The two aspects are intimately connected.

IS FORMAMIDE A PLAUSIBLE PREBIOTIC PRECURSOR?

The nature of the chemicals that played the role of prebiotic precursors on primitive Earth is still a debated argument. In a general approach to the problem, the following physical and chemical properties of the simple organic compounds under consideration should be taken into account.
Namely: (i) the relative abundance of the starting biogenic materials, to be considered a pre-requisite for the early onset (Dunlop et al., 1978; Lazcano and

Miller, 1994; Westall et al., 2001; van Zuilen et al., 2002) of genetic processes on this planet; (ii) their stability; (iii) their ability to react to give more complex structures following reproducible pathways. The formation of precursors based on simple chemical processes, and the *quasi*-simultaneous presence of all the building blocks to be used for the assembling of informational molecules are other important requisites.

(i) **Availability.** Formamide (H_2NCOH) meets the required criteria of abundance and diffusion in the Universe. The analysis of the molecular composition of comets-asteroids and of the interstellar clouds shows that the compounds made of the 4 more common and biologically relevant elements H, O, C and N (excluding He) are isocyanate HNCO and formamide H_2NCOH (Millar, 2004). Formamide was detected in the gas phase of interstellar medium (Crovisier, 2004), in the long period comet Hale-Bopp (Bockelee-Morvan et al., 2000), and tentatively in the solid phase of grains around the young stellar object W33A (Schutte et al., 1999). Possible formamide production under *Europa*-like conditions was observed (Hand, K.; Carlson, R. W., Department of Geological & Environmental Sciences, Stanford University; personal communication, July 2006).

(ii) **Stability.** Formamide meets the required criteria of stability. This topic must be considered in connection with hydrogen cyanide (HCN) chemistry. Since the pivotal experiment by Orò (Orò, 1960) on the synthesis of adenine from HCN, numerous studies were devoted to assess the role of this compound in the origin of primordial nucleic acids (Orgel, 2004). Nevertheless, two problems remain unsolved in the prebiotic relevance of HCN chemistry: (i) the thermodynamic instability of HCN under hydrolytic conditions, (ii) the narrow panel of nucleobases, limited only to purines, that can be formed by its condensation process. In the perspective of this latter observation, an all-purine precursor of nucleic acids was proposed, in which the pyrimidines present in extant nucleic acids would be post-enzymatic substitutes for their isoelectronic and isogeometric purines (Wächtershäuser, 1988) HCN is a gas under a wide range of environmental conditions. Thus, HCN chemistry in homogeneous solution

(the largely accepted chemical prebiotic *scenario* on the primitive Earth) firstly requires absorption in water. After the adsorption process polymerization and hydrolysis of HCN compete, the results being determined by its concentration. The two reactions are equivalent at concentrations of HCN between 0.01 and 0.1 M (between pH 8 and 9). Hydrolysis to formamide (Scheme 1, equation A) predominates in dilute solutions while polymerization takes over at higher concentrations (Sanchez, 1967). The steady state concentration of HCN in the primitive ocean was calculated, on the basis of the estimated rates of its production and hydrolysis, to be at pH 7 4 x 10^{-12} M at 100°C and 2 x 10^{-5} M at O°C. These concentrations are far too low for polymerization to nucleobases to occur, thus favouring hydrolysis to formamide (Stribling and Miller, 1987; Miyakawa et al., 2002).

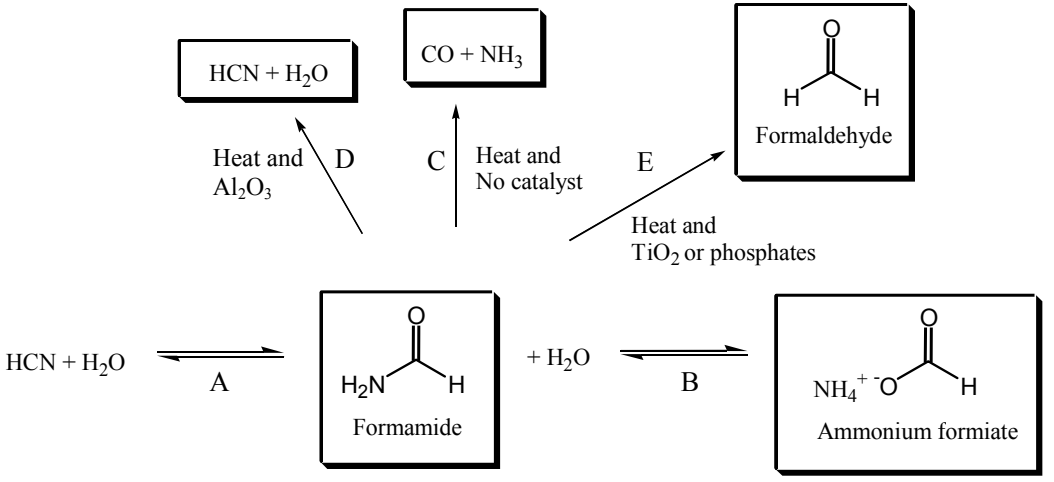
Since HCN is more volatile than water it cannot be concentrated by simple evaporation at pH lower than its pKa (9.2 at 25°C) (Shapiro, 2002). This suggested eutectic freezing as a means for HCN to reach the sufficient concentration for polymerization (Miyakawa et al., 2002).

In the same study, the hydrolysis rate (and the steady state concentration) of formamide to ammonium formate (Scheme 1, equation B) was also estimated as 2×10^{-18}, 1×10^{-15} and 1×10^{-9} M, at 200, 100 and 0 °C respectively, assuming that in the primitive ocean formamide was formed only by HCN hydrolysis.

On the basis of these data the authors suggest that "it is unlikely that formamide could have served a significant role in the prebiotic chemistry", a quite definitive sentence for this compound!

However, this assumption does not take into consideration that:

- formamide can be formed from prebiotic compounds largely diffused on the primitive Earth other than HCN



Scheme 1: Basic Formamide Chemistry (Saladino et al., 2007)

- formamide is liquid under a wide range of temperature and pressure values, with a boiling point of 210°C and very limited azeotropic effects (ECT Kirk-Othmer Encyclopedia of Chem. Techn., 1978)

Thus, at difference from HCN, formamide in a drying lagoon model can be easily concentrated, increasing its stability upon concentration and providing the adequate concentration for polymerization to nucleobases to occur. The hydrolysis of formamide in water was revisited by studying the solvent deuterium kinetic isotope effect. This analysis provided a value of the constant k_{hyd} of 1.1×10^{-10} s^{-1}, corresponding to a $t_{1/2}$ of ~200 yr at 25°C and pH 7 (Slebocka-Tilk et al., 2002).

(iii) **Reactivity.** As an organic compound able to generate "*in situ*" many other simple chemicals useful for the synthesis of nucleobases formamide can be

considered as a multifunctional prebiotic precursor. The ratio of the afforded precursors depends on the specific environmental conditions.
At 190-210 C° under atmospheric pressure formamide thermally decomposes either to ammonia (NH_3) and carbon monoxide (CO) (Scheme 1, equation C) or to HCN and water (Scheme 1, equation D). The formation of HCN is usually favoured in the presence of suitable catalysts, i.e., with aluminum oxides the yield at temperatures between 400°C and 600°C is > 90%, while in the absence of catalysts the reaction forming NH_3 and CO predominates (ECT Kirk-Othmer Encyclopedia of Chem. Techn., 1994).
The formation of formaldehyde, potentially leading to prebiotic synthesis of sugars, is favoured in the presence of titanium dioxide or phosphate, when formamide is heated (Scheme 1, equation E). Further decomposition products are also detected. These include polymeric hydrogen cyanide derivatives (Elvers et al., 1989) potentially producing nucleobases under hydrolytic conditions. Due to its high dielectric constant formamide (Faebe, 1990) is in addition an excellent solvent for both metal oxides and inorganic salts, which can act as catalysts during the condensation processes to nucleobases.
Thus, the composition of a reaction mixture based on formamide as the main component is tuned by the composition of the environmental reactor providing, at difference from HCN, all the prebiotic precursors necessary for the synthesis of both purine and pyrimidine nucleobases. The composition of the panel of the prevailing products depends on the specific physical and chemical properties of the catalysts present in the reaction medium.

Purpose of the Work

Formamide provides a chemical frame potentially affording all the monomeric components necessary for the formation of nucleic polymers. In the presence of the appropriate catalysts and by moderate heating formamide yields a complete set of nucleic bases and acyclonucleosides. This latter class of compounds is the step towards next level of complexity, potentially leading to informational

macromolecules by the release of formaldehyde useful for the formose condensation. Formaldehyde is converted into a mixture of monosaccharides by an aldol-like reaction. In a dilute solution of formaldehyde, ribose and 2'-deoxyribose may be formed by the action of ultraviolet light. This observation is of particular prebiotic relevance because of the known difficulty of building under prebiotic conditions the ß-glycosidic bond between separately synthesized nucleobases and sugars. Formamide acts as a multifunctional prebiotic precursor. The synthesis of nucleic bases from formamide, their formamide-based phosphorylation and their hypothetical but plausible formamide-triggered polymerization would remain a futile cycle of syntheses/degradations if the eventually formed polymers would not find themselves in the thermodynamic conditions allowing their survival as macromolecules. In this respect, sets of physico-chemical conditions are identified in which the polymeric state of phosphorylated nucleosides is favored over the monomeric one, for both the ribo and the deoxyribo systems. This finding establishes the principle that thermodynamic niches which differ for DNA and RNA exist favoring the otherwise intrinsically unstable polymeric molecular forms. RNA quite likely predated DNA in the development of biochemistry. However, given their marked differences, when considering the properties that might have influenced their origin, both DNA and RNA have to be analyzed, and compared. Based on these considerations, a general analysis of the stability of the phosphoester bonds, was performed, in ribo- and deoxyribo oligomers in the presence of minerals that have shown catalytic properties in the formamide/nucleic bases system. The stabilizing effect of soluble phosphates (Na_3PO_4, or $Na_4P_2O_7$, or $Na_5P_3O_9$) and organic phosphates, on RNA and DNA, was analyzed. A narrow pH range was identified in which complex sequences resist degradation markedly more than monotonous ones, thus potentially favouring the evolution of sequence-based genetic information. Given that the founding property of a polymer is to maintain its polymeric form and its sequence information, these findings support the view that the evolution of pre-genetic molecular information occurred based on the intrinsic properties of nucleic polymers, possibly in liquid-phase acellular

environments. It is not known how the nucleic bases were made available in sufficient concentrations and in acceptably equilibrated pools, nor how they chemically evolved into nucleosides, nor which activation mechanism allowed the monomeric units to polymerize in a process that in a pre-enzymatic world would have necessarily been spontaneous and self-sustaining. The hypothesis that these specific attributes of formamide allowed the onset of prebiotic chemical equilibria capable of Darwinian evolution is discussed.

Results

THE SYNTHESIS OF NUCLEIC PRECURSORS FROM FORMAMIDE

Nucleic bases and acyclonucleosides

We have observed that formamide has the unique property of condensing into both purine and pyrimidine nucleobases simply upon heating at 110-160°C in the presence of largely diffused metal oxides and minerals (Saladino et al., 2001; 2003; 2004; 2005a,b; 2006b) (Costanzo et al., 2007b). The products obtained are listed in Table 1, crossed with the catalysts tested and grouped as a function of (approximate) increasing complexity. Purine is the only compound obtained by heating formamide in the absence of catalysts. The most relevant aspects of this large ensemble of products are:

- the panel of compounds obtained in the presence of each catalyst is 'clean'.

Only few products are observed, in certain cases the synthesis being highly specific, as in the case of the phosphate mineral pyromorphite yielding exclusively cytosine or in the case of childrenite yielding almost only *N*-formylglycine. In other instances richer panels of products are obtained, as with pyrophosphate $Na_4P_2O_7$ yielding (in addition to purine) adenine, hypoxantine (a bioisoster of guanine), uracil, cytosine, *N*formylglycine and carbodiimide; and with TiO_2 yielding (in addition to purine) adenine, N9-formylpurine, N9-N6-diformyladenine, cytosine, thymine and 5-hydroxymethyluracil.

- Quite interestingly TiO_2 also catalyzes the synthesis of purine acyclonucleosides (not reported in Table 1, see Saladino et al., 2003). The mixture of NHCHO and formaldehyde (products heating formamide in the presence of titanium dioxide) is resposible for the formation of purine acyclonucleosides, probably by a formose-like condensation of an activated formaldehyde on the exocyclic formyl moiety of newly synthesized formylpurine and adenine derivatives. Under formose condensation formaldehyde is converted into a mixture of monosaccharides by an aldol-like reaction. Several sugars, including ribose and deoxyribose, may be formed by the action of uktraviolet

This observation is of particular prebiotic relevance because of the known difficulty of building under prebiotic conditions the β-glycosidic bond between separately synthesized nucleobases and sugars (Fuller et al., 1972). Anyhow, even in the instance of relatively higher complexity mixtures the products profiles keep their character of neatness and do not usually contain degradation products nor additional classes of compounds.

Glycine and carbodiimide

The α-amino acid derivative *N*-formylglycine was detected in formamide-based syntheses catalyzed by phosphate-minerals, often accompanied by carbodiimide (Saladino et al., 2006c). The synthesis of carbodiimide, which is an important agent for the condensation of aminoacids into peptides, could be responsible for the formation of formylglycine from *in situ* generated glycine (Saladino et al., 2006c), suggesting a role for the formamide-phosphate system in the prebiotic synthesis of peptides.

Intermediates of the synthetic pathways for components of extant nucleic acids are also observed, 4-aminoimidazole-5-carboxamide(AICA), 5-hydroxymethyluracil and 4-formylaminoimidazole-5-carboxamide(f-AICA). The chemical mechanisms onto which all these syntheses are based are described and critically discussed in Saladino et al., 2004; 2005a,b; 2006b; 2007.

Table1: Catalysed synthesis of nucleic acid components and precursors from formamide.

Product:

1 = Urea; 2 = Carbodiimide; 3 = N-Formylglycine; 4 = Hydroxypyrimidine;
5 = 4(3H)-Pyrimidinone; 6 = Uracil; 7 = 5-Hydroxymethyluracil;
8 = Dihydroxyuracil; 9 = Cytosine; 10 = Thymine; 11 = Parabanic acid;
12 = AICA: 5-Aminoimidazole-4-carboxamide;
13 = fAICA: 5-Formamidoimidazole-4-carboxamide; 14 = Purine;
15 = N^9-formylpurine; 16 = Adenine; 17 = N^9,N^6-Diformyladenine;
18 = Hypoxanthine.

Product	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CATALYST																		
Silica	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	+	-	-
Alumina	-	-	-	+	-	-	-	-	+	-	-	-	-	+++++	-	+	-	-
Kaolin	-	-	-	+	-	-	-	-	-	-	-	-	-	+++++	-	-	-	-
Zeolite	-	-	-	+	-	-	-	-	+	-	-	-	-	+++++	-	+	-	-
$CaCO_3$	-	-	-	-	-	-	-	-	-	-	-	-	-	+++++	-	-	-	-
KP-10 Clay [a]	-	-	-	-	-	+	-	-	+++	-	-	-	++	++++	+++	++	-	+
K-30 Clay[a]	-	-	-	-	-	+	-	-	+++	-	-	-	++++	+	++++	++++	-	-
KSF Clay [a]	-	-	-	-	-	+	-	-	+++	-	-	++++	+++	++++	+	+++	-	+
Al-PILC Clay [a]	-	-	-	-	-	+	-	-	+++	-	-	+++	+	+++	+	+	-	+
TiO_2	-	-	-	-	-	-	+	-	+	+	-	-	-	+++	+++	++	+	-
$MgFeSiO_4$	+	-	-	-	++	+	-	-	+++	-	-	-	-	+	-	-	-	-
$Mg_{1.5}Fe_{0.5}SiO_4$	+	-	-	-	++	-	-	-	++++	-	-	-	-	-	-	-	-	-
$Mg_{0.5}Fe_{1.5}SiO_4$	-	-	-	-	+++++	+	-	-	+++	-	-	-	-	-	-	-	-	-
Fe_2SiO_4	+	-	-	-	+++++	+	-	-	+++++	-	-	-	-	+	-	-	-	-
Mg_2SiO_4	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

Table1 (continued): Catalysed synthesis of nucleic acid components and precursors from formamide.

Product:
1 = Urea; 2 = Carbodiimide; 3 = N-Formylglycine; 4 = Hydroxypyrimidine;
5 = 4(3H)-Pyrimidinone; 6 = Uracil; 7 = 5-Hydroxymethyluracil;
8 = Dihydroxyuracil; 9 = Cytosine; 10 = Thymine; 11 = Parabanic acid;
12 = AICA: 5-Aminoimidazole-4-carboxamide;
13 = fAICA: 5-Formamidoimidazole-4-carboxamide; 14 = Purine;
15 = N^9-formylpurine; 16 = Adenine; 17 = N^9,N^6-Diformyladenine;
18 = Hypoxanthine.

Product	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CATALYST																		
Na_3PO_4	+	+	++	-	-	+	-	-	++++	-	-	-	-	+++	-	-	-	-
$Na_4P_2O_7$	-	+	+	-	-	+	-	+	+++	-	-	-	-	++	-	+	-	+
$Na_5P_3O_9$	-	+	++	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Turquoise[c]	-	-	+	-	-	-	-	-	++	-	-	-	-	++	-	-	-	-
Childrenite[c]	+	-	+++++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ludlamite[c]	-	-	+++++	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Vivianite[c]	+	-	++++	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Vauxite[c]	-	-	++++	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Lazulite[c]	-	-	+++	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-
Hureaulite[c]	-	+	-	-	-	+	-	-	++	-	-	-	-	+	-	-	-	-
Augelite[c]	-	-	+++	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Wavellite[c]	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Libethenite[c]	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Pyromorphite	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-

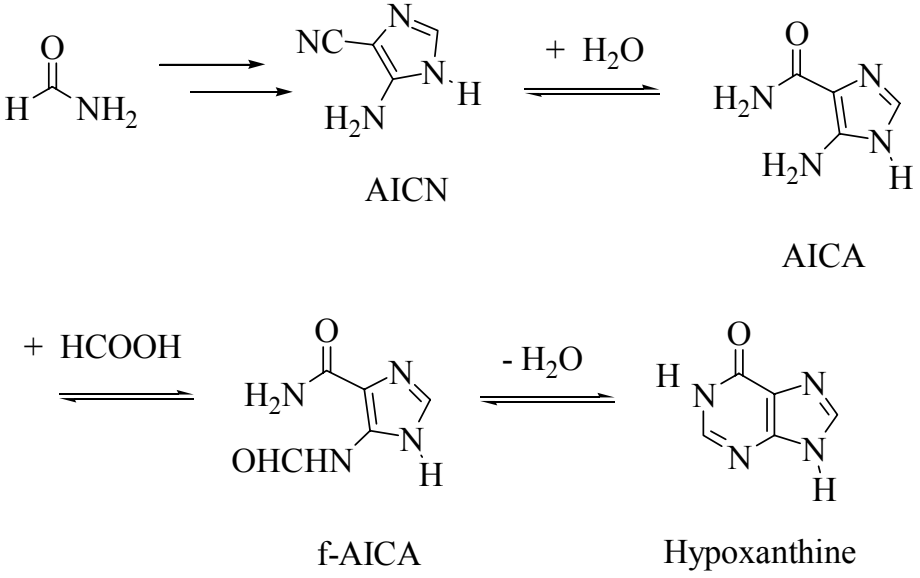
Legend to Table 1. [a]: Montmorillonite clays, [b]: Cosmic Dust Analogues, [c]: phosphate minerals. Data from [12] for the syntheses catalyzed by silica, alumina, kaolin, zeolites, and $CaCO_3$; from [13] for TiO_2; from [15] for olivines; from [16] for the soluble and mineral phosphates, as listed; mg of product per gram of formamide. +: 0.1-5.0 mg; ++: 5-10 mg; +++: 10-20 mg; ++++: 20-40 mg; +++++: >40mg.

The chemomimesis concept as a selector of prebiotic precursors

As mentioned above, the identity of the first prebiotic precursors of nucleic acids is still an argument of debate. On the other hand, the analysis of the mechanism of reaction of simple organic molecules reveals instances in which key intermediates are also produced corresponding to those observed in extant biological pathways. The concept of chemomimesis applies to these correspondence. This term, first introduced by Eschenmoser and Loewenthal in 1992 (Saladino et al., 2007), generally refers to a chemical reaction pathway that can be used as a template for the enzymatic processes that will appear later in

evolution, yielding the same final products. This property can in principle distinguish two classes of prebiotic precursors: the precursors that are able to generate a chemomimetic process from those that are not. Formamide chemistry shows interesting cases of chemomimesis.

Scheme 2



As an example, 5-aminoimidazole-4-carboxamide (AICA) and 5-formamidoimidazole-4-carboxamide (f-AICA), obtained in high yield in addition to hypoxanthine upon heating formamide in the presence of montmorillonites (Table 1), are also key intermediates (as ribonucleotide-5'-monophosphates) in the last steps of the extant biosynthesis of inosine-5'-monophosphate (IMP), the main route to purine nucleotides in the cell (Scheme 2). Similarly, the addition of formaldehyde to a preformed uracil scaffold during the synthesis of thymine from formamide and TiO_2 is a key step for the introduction of the methyl moiety, in agreement with the extant biosynthesis of thymidine. In this reaction a

formaldehyde unit is added to uridine, masked as the activated methylene unit of methylene tetrahydrofolate (MTHF) to give 5-hydroxymethyluracil-5'-monophosphate(HMU-5'-monophosphate).Thymidine will be obtained by successive hydride shift rearrangement. The possibility that early chemical events played the role of templates for the development of more complex (but also more efficient and selective) enzymatic pathways is a fascinating concept to be further evaluated in the study of the molecular evolution of informational polymers.

THE SOURCE OF PHOSPHATE IS A PROBLEM.

Early studies on the condensation of water-soluble phosphates to polyphosphates and on the phosphorylation, condensation or polymerization of biomolecules with polyphosphates have been reviewed (Schwartz, 1997; Yamagata et al., 1991). Most of the phosphorus in the early Earth would have been in the form of water insoluble minerals like apatites. Therefore, the origin of the water-soluble (poly)phosphates required for prebiotic evolution has long been a mystery (Schwartz, 1997).Yamagata et al. (Yamagata et al., 1991) showed that volcanic activity produces water-soluble phosphates through partial hydrolysis of P_4O_{10}, providing at least a partial solution to their origin. However, as pointed out, phosphates from hydrolysis of polyphosphate would precipitate to the sea bed as insoluble salts. The phosphorylation of biological molecules has been explored through several different routes. Phosphonic acids have been proposed as a source of biophosphates (De Graaf et al., 1997). For the phosphorylation of nucleosides, two early reports described the preparation of uridine phosphates by heating uridine with inorganic phosphates in an aqueous environment (Beck et al., 1967) and the effects of condensing agents on this reaction (Lohrmann and Orgel, 1968). However, the inefficiency of the system due to the competition of water with the nucleoside was pointed out by the authors. Additionally, cyclization of uridine 2′:3′ phosphate occurred under the same conditions but in much greater yield (Lohrmann and Orgel, 1968) by efficient intramolecular

esterification. A possible solution to abiotic nucleotide formation provided by solid state chemistry was also proposed (Reimann and Zubay, 1999) (Zubay and Mui, 2001). The alternative offered by phosphorylation in organic solvents, notably in formamide, was described in a series of pioneering studies by Schoffstall (Schoffstall , 1976; Schoffstall et al., 1982; Schoffstall and Liang, 1985; Schoffstall and Mahone, 1988). Building on these latter observations and on the large availability of phosphates in mineral form, we report the efficient phosphorylation of nucleosides occurring in formamide on numerous phosphate minerals. Consequently, the two alternatives mentioned above are not necessarily in contradiction: activated monomers can form in prebiotic conditions in a liquid, non-aqueous environment in the presence of phosphate minerals in conditions compatible with the thermodynamics of polymerization.

ABIOTIC NUCLEOSIDE PHOSPHORYLATION

In the presence of formamide, crystal phosphate minerals may act as phosphate donors to nucleosides, yielding both 5' and, to a lesser extent, 3' phosphorylated forms.

With the mineral Libethenite the formation of 5'AMP can be as high as 6% of the adenosine input and last for at least 10^3 hrs.

At high concentrations, soluble non-mineral phosphate donors (KH_2PO_4 or 5' CMP) afford in addition to 5' and 3' AMP also 2' and 2':3' cyclic AMP. The phosphate minerals analyzed were Herderite $Ca[BePO_4F]$, Hureaulite $Mn^{2+}_5(PO_3(OH)_2(PO_4)_2(H_2O)_4$, Libethenite $Cu^{2+}_2(PO_4)(OH)$, Pyromorphite $Pb_5(PO_4)_3Cl$, Turquoise $Cu^{2+}Al_6(PO_4)_4(OH)_8(H_2O)_4$, Fluorapatite $Ca_5(PO_4)_3F$, Hydroxylapatite $Ca_5(PO)_4OH_3$, Vivianite $Fe^{2+}_3(PO_4)_2(H_2O)_8$, Cornetite

$Cu^{2+}_3(PO_4)(OH)_3$, Pseudomalachite $Cu^{2+}_5(PO_4)_2(OH)_4$, Reichenbachite $Cu^{2+}_5(PO_4)_2(OH)_4$, Ludjibaite $Cu^{2+}_5(PO_4)_2(OH)_4$) (Costanzo et al., 2007a).

Based on their behaviour in the formamide-driven nucleosides phosphorylation reaction these minerals can be classified:

1) inactive,
2) low-level phosphorylating agents,
3) active phosphorylating agents.

Instances were detected (Libethenite and Hydroxylapatite) in which phosphorylation occurs onto the mineral surface, followed by release of the phosphorylated compounds. Libethenite and Cornetite markedly protect the β-glycosidic bond.
As a prerequisite to the analysis of the phosphorylation (or otherwise) of adenosine by phosphate minerals, the kinetics of free phosphate release (or its absence) were determined.
The molybdenum-blue colorimetric assay allowing only the detection of orthophosphate (Chen et al., 1956) was the method of election. Given that the phosphate minerals studied all contain single-phosphorus units the method is adequate.
The ground mineral material was treated at 130°C in 100% formamide and samples were analyzed at various time intervals. Fig. 1 shows examples of the two typical different behaviors: Herderite $Ca[BePO_4F]$ which did not released phosphate, and Hureaulite $Mn^{2+}_5(PO_3(OH)_2(PO_4)_2(H_2O)_4$ which steadily released phosphate as a function of time.

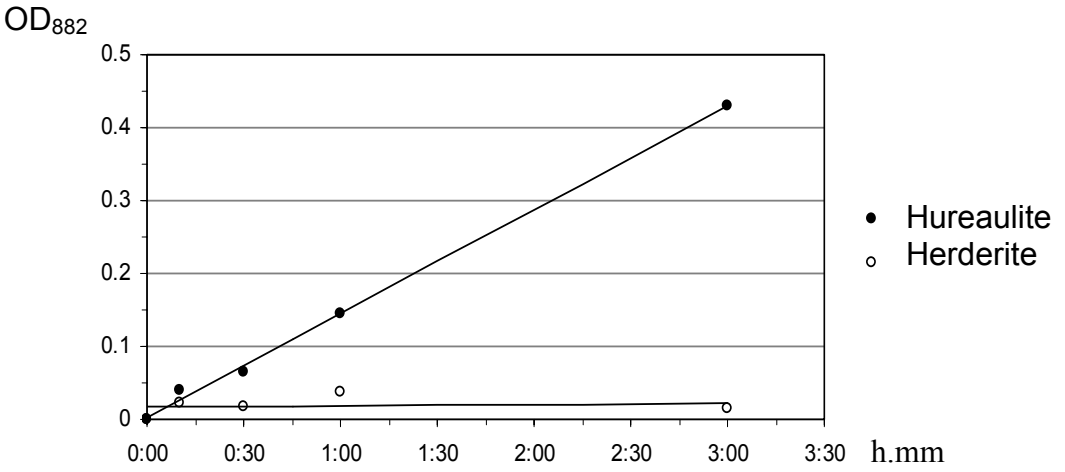


Fig.1: Hureaulite $Mn^{2+}_5(PO_3(OH))_2(PO_4)_2(H_2O)_4$,release: 0.107µg/ml·hrs^{-1} Herderite $Ca[BePO_4F]$, no release (Costanzo et al., 2007a).

Minerals were pretreated (130°C, 72 hrs, in pure formamide), then temperature was lowered at 90°C and adenosine added. Samples were taken at various times and the reaction products analyzed by HPLC. Minerals of class 1 (Herderite, Pyromorphyte, Turquoise, Fluorapatite, Vivianite) release no or minimal (≤0.01 µg/ml) amounts of phosphate and phosphorilation doesn't occurs. Class 2 minerals do not presumably take active part in the phosphorylation process, their function being quite likely limited to a passive release of phosphate in the medium so phosphorylation is at very low levels, as exemplified by Hureaulite (Fig. 2) and Pseudomalachite $Cu^{2+}_5(PO_4)_2(OH)_4$ (data not shown). In this case the products identified are limited to the most abundant 5'AMP, while only traces of other compounds were observed.

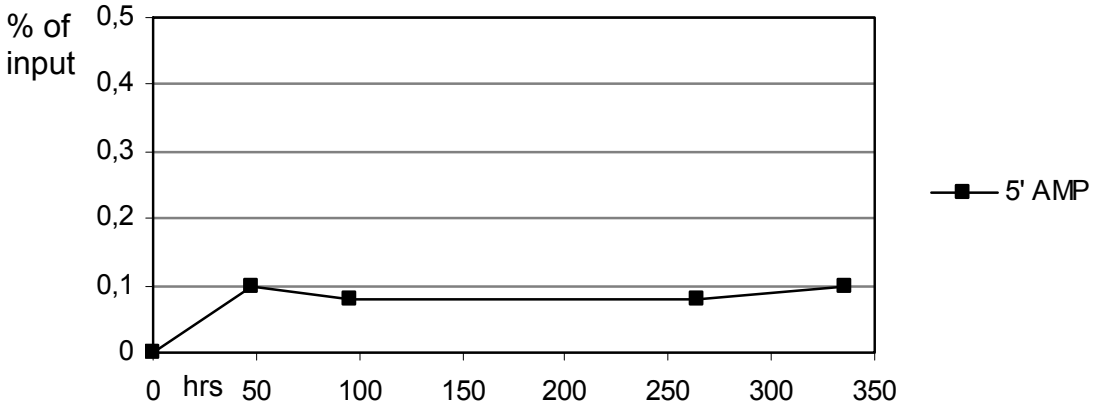


Figure 2. Formation of 5'AMP from adenosine in formamide/Hureaulite at 90°C for the indicated times (abscissa). Hureaulite pre-treatment: 72 hrs at 130°C (Costanzo et al., 2007a).

A group of minerals (class 3) was identified which are characterized by an active behavior: Libethenite $Cu^{2+}_2(PO_4)(OH)$, Ludjibaite $Cu^{2+}_5(PO_4)_2(OH)_4$, Reichenbachite $Cu^{2+}_5(PO_4)_2(OH)_4$, Cornetite $Cu^{2+}_3(PO_4)(OH)_3$ and Hydroxylapatite $Ca_5(PO_4)_3OH$.

Fig. 3 shows the phosphorylation reactions, carried out as for the previous samples, with an additional analytical step. Two parallel samples were treated as follows: both were pretreated at 130°C (72 hrs). One sample was centrifuged three times to remove the mineral, leaving the released phosphate in the formamide super (dubbed as "solute"). The other sample containing the mineral was analyzed as such in the standard conditions (90°C, 100% formamide). The two samples were analyzed separately by HPLC.

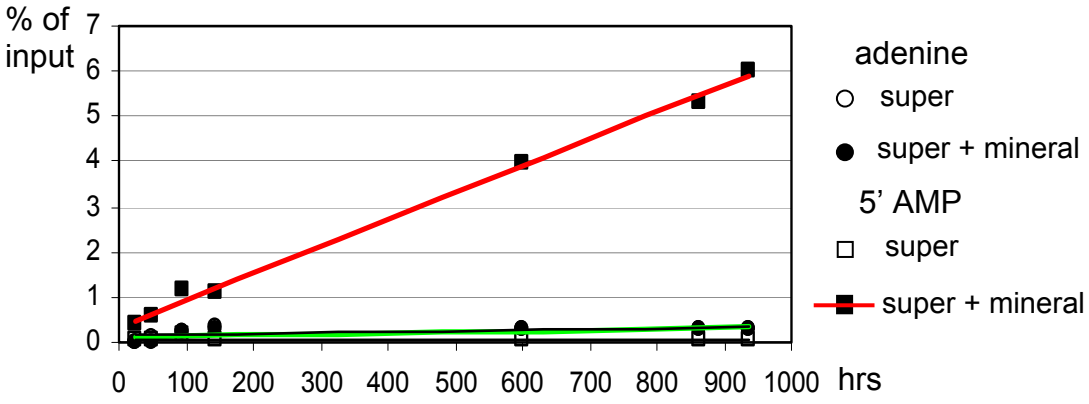


Figure 3. Formation of 5' AMP from adenosine in formamide/Libethenite at 90°C for the indicated times (abscissa). Libethenite pre-treatment: 72 hrs at 130°C (Costanzo et al., 2007a).

In the case of Libethenite the mineral-containing fraction affords a major production and release of 5'AMP, regularly continued for about 1000 hours. The release was detected of adenine (4.0%), of 3'AMP (1.3%), of 2':3' cyclic AMP (0.92%) (data not shown). Thus, Libethenite actively promotes phosphorylation, selectively releasing from its surface one of the formed compounds (5'AMP) and keeping in bound but releasable form other products of the reaction. The solute-containing supernatant from Libethenite yields only trace amounts of 5'AMP, as observed for Class 2 minerals. The four minerals identified as Class 3 display pseudo-catalytic activity. The mineral must be present in order for the phosphorylation reaction to occur. Mineral surface phenomena are here a *bona fide* required part of the reaction, as shown by the fact that the pre-released phosphates ("solute") are almost inert and that the presence of the mineral is necessary for the phosphorylation process to occur at high pace. However, given

that the phosphate component of the mineral is transferred to the recipient adenosine, these minerals cannot be classified as catalysts. The catalyst function is exerted by formamide. The phosphorylation of adenosine by two different inorganic phosphate donors was analyzed for the sake of comparison with previously reported nucleoside phosphorylation systems (Beck et al 1967; Schoffstall and Mahone, 1988; Lohrmann and Orgel, 1971) and in order to define the kinetics and the products regio-selectivity of the phosphate reactions in formamide. Adenosine was reacted in water or in pure formamide at 90°C for increasing time in the presence of the appropriate phosphate donor (KH_2PO_4 or 5'CMP). The products were analyzed by HPLC (Fig. 4).

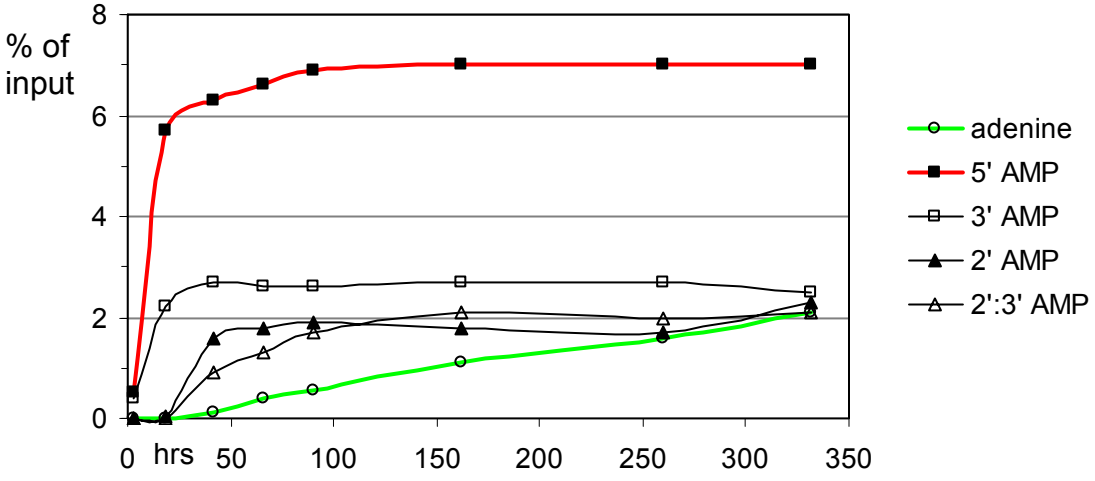


Fig. 4: Adenosine phosphorylations by KH_2PO_4 (or by 5'CMP data not shown). Adenosine was reacted (100% formamide, 90°C, as detailed in Methods) with KH_2PO_4 for the indicated times (abscissa) and analyzed by HPLC. The products afforded are indicated in the legend on the right side. Ordinate: % of the product relative to adenosine input. (Costanzo et al., 2007a).

Adenosine is phosphorylated in different positions and the order of appearance of each species is governed by its rate of formation, with the last to form being the 2':3' cyclic species. The differential rates of degradation (2'AMP > 3'AMP > 5'AMP > 2':3' cyclic AMP), measured on the order of minutes, and the species-specific rates of rephosphorylation, measured on the order of hours, determine the steady state equilibrium of the pool of phosphorylated nucleosides. A similar overall behavior is observed in the specular system of cytidine phosphorylation by KH_2PO_4 or by 5' AMP. The primary difference is the higher total amount of phosphorylated forms in the Cytosine system (21%) relative to the Adenine system (16%). Thus, the overall rate of phosphorylation is determined more by the acceptor than by the donor. In water no phosphorylated forms were observed (data not shown), the only reaction taking place being the cleavage of the β-glycosidic bond and the consequent release of adenine. As reported (Saladino et al., 2006), the half-life of this bond at 90°C in water is $4.5 \cdot 10^2$ hrs. Thus, the active component of the phosphorylation reaction is formamide.

The interest of the 2':3' cyclic phosphate nucleotide

The more stable phosphorylation product in the formamide system is the 2':3' cyclic form. The facile formation and the reactivity of this specific cyclic form make it particularly interesting in the perspective of polymer chain formation. It was actually shown (Millar, 2004; Saladino et al., 2001) that 3',5'-linked hexaadenylic acid with a 2':3' cyclic phosphate terminus couples on a polyuridylic acid template in the presence of ethylene diamine to form the dodecamer and octadecamer (Lohrmann and Orgel, 1968). The bond produced was largely that of the 2',5' isomer, but about 5% of 3',5' bonds also formed. The same authors observed (Millar, 2004) that upon annealing with a 3',5'-linked complementary poly U strand the stability of the 2',5' bond becomes about 900-fold lower than that of the 3',5' bond. This helical conformation-induced selective instability rapidly leads to a majority of the "natural" 3',5' phosphoester bonds. The presence of mineral phosphates (which also catalyze the condensation of formamide into a large set of nucleic precursors –Akhter and Alawi, 2003) could have allowed, within the same chemical frame, the next step towards

polymerization: nucleoside phosphorylation (Verlander et al., 1973). Activated nucleic monomers can form in a liquid non-aqueous environment in conditions compatible with the thermodynamics of polymerization, thus providing a solution to the standard-state Gibbs free energy change ($\Delta G°$) problem, the major obstacle for polymerizations in liquid phase in plausible prebiotic scenarios.

PREBIOTIC SYNTHESIS OF OLIGONUCLEOTIDES

The first attempts of prebiotic synthesis of oligonucleotides were mainly directed to design the optimal conditions for the formation of the phosphate ester bond between preformed nucleotide derivatives. In principle, the prebiotic synthesis of the oligonucleotide chain can be performed more easily in the presence of a template. In this case, molecular recognition processes based on specific hydrogen bond interactions between complementary nucleobases can direct the monomer in the correct spatial position to form the phosphodiester bond with low energy requirement. Based on this model, Orgel described the first selective polymerization of adenosine-5'-monophosphate (5' AMP) and guanosine-5'-monophosphate (5' GMP) in the presence of complementary oligonucleotides as templates (polyuridylic and polycytidylic acids, respectively) using different condensing agents (Sulston, et al., 1969; Schneider-Bernloehr et al., 1968; Orgel, 1968) 2',5'-phosphodiester linkages were predominantly formed.

These experiments marked an important advancement in the prebiotic synthesis of oligonucleotides, introducing the use of activated nucleotides, mainly on the 5'-position of the sugar, for the polymerization process. In the 1970s, Orgel described the first example of an efficent prebiotic synthesis of oligonucleotides using together the template and the activated nucleotides approaches (Osterberg et al., 1973). The phosphate activating group, that was usually a good leaving group such as imidazole and 2-methylimidazole, played a relevant role in driving the polymerization toward longer oligomers (Weimann et al., 1968). In this context, imidazolide derivatives of ribonucleotide diphosphates were used for the polymerization of oligonucleotides linked by pyrophosphate bonds (Schwartz

and Orgel, 1985). Noteworthy, the prebiotic synthesis of oligonucleotides in the presence of a template can be selectively catalyzed by minerals which were widely diffused on the primitive Earth, such as montmorillonite clays (Ferris et al., 1984). Ferris described several examples of montmorillonite catalyzed synthesis of oligonucleotides (Ferris and Ertem, 1992a,b; Ferris and Ertem, 1993; Ferris, 1993). Linear and cyclic oligonucleotides containing both 3',5'- and 2',5'-phosphodiester bonds, and pyrophosphate bonds ($N^{5'}$ppN bonds), were for instance synthesized in acceptable yield by condensation of 5'-phosphorimidazolide cytidine (ImpC) on montmorillonite. The presence of template doesn't reveal the first prebiotic polymerization event. In 1973 Orgel and co-workers observed that adenosine cyclic 2',3'-phosphate, mainteined in a dry-state and in alkaline conditions at moderate temperature, self-polymerization to give oligonucleotides chainlenght up to at least 6 (Verlander et al., 1973). Hydrothermal environments present on the sea floor of the primitive Earth might also have served as a local reactor for the synthesis of oligonucleotides from nucleotides (Matsuno, 2000). These environments are characterized by the presence of a constant temperature gradient between the hot vents and their surroundings, where synthetic and degradative pathways can be operative (Matsuno, 1997). In this context, the interface between the hot and the cold region deserves special attention. In fact, in a scenario in which the mass transfer across the interface is faster than the degradative processes, products obtained at high temperatures may survive long enough to be used in the molecular evolution process. Matsuno reported the first example of synthesis of oligonucleotides from nucleotide monophosphates in the absence of condensing agents in a simulated hydrothermal environment (Ogasawara et al., 2000).

UNITARY CHEMICAL FRAME

What is actually still lacking is a unitary chemical frame: if polymers had to form, the system had to contain all the necessary starting components. If it is true that life is a robust phenomenon, as it appears from its early onset, the

polymerizations had to occur based on a single unitary process and starting from a simple precursor. In principle, the ideal prebiotic precursor is a one-carbon fragment compound that is able to contemporarily synthesize nucleobases and sugars, acting also as condensing agent for the phosphorylation process. The formamide-based efficient production of large panels of both purines and pyrimidines nucleobases and acyclonucleosides, combined with its ability to catalyze the phosphorylation of nucleosides, could provide one plausible solution to the problem of global availabilities. The great versatility of synthesis afforded by common and prebiotically available catalysts adds to this plausibility. In a prebiotic perspective, what is the reason why nucleobases, possibly in their nucleoside forms, would polymerize and, following their very combination into linear macromolecules, give rise to next level of informational complexity?

In the words of Watson and Crick referred to DNA: "it has not escaped our notice" that this type of informational macromolecule and its structural property of complementary specific base pairing "immediately suggests a possible copying mechanism for the genetic material" (Watson and Crick, 1953). The prebiotic perspective changes into a biotic one if a molecular Darwinian selection process comes into play. The selective advantage is intrinsic in the physico-chemical attributes of the system: that it simply consists of the increased survival of the components upon polymerization. An extensive and unitary analysis of this fact has not been performed. However, numerous observations have been reported since: the rate of hydrolysis of free deoxynucleosides is 10–50 times higher relative to the rate of cleavage of N-glycosyl bonds in single-stranded DNA (Shapiro and Kang, 1969); the rate of hydrolysis of Nglycosyl bonds in deoxynucleosides is higher that in deoxynucleotides, which is higher than in DNA (Garrett and Mehta, 1972); depurination is 4 times faster in single- versus double-stranded DNA (Lindahl and Karlström, 1973).

Although performed in different systems and with different techniques, taken together these observations indicate that upon polymerization the stability of the starting monomers changes.

Whether this fact is sufficient to provide a Darwinian advantage for the survival

of polymers versus the survival of monomers.

NATURAL SELECTION IS THE STABILITY OF CHEMICAL BONDS: NEED OF THERMODYNAMIC NICHES

Let us start from the classical, far-sighted words by Darwin "… *we could conceive in some warm little pond, with all sort of ammonia and phosphoric salts, light, heat, electricity, etc,…*" (Darwin, 1888). Let us also assume the presence of formamide in the pond. Let us also imagine that these liquids fill crevices in rocks made of any of the catalysts (or of a combination thereof), possibly of clays and/or olivines of terrestrial and/or sidereal origin. At moderately hot temperatures (in our experimental settings between 90°C and 160°C, Saladino et al., 2001, 2003, 2004, 2005a,b; 2006a,b; 2007) this pristine little pond would quickly be enriched by nucleic bases and, if TiO_2 was also present, by sugar chains growing onto them (Saladino et al., 2003). How widespread might have been the photochemistry at the basis of this latter process has not been thoroughly studied; however, it can be confidently assumed that additional catalysts might have also been able to carry out similar synthetic processes. In hot formamide nucleic bases are rapidly degraded (Saladino et al., 1996) and the synthesis/degradation cycle would remain totally sterile, in spite of the robustness of the formamide-based synthetic reactions. A way-out from such a futile cycle could have been found if two conditions were met: an equally robust and simple polymerization process and the existence of a set of conditions resulting in a thermodynamic niche in which the survival of the polymer is favoured. We have not completely studied the role of formamide in the abiotic polymerization process. Its plausibility and possible robustness are hinted at by the pioneering observations by Schoffstall and co-authors who described the facile and high-yield phosphorylation and trans-phosphorylation processes of nucleic bases by formamide and various inorganic phosphates (Schoffstall et al., 1976; 1982; 1985; 1988). We have confirmed their observations using also organic phosphates (Costanzo et al., 2007a), and found that the yields of *de novo*

phosphorylated nucleosides may be quite high and position selective (detailed in the section: abiotic nucleoside phosphorilation). No matter how hypothetical, this scheme relies on chemically and thermodynamically (Van Holde, 1980) sound assumptions. Let us further imagine that water slips into the dry (Van Holde, 1980) phosphate-clay crevice in which the nucleic bases have formed (Saladino et al., 2001, 2003, 2004b, 2006c; Costanzo et al., 2007b) and have been (trans)-phosphorylated (Costanzo et al., 2007a), thus binding one to the other. The question is: how long will the polymers survive in the water solution in which they dissolve and in which they are supposed to endure for a while long enough to replicate and start their evolutionary endeavour? The exploitment of the set of conditions (here dubbed "thermodynamic niche") favouring the survival of the phosphoester bond when embedded in a polymer is considered the initial Darwinian property necessary to allow accumulation and evolution of chemical ur-genetic information (Saladino et al., 2006b).

DNA AND DEOXYMONOMER

The half-life values of the phosphoester bonds were analyzed in detail both in deoxyribo nucleosides and nucleotides, and in the DNA chain (Saladino et al., 2005c) in a partial panel of conditions similar to those in which all the processes of formamide chemistry do occur: temperatures up to 110°C and formamide content in water between 0 and 100%. We have defined (i) the stability of the 3'- and the 5'-phosphoester bonds in a model deoxyoligonucleotide as a function of temperature and of the water/formamide w/w ratios and (ii) the stability of the 3'- and the 5'-phosphoester bonds in monophosphate deoxynucleosides under a corresponding set of conditions. The comparison of these two sets of data provides information relevant to the monomer-*versus*-polymer stability bias. The degradation pathways of the deoxy polymer chain, the Hydrolysis via Nucleobase Substitution (HNS) pathway prevailing in water, the Hydrolysis via Nucleobase Degradation (HND) pathway prevailing in formamide. The fast hydrolytic degradation of DNA and the regularity of the cleavage ladders are evident (Fig. 4 panel A).

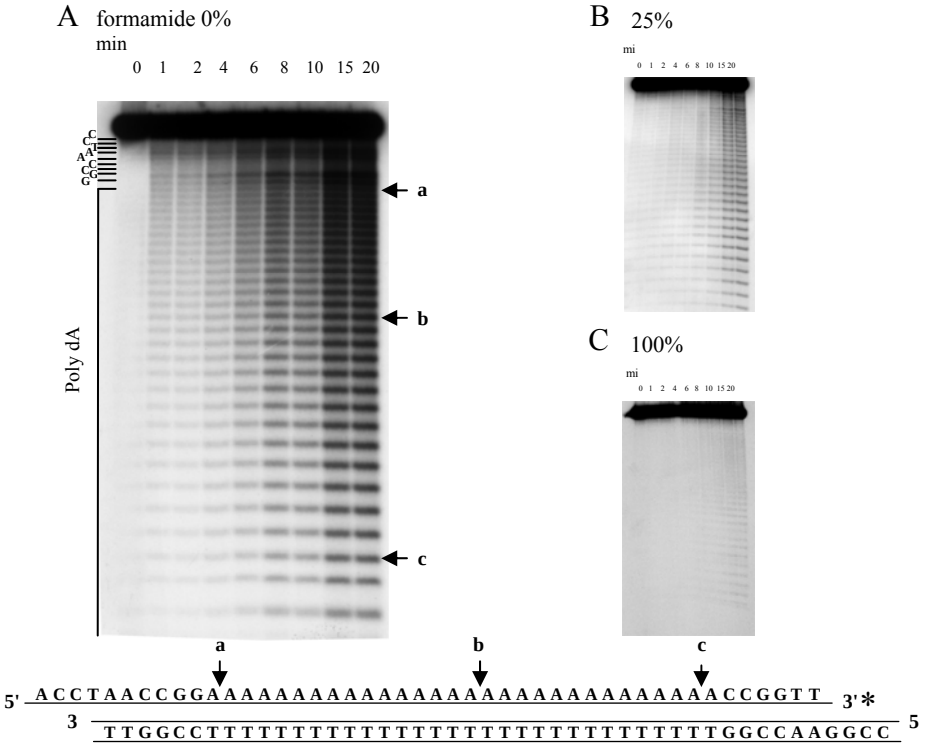


Fig. 4: Degradation of a 3'-labeled 46-baselong oligonucleotide containing a 30-bp-long dA stretch and mixed sequence extremities. The oligo was incubated at 90°C for the time indicated on top of each lane in H_2O (A), in 25% (B), or 100% formamide (C) and processed. The a, b, and c letters point to the positions whose cleavage kinetics was determined.

Depurination is known to be faster than depyrimidination (Saladino et al., 1996; 1997) (Kochetov et al., 1972). The degradations reported in the accompanying panels (B and C) performed in the presence of increasing amounts of formamide

show that as a general trend formamide appreciably protects DNA from the hydrolytic process. The variations from this trend, namely at low formamide concentration and at high temperatures, are established. The average $t_{1/2}$ of the 3'-phosphoester bond under these conditions (90°C, H_2O) is 142 ± 24 min. *Panel B* describes the effect of increasing formamide on the 3'-phosphoester cleavage reported above: enhancement by the low concentrations and protection by the higher ones. For the 5'-bond the same analysis was performed on the same oligo, labeled at the 5'-extremity (see "Methods"): the average half-life of the 5'-phosphoester bond is 230 ± 37 min (90°C, H_2O). A detailed analysis was performed of the effect of temperature and formamide on the stability of the DNA chain. At the fixed time of 8 h, the kinetics of degradation was analyzed for the temperatures of 40, 50, 60, 70, 75, 80, and 90°C at formamide concentrations of 0, 10, 25, 33, 66, and 100%. Fig. 5 shows a series of scanning profiles obtained from this electrophoretic analyses presented as a function of increasing temperature (vertical) and increasing formamide % (horizontal). The first vertical row from the left (H_2O, 0% formamide) describes the effect of increasing temperature in enhancing the rate of hydrolysis. The diagnostic feature inside each panel is the displacement of the degradation profile from the origin of the scanned lane on the left (the left most peak corresponding to the uncleaved sample) toward the bottom of the lane (the right side of the plot). The results are interpreted according to the two following different degradation mechanisms. At temperatures 100°C or above and in the absence of water, formamide reacts with both purine and pirimidine nucleobases by nucleophilic addition at position C(8) and position C(4) and C (6), respectively (Saladino et., 1996 for purine; Saladino et al., 1997 for pyrimidines), causing the opening of the imidazole or pyrimidine rings and the ensuing cleavage of the β-glycosidic bond. The β-eliminations of both the 3'- and the 5'-phosphoester bonds and the consequent cleavage of the polymer follow (Saladino et al., 1996; Saladino et al., 1997). The cleavage at 3' is faster than that at 5' (Saldino et al., 1996; Negri et al., 1996). This reaction pathway can be referred to as Hydrolysis following Nucleobase Degradation (HND). In water the hydrolytic cleavages of the oligonucleotide occur following

the direct cleavage of the β-glycosidic bond (mainly leading to the removal of the non-degraded base). This mechanism can be referred to as Hydrolysis following Nucleobase Substitution (HNS).

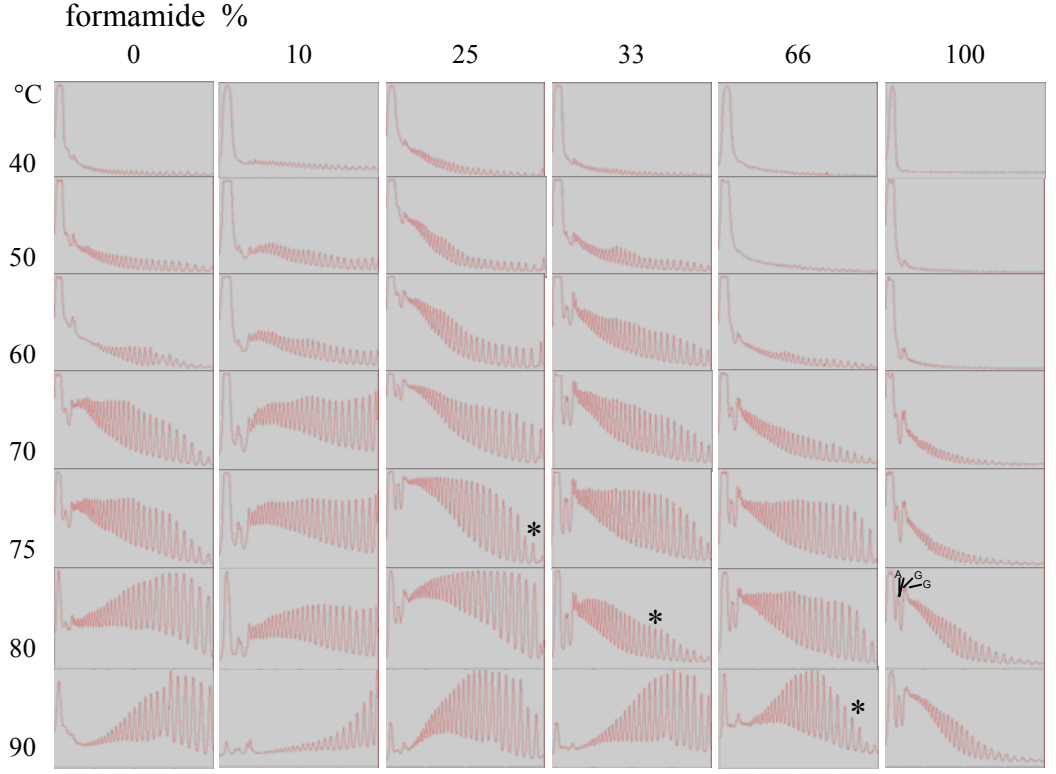


Fig. 5: Scanning profiles of electrophoresis analyses The reactions were run at the formamide concentrations indicated on top and at the temperatures indicated on the left (Saladino et al., 2005c).

Inspection of the profiles of the samples (Fig. 5) treated at increasing concentrations of formamide revealed its increasingly protective effect against HNS hydrolysis, as evident in the upper horizontal rows of panels (40 and 50°C, increasing formamide from left to right). The last vertical row on the right (100% formamide, increasing temperatures from top to bottom) shows patterns caused by β-elimination cleavage due to the HND pathway. This latter reaction is markedly sequence-dependent as indicated in the "80°C-100%" panel. The order of cleavage is G ≥A > C » T (Saladino et al., 1996; Negri et al., 1996). HNS hydrolysis decreases from left to right and, in central panels, from top to bottom, as shown by the intermediate behavior observed at intermediate conditions, where the two mechanisms coexist. HPLC analyses are performed to establish the effect of formamide on the stability of the β-glycosidic bond in deoxymonomers. Measurements were performed as a function of formamide concentration in water and of temperature. HPCL analysis of the degradation of deoxyadenosine indicate that the half-life of this bond at 90°C in water is 8.5 ± 10^3min. Increasing concentrations of formamide first decrease and then (>25%) markedly increase the stability of this bond. This double effect exerted by increasing formamide on the stability of the β-glycosidic bond, observed both in the deoxyadenosine and in the deoxyadenosine monophosphate. A detailed analysis of the numerous effects of formamide on the 5'-phosphoester bond of 5'-dAMP is shown in Fig. 6. Formamide strongly stimulates the disappearance of the 5'-dAMP (blue line) due to the cleavage of the 5'-phosphoester bond, resulting in the increasingly earlier formation of the nucleoside (red). An additional effect is the delayed appearance of the base (green) due to the slower cleavage of the β-glycosidic bond. The fact that formamide strongly protects the β-glycosidic bond is shown by the increasing distance between the red and green curves as a function of the increasing concentration of formamide. The same analysis are performed for the 3'-monophosphate form of deoxyadenosine as a function of both temperature and formamide concentrations. Increasing formamide decreases the half-life of the 3'-phosphoester bond. Interestingly, the presence of the phosphate group in 3' stabilizes the β-glycosidic bond.

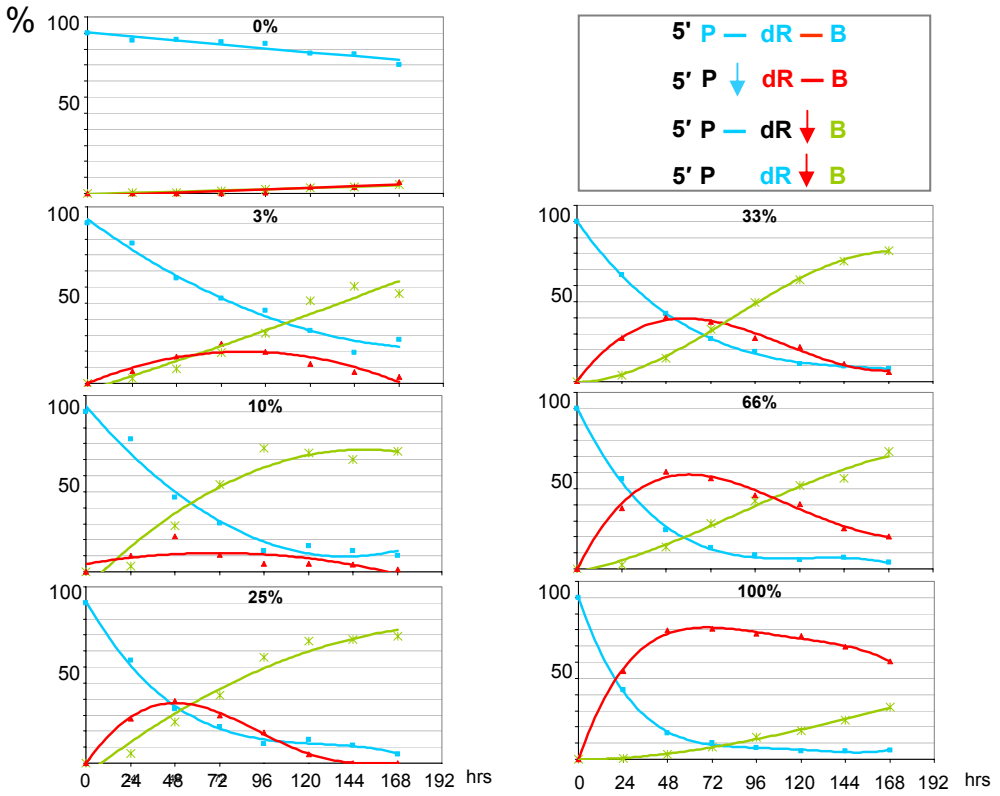


Fig. 8: HPCL analyses of the degradation of 5'- monophosphate deoxyadenosine (Saladino etal., 2005c).

The half-lives of the β-glycosidic bond and of both the 3'- and 5'-phosphoester bonds as a function of increasing formamide are summarized in Fig. 9, and 10A e B, respectively. The β-glycosidic bond (Fig. 9) is cleaved faster in the presence of low concentrations of formamide and then is markedly protected. This effect

occurs for the β-glycosidic bond in both adenosine and on its phosphate forms. The half-life values in Fig. 9 for the 5'-dAMP plot are taken from the degradations reported in Fig. 8. The overall trend is respected in both cases (as expected) with one important notation, the half-lives of the β-glycosidic bond are constantly longer in the phosphate form. In water the effect is quite strong.

The 3'- and the 5'-phosphoester bonds are cleaved faster in the presence of formamide, the effect being quite strong for the 5'-phosphoester bond, less so for the 3' one. The stability in water of the 5'-phosphoester bond is 10-fold higher ($t_{1/2} = 27 \cdot 10^3$ min) than that at 3' ($t_{1/2} = 2.6 \cdot 10^3$ min).

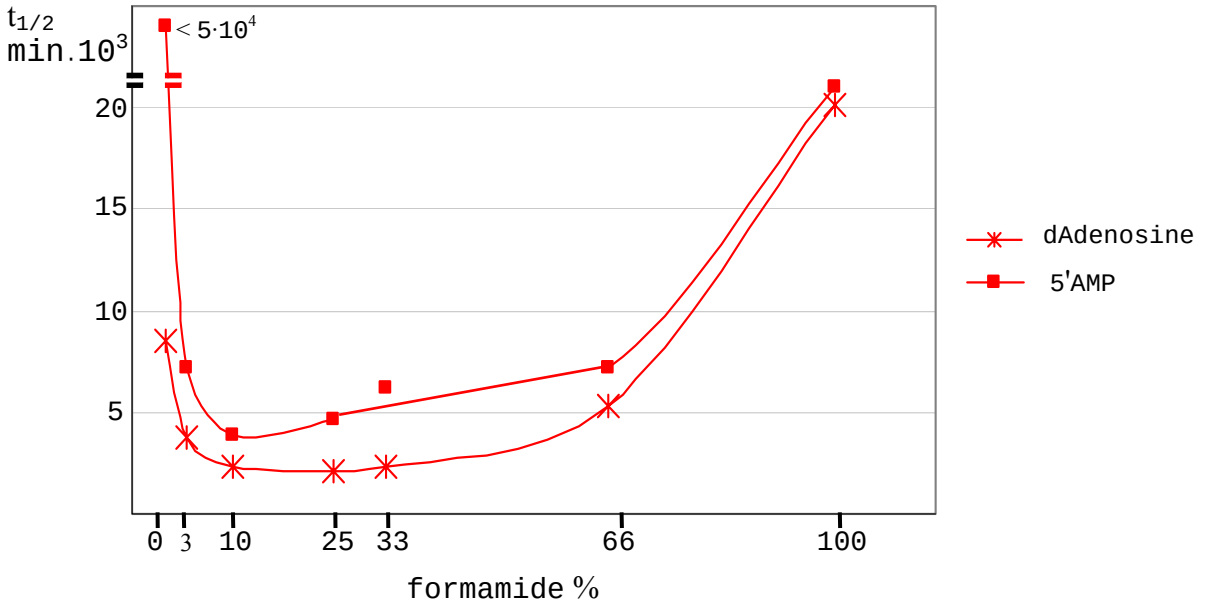


Fig. 9: The stability of the β-glycosidic bonds as a function of formamide concentration. The half-lives (min ± 10^3) of deoxyadenosine (dAdenosine) and of 5'-dAMP are calculated from the experiments reported in Fig. 8.

The data reported above on the stability of the β-glycosidic and phosphoester bonds in the monomers and polymers reveal strong and differential sensitivity (Saladino et al., 2005c). Independent of the detailed chemical mechanisms involved, the plot of the half-life values of the 5'-phosphoester bonds in the monomer 5'-dAMP and in the oligomer as a function of formamide concentration (Fig. 10*A*) reveals a relevant property: formamide *decreases* the half-life of the 5'-phosphoester bond in the monomer and *increases* that of the same bond in the polymer. The effect is quite strong: from a $t_{1/2}$ of $28.5 \cdot 10^3$ min for the AMP and of $0.23 \cdot 10^3$ for the polymer (ratio = 125), the situation is reversed starting at 75% formamide. In formamide (100%) the same bond becomes *more stable in DNA* than in the precursor monomer. For the 3'-phosphoester bond the phenomenon and the trend are similar, although somewhat less marked: $2.55 \cdot 10^3$ *versus* $0.14 \cdot 10^3$ (ratio = 18.3) (Fig. 10*B*).

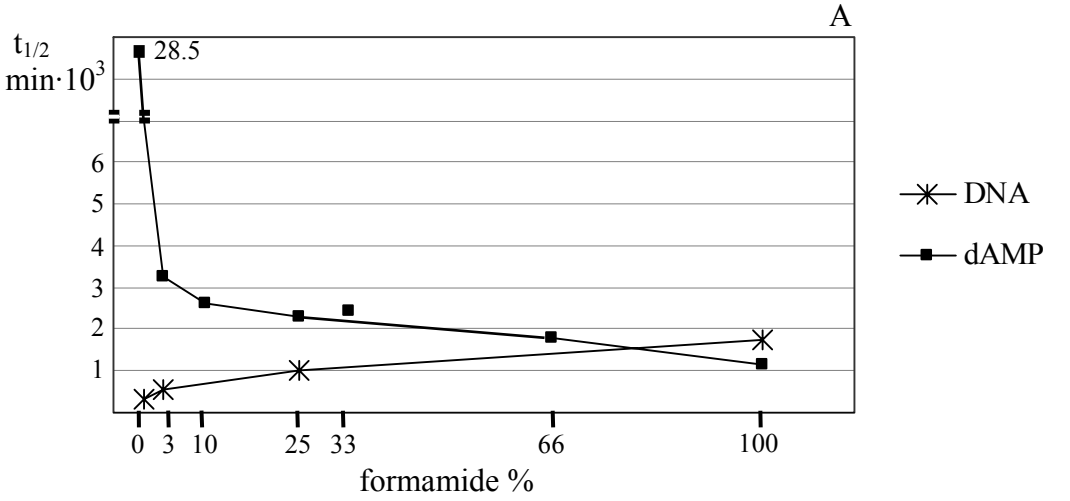


Fig. 10A: Summary comparison of the stability of the 5'-phosphoester bonds in the polymer and in the monomer (Saladino et al., 2005c).

In this physico-chemical frame, polymerization would obviously be forbidden. Formamide reverses these stability parameters, potentially allowing the survival of the polymeric form.

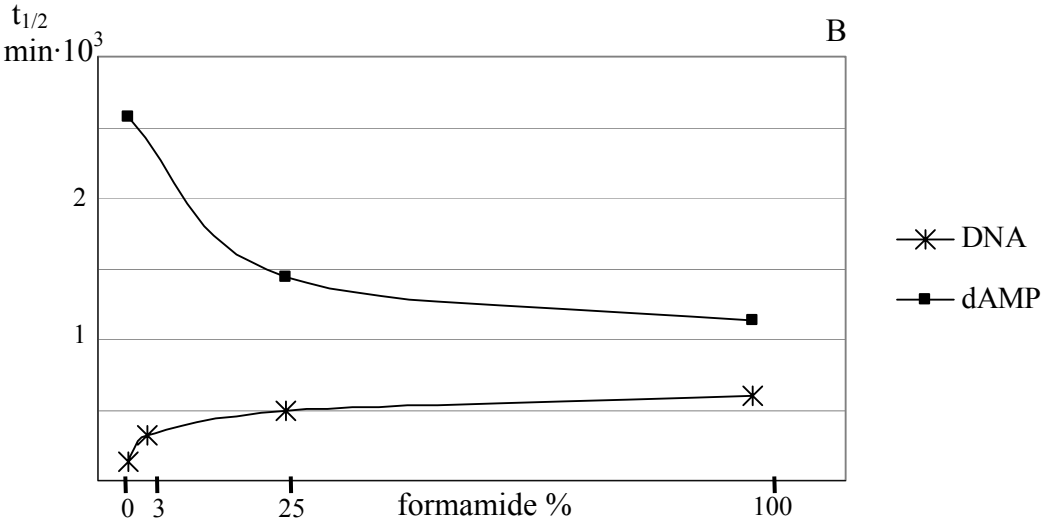


Fig. 10B: Summary comparison of the stability of the 3'-phosphoester bonds in the polymer and in the monomer. The $t_{1/2}$ values of the bonds when embedded in the oligonucleotide are taken from digestion experiments similar to those reported in Fig. 4 (for the 3'-phosphoester bond) and from similar experiments (not shown) for the 5'-phosphoester bond (Saladino et al., 2005c).

In conclusion, a limited but well defined ensemble of conditions was identified in which the 5'-phosphoester bond is more stable in DNA than in dAMP: >75% formamide, 90°C. The polymer, or even a short oligomer, would not survive for long in these conditions because of its repetitive nature. However, these

measurements and findings provide the basic assay for the evaluation of the conditions, of the catalysts, of the shielding surfaces, and of the physico-chemical environment that could sufficiently further enhance the stability of the polymers relative to that of the monomers enough to allow their survival, replication, and evolution.

THE TOP-DOWN APPROACH: DEGRADING BY FORMAMIDE IN THE PRESENCE OF CATALYSTS

We have experimentally identified formamide as a compound that at temperatures around 100°C selectively degrades the DNA bases in the following order of decreasing reactivity: G>A>C>T (Saladino et al., 1996; 1997). the most favorable set of conditions using this precursor for the synthesis of nucleic bases would be high formamide concentration, anhydrous conditions, the presence of a catalytic system, a local stable microenvironment, and a temperature higher than 100°C and lower than 180°C. Imbalance of the precursors is a problem as serious as their instability. The two properties are connected.

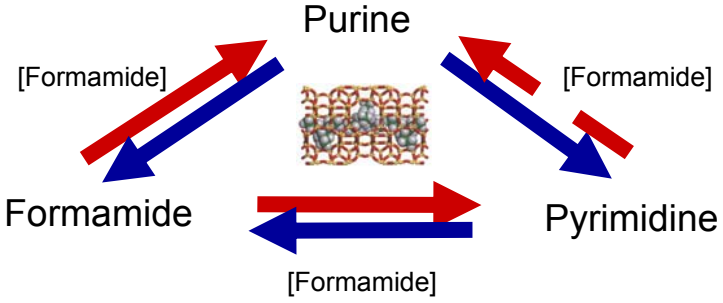


Fig.11: In the presence of formamide, additional formamide is recovered from the degradation of purine and pyrimidine bases (solid outer arrows). Synthetic reactions also occur (inner arrows) leading from formamide to purines and pyrimidines. Balance of the precursor could lead to polymerization(Saladino et al., 2004c).

This is a consequence of the fact that DNA in solution is chemically stabilized by its double-stranded structure and that a double strand requires for optimal pairing equal amounts of purines and pyrimidines. Pool equilibration is greatly favored by the existence of a cycle of the type described in Figure 11. Formamide condenses into a base; then, in the continued presence of formamide, the base is degraded to release the precursor back into the medium. This process prevents the precursor formamide from being locked into the base that happens to be produced more efficiently and that, for the very reason of its relative abundance, would remain unutilized in base pairings. Thus, cycling may provide a solution to the instability problem caused by the very property of formamide of being at the same time an operator of the construction and of the degradation of nucleic bases. We have reported that relative abundances change depending on the catalyst present; we have analyzed the effects on the degradation of nucleosides and of polynucleotides under the same experimental conditions that it serves as a catalyst for formamide condensation into purines, pyrimidines and acyclonucleosides (Saladino et al., 2003).

TiO_2

The differential effects of TiO_2 on the degradation of polynucleotides were studied for homogeneous or mixed-sequence polymers. Starting from the highly differential sensitivity of the DNA-bound bases (G>A>C>T, as in Negri et al., 1996) in its absence, the presence of a threshold concentration (3 mg/ml) of TiO_2 brought the sensitivity of the four bases to similar levels. Together with the observed effects on the degradation of nucleosides (Saladino et al., 2003), these results reveal a role of this specific catalyst in equilibrating the pool as shown in fig.12. Here are describes the modification of the sensitivity of the four bases to formamide: decreased degradation of DNA-embedded guanine (G) and adenine. (A) as a function of the increasing concentration of TiO_2 , and the opposite effect on thymine (T) and cytosine (C). The plot shows the relative sensitivity (ordinate) of the four bases as a function of the increasing concentration of TiO_2 (abscissa). The equilibration effect is evident.

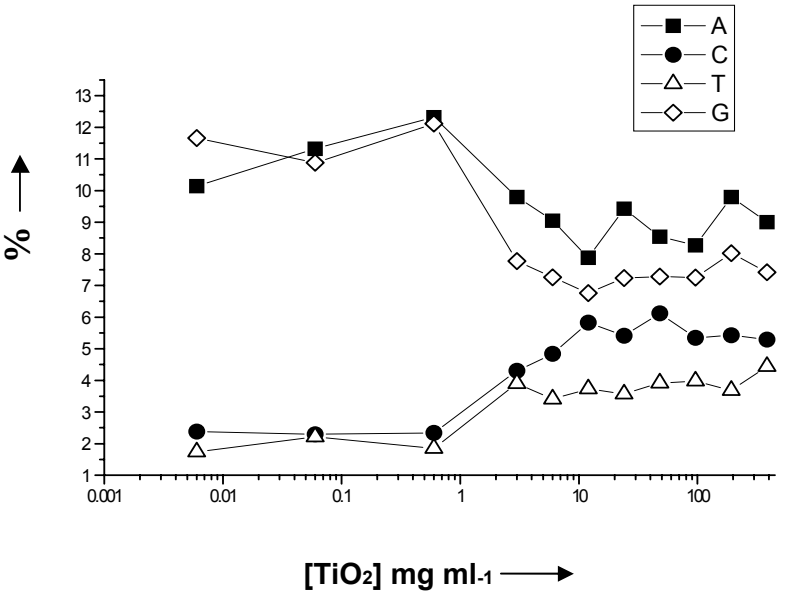


Fig. 12: The relative sensitivity (ordinate) of the four bases as a function of increasing concentration of TiO_2 (abscissa). The values reported in ordinate represent the average sensitivity (%) to cleavage of the each indicated base when embedded in DNA and cleaved by formamide in a condition of "less than one cleavage" per DNA molecule. Experimental details in Saladino et al., 2003.

Montmorillonites

Differentially affect the rate of degradation of nucleobases when embedded in 2'-deoxyoligonucleotides; namely, montmorillonites protect adenine and guanine from the degradative action of formamide, while thymine degradation is enhanced. The oligonucleotide backbone reactivity to formamide is also affected; this shows that the interaction with montmorillonites modifies the rate of abstraction of the Hα and Hβ protons on the sugar moieties (Fig. 13). We also observe that montmorillonites tune the reactivity of formamide with 2'-deoxyoligonucleotides (Saladino et al., 2004b). Two major models of interaction

between oligonucleotides with clays have been suggested. In the first, the oligonucleotides are partially adsorbed and bound to the mineral surface, a part of the molecule interacting on the edges of the clay. The second model claims that one end of the oligonucleotide is bound to the clay surface, while the other part remains unbound (Franchi et al., 2003). At the nucleobase level, purines interact with the clay surface more efficiently than pyrimidines. Thus, we can only hypothesize that the interaction of the oligonucleotide with montmorillonites modifies the sugar packaging modes. This would favour or disfavour a specific β-elimination process (i.e., 3'- versus 5'-cleavage) depending on the nature of the nucleobases proximal to the reactive site and on the type of interaction with metal ions on the clay surface.

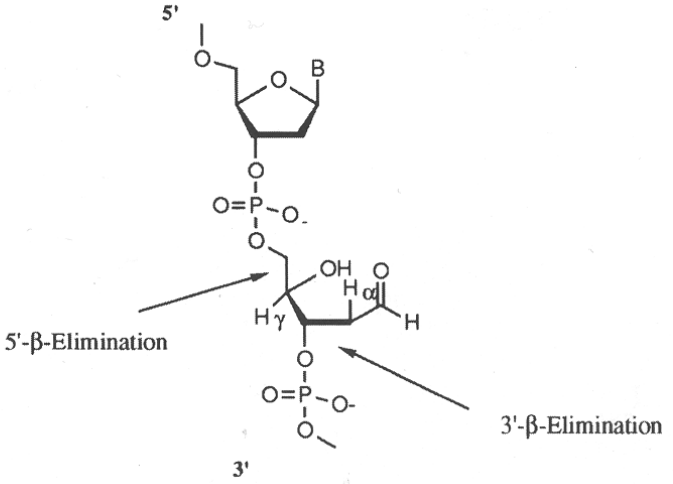


Fig. 13: The cleavage of the 3'- and 5'-phosphodiester bonds by formamide. Schematic representation of the degradation of the sugar moiety indicating the two different β-eliminations (Saladino et al., 2004b).

Cosmic Dust Analogues (CDAs)

Based on results from space missions, space and ground-based observations, and laboratory analyses of interplanetary dust particles (IDPs) (Molster, F.J) "fluffy" grains of amorphous olivines were synthesized in the laboratory (CDAs were produced by fast condensation of target vaporized materials by high-energy Nd:YAG laser ablation. Laser targets were oxide mixtures (SiO_2, MgO, FeO) (Saladino et al., 2005b).

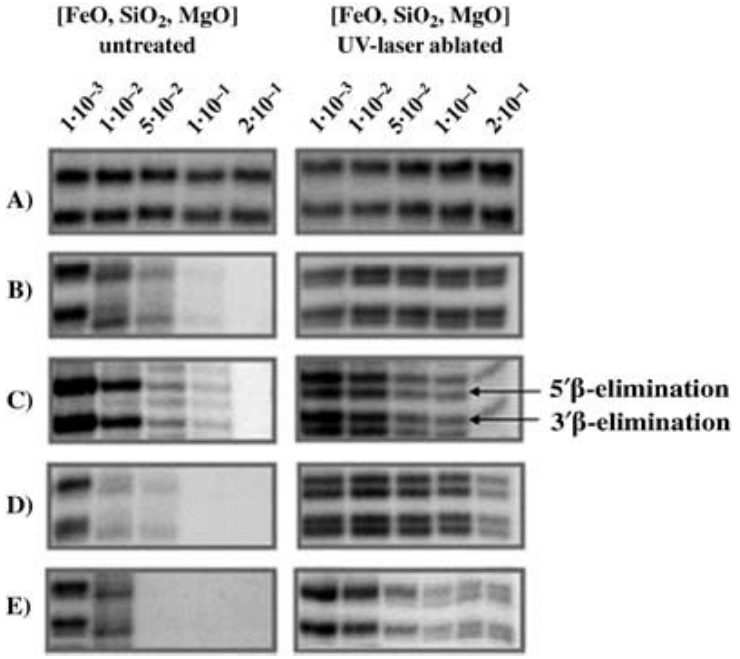


Fig.14: CDA olivines modify the degradative reactions of formamide on DNA. A) Fayalite Fe_2SiO_4; B)$Mg_{0.5}Fe_{1.5}SiO_4$; C) olivine $MgFeSiO_4$; D)$Mg_{1.5}Fe0_{0.5}SiO_4$; E) forsterite Mg_2SiO_4 ; The bands correspond to positions 15 (upper band) and 16 (lower band) of the poly-A region (Saladino et al., 2005b).

They were synthesized as CDAs in order to reproduce the chemical composition and morphology of silicate dusts expected in different astronomical environments. The olivines prepared as CDAs produce quite different effects: marked loss of base cleavage selectivity even at low concentration; strong alteration of the 3'/5' cleavage ratio with marked increase of 5' cleavage; weak protection effect. Figure 14 compares five different compounds (A–E) as a function of concentration. They were tested as untreated mixture of oxides or as CDA compounds. In summary, the effect of CDA olivines on DNA attack by formamide consists of the alteration of base selectivity (G>A>C»T), and modification of the rate of β-elimination of Hα/Hγprotons.

Phosphates

A strong, phosphoester bond-specific protection effect by phosphates was observed (Saladino et al., 2006c).

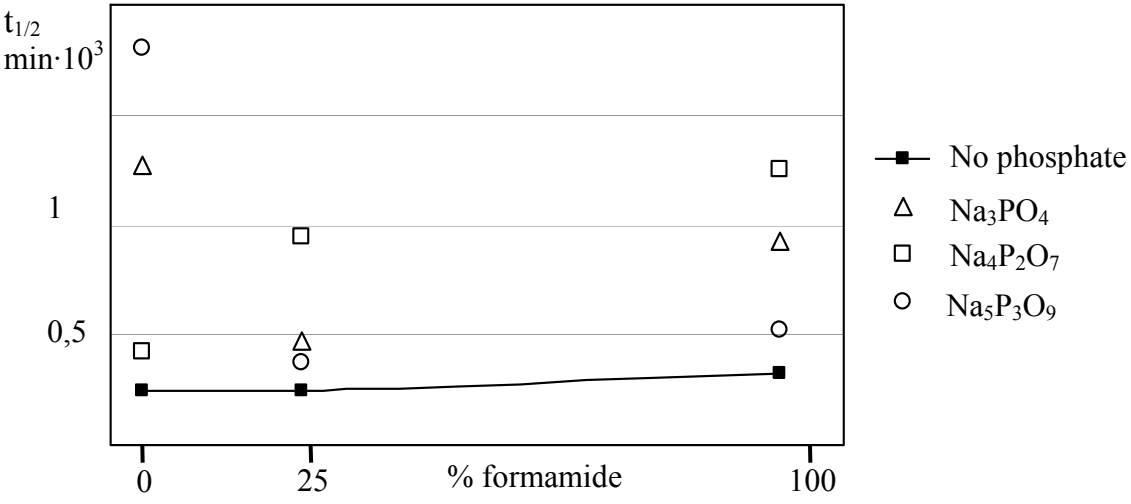


Fig. 15: Soluble phosphates protect the phosphodiester bonds of DNA. The degradation kineticsof the DNA bonds was analyzed on the 5'-labeled oligomer 5'-ACCTAACCGG [A]30 CCGGTT -3' (Saladino et al., 2006c).

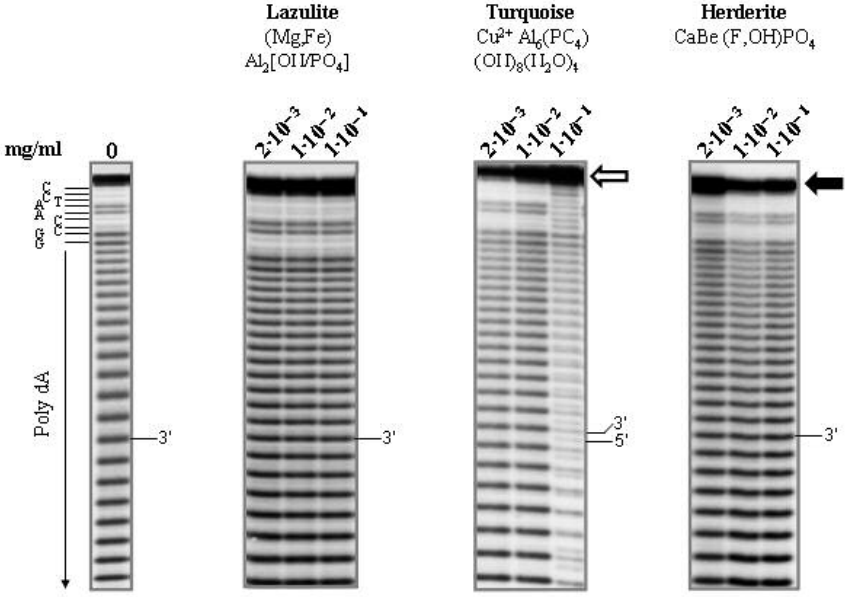
Soluble phosphates exerted a strong protection versus these DNA cleavage reactions and changed the 5' versus 3' cleavage specificities, as shown by the modification of the cleavage patterns (Fig. 15). The triphosphate protects in water thus revealing its interference with the HNS (Hydrolysis following Nucleobase Substitution that preferentially cleaves at 3') degradation mechanism. The triphosphate also interferes with the HND (Hydrolysis following Nucleobase Degradation that cleaves with comparable intensities at both 3' and 5') degradation mechanism, completely preventing the cleavage of the 5'-phosphoester bond, but not the cleavage of the 3' one. Both mono- and diphosphates (Na_3PO_4 and $Na_4P_2O_7$) behaved similarly (Ciciriello et al., 2007). The average half-life of the 5'- phosphoester bonds in the DNA oligomer was determined as described in Saladino et al., 2005c.

Phosphate minerals

In water when deoxyoligonucleotides are treated in the presence of the indicated amounts of each mineral, this effect-on-stability assay produced three types of results: 1) no (or very poor) effect. 2) Protection. 3) Stimulation of the cleavage. We observed: *no effect* for Laueite, Purpurite, Eosforite; *protection* by Lazulite, Herderite, Pyromorphite, Hydroxylapatite, Fluorapatite, Sholzite, Childrenite, Augelite, Calcioferrite, Wavellite, Vauxite, Ludlamite; *stimulation* by Variscite, Strengite, Wardite, Hureaulite, Tarbuttite, Turquoise, Cacoxenite, Vivianite, Libethenite, Anapaite.

The effect of phosphate minerals was tested as above on the 46-mer oligo in formamide yielding the “single band” ladder-like degradation profile (Fig. 16, leftmost lane). This condition allows to test the possible induction of double cleavages by the crystal minerals. Figure 6 shows that three different effects are induced on the destabilization of the DNA chain by formamide. The minerals are listed in order of increasingly protective (empty arrow) or degradative (solid arrow) activity. At the contrary of the effect observed in water, in formamide the majority of minerals exerted a protective effect. The degradation of DNA by formamide is sequence-selective (Saladino et al. 1996; 1997; Negri et al., 1996), the order of attack being G≥A>C>>T (see also the control lane in Fig. 16).

Several phosphate minerals among those exerting no overall effect (namely: Hureaulite) or a protective action (namely: Laueite, Childrenite, Vivianite) caused a loss of the sequence selectivity towards formamide attack. Figure 17 shows four examples of this phenomenon, as indicated by the triangles on the upper right side of the four panels. Cleavage at purines is not indicated for graphical simplicity. Remarkably, two minerals (Childrenite and Vivianite) stimulate the degradation in correspondence of the otherwise most resistar (Negri et al., 1996) thymine residue (Ciciriello et al., 2007).



No effects: Fluoroapatite, Hureaulite, Hydroxylapatite, Lazulite, Variscite, Wardite, Wavellite

Protection: Eosphorite, Purpurite, Pyromorphite, Tarbuttite, Calcioferrite, Anapaite, Scholzite, Childrenite, Augelite, Vauxite, Libethenite, Cacoxenite, Strengite, Turquoise, Laueite, Ludlamite, Vivianite

Stimulation: Herderite

Fig. 16: *The effects of phosphate minerals on the instability of DNA molecules in*

formamide. Reactions were performed in 100% formamide at 110°C for 30 min in the absence (first lane) or in the presence of the indicated amounts of the indicated mineral. The arrows point to the protection (empty arrow) or to the stimulation (solid arrow) effects.

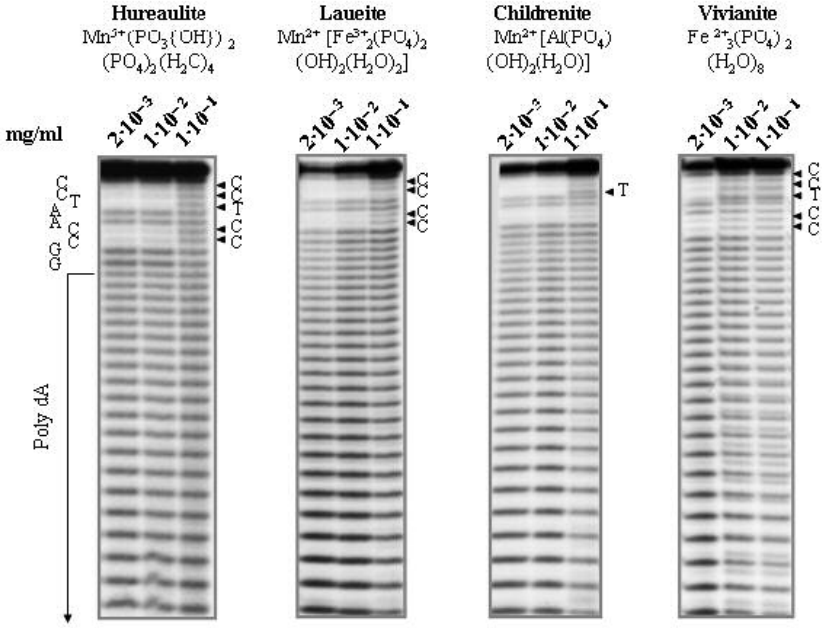


Fig. 17: Phosphate minerals induce loss of sequence selectivity in the attack by formamide. Conditions as in Figure 16. The appearance of the bands corresponding to pyrimidine residues indicates the loss of sequence-specificity caused by the indicated mineral. Similar assays were performed with the other 22 minerals (not shown) (Ciciriello et al., 2007).

The thermic and chemical extension of the identified survival niches is narrow and the protection effect is limited (ibidem). Complex polymers would not remain such for long. Hence the relevance of helper molecules or of conditions in which nucleic acids polymers could be further stabilized, thus avoiding or delaying the otherwise futile cycle of polymerizations/depolymerizations. RNA as DNA is important in the development of prebiotic chemistry. However, given their marked differences, when considering the properties that might have influenced their origin, both DNA and RNA should be analyzed, and the results compared. Based on these considerations, we have undertaken a general analysis of the stability of the phosphoester bonds also in ribo-oligomers and monomers (Saladino et al., 2006a).

RNA AND RIBOMONOMER

In the origin of the informational polymers, their survival as macromolecules is even more problematic than the polymerization process itself. Do physico-chemical conditions exist, providing kinetic and/or thermodynamic advantage to the otherwise intrinsically more unstable polymeric form over the monomer? We focus here on RNA and describe a systematic comparison of the stability of the phosphoester bonds in the precursor monomers *versus* the stability of the same bonds when present in RNA, under a wide range of temperatures and water/formamide ratios. The stability of the β-glycosidic bond in the same set of conditions was also determined. For oligonucleotides, the half-lives of the phosphoester bonds were determined from the rate of disappearance of the band representing the intact 20-mer molecule. Cleavage of RNA normally requires participation of the 2'-OH group as an internal nucleophile (Morrow et al., 1995) by two “nucleophilic cleavage” events: the trans-esterification and hydrolysis reactions (Fig. 18). During the trans-esterification (pathway a), the 2'-OH nucleophile attacks on tetrahedral phosphorus (V) affording a 2',3'-cyclic monophosphate I, which in turn can be hydrolyzed to a mixture of 3'- and 2'-phosphate monoester II and III, respectively (pathway b). Both steps can be catalyzed by a large range of chemical species, including protons, hydroxide,

nitrogen derivatives, and metal ions. On the basis of this known mechanism two operative assumptions were made.

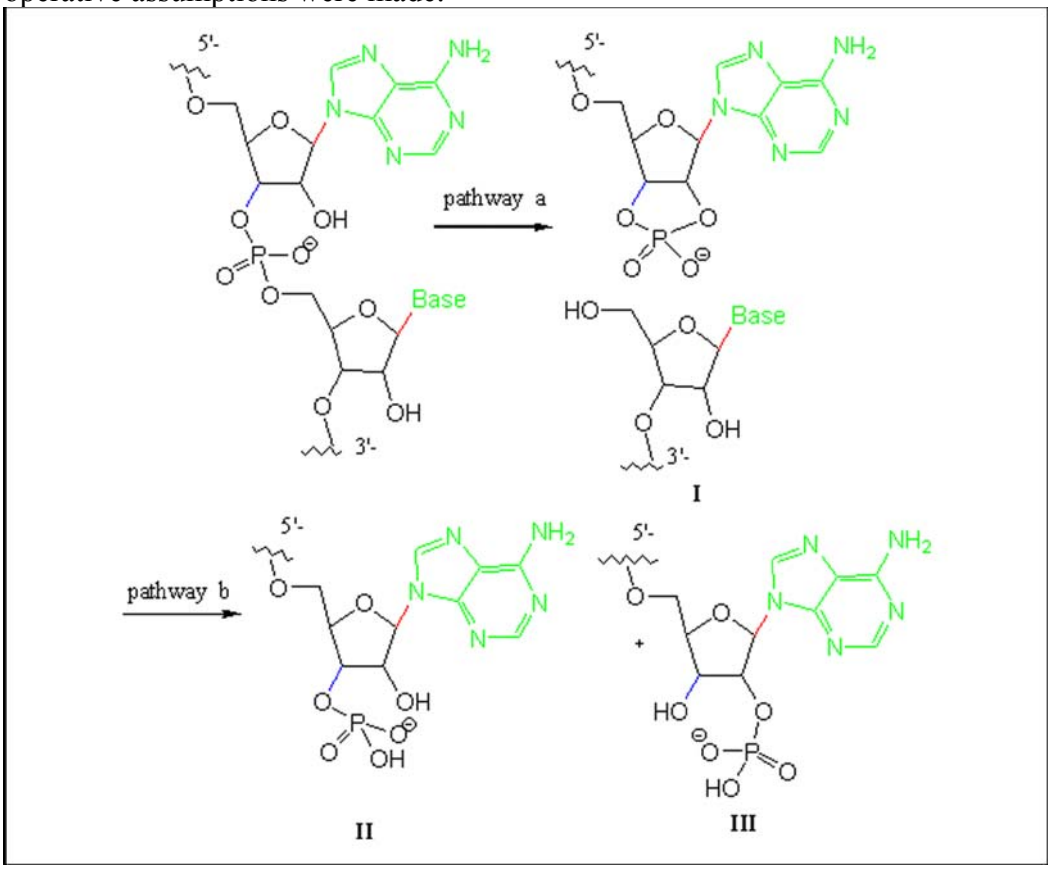


Fig. 18. Mechanism of RNA degradation (Saladino et al., 2006a).

1) It was assumed that the cleavage of the 3'-phosphoester bond is largely moreeffective than the cleavage of the 5'-one and that for practical calculation

purposes the 5'-phosphoester bond is not cleaved. In support, the cleavage of the 5' terminally labeled RNA molecule only shows the standard cleavage of the 3'-bond (see Fig. 19), whereas the double bands typically produced by 3'- and 5'-cleavages occurring in the same population of molecules were never detected.

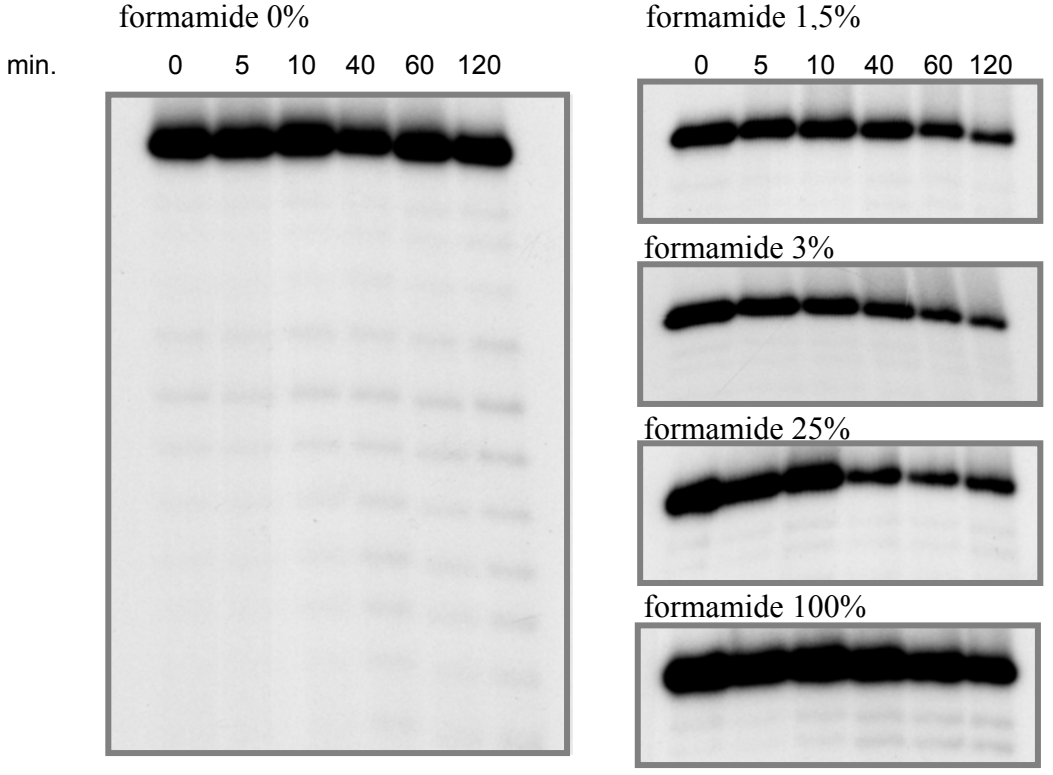


Fig. 19. Degradation kinetics of the 5'-labeled P1 RNA as a function of the formamide concentration reported on top of each panel. Samples were treated for the indicated time (min) at 70°C. A fully run panel is shown only for the pure water. Only the full-length front is shown for the others (Saladino et al., 2006a).

This contrasts with DNA (Saladino et al., 2005c), where under several conditions both cleavages do occur, giving rise to the diagnostic double bands.
2) In the ribooligonucleotide studied here, the breakage of the phosphodiester chain is not sequence biased under the conditions used. This sequence may have, like many other RNAs, preferential breakage sites (La Neve et al., 2003; Gioia et al., 2005) that require special defined conditions to be cleaved. Outside the special cleavage conditions this RNA is stable (La Neve et al., 2003). These conditions are well characterized (La Neve et al., 2003; Gioia et al., 2005) and are different from the ones here. The half-life of the oligonucleotide was determined with standard graphical procedure from plots of the % disappearance of the intact 20-mer molecules. Given that one disappearing molecule represents one cleavage, and given the two assumptions reported (no 5'-cleavage, no sequence bias), the half-life of a 3'-phosphoester bond in the ribooligonucleotide is given by the half-life of the oligonucleotidi ×19 (that is: the number of 3'-phosphoester bonds in the 20-mer). HPLC analyses are performed to establish the effect of formamide on the stability of the β-glycosidic bond in ribomonomers. Measurements were performed as a function of formamide concentration in water and of temperature. In addition to its interest *per se*, determination of the stability of this bond is a prerequisite for the analysis of the stability of phosphoester bonds in nucleotides and in oligonucleotides. In the adenosine's HPLC experimental layout, no peaks other than those of adenosine and adenine are observed. Thus, no other cleavage than that of the β-glycosidic bond occurs under this range of conditions. The half-life of this bond at 90°C in water is $> 5 \times 10^5$ min. Increasing concentrations of formamide first decrease and then (> 25%) increase the stability of this bond. Fig. 20 shows this double effect exerted by increasing formamide on the stability of the β-glycosidic bond. Fig. 20 also shows the $t_{1/2}$ of the same bond in both 5'- and 3'-adenosine monophosphates. Interestingly, in the presence of a phosphate group in both 3'- and (less so) in the 5'- position, the half-life of the β-glycosidic bond is strongly decreased in water and it is progressively protected by increasing formamide concentrations. The $t_{1/2}$ of the β-glycosidic bond in H_2O is 0.14 ± 10^4 min for the

3'-AMP and 0.21 ± 10^4 min for the 5'-AMP (see legend to Fig. 20). At formamide > 33% the high stability (> 5 ± 10^5 min) of this bond is re-attained. These effects are markedly sensitive to temperature (Fig. 20, middle and lower panels).

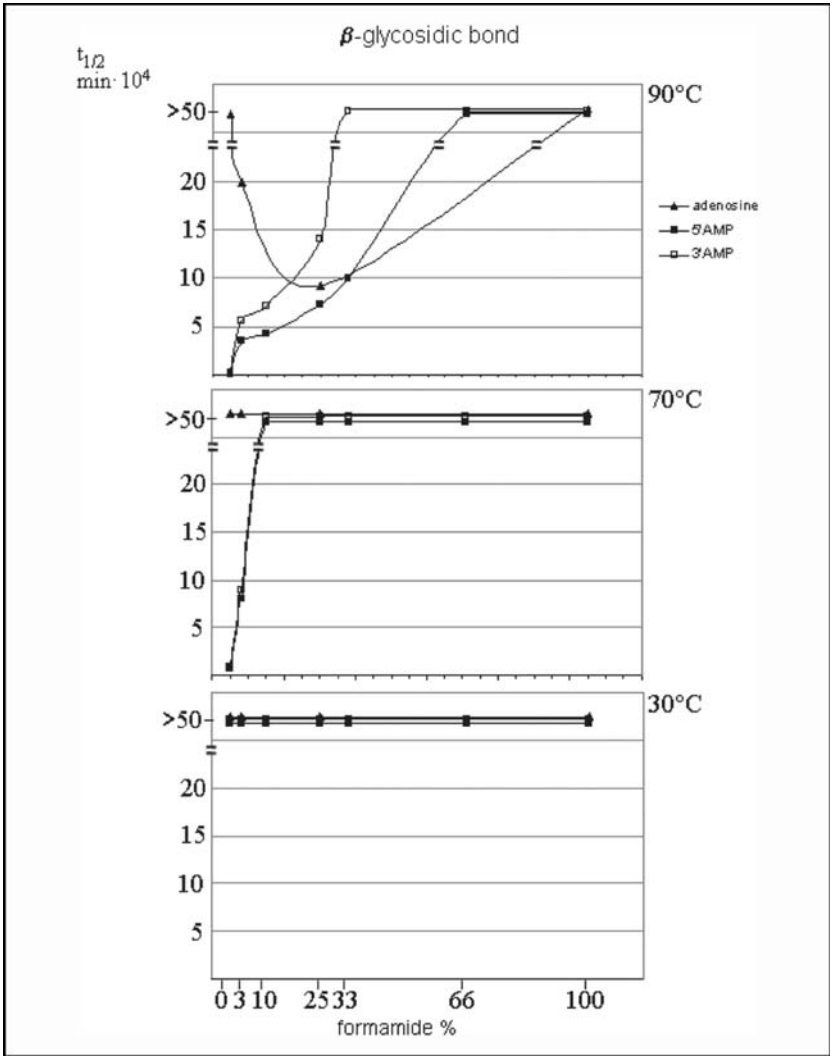


Fig. 20: The stability of the β-glycosidic bond in adenosine, in 3'-AMP, and in 5'-AMP as a function of formamide concentration. The half-lives (min $\times 10^4$*) are calculated from the HPLC of adenosine, 3'-AMP, and 5'-AMP. The three panels show results obtained at the indicated temperatures of 90°C, 70°C, and 30 °C. The numerical values of the lowest data points (which are not graphically resolved) are 0.21*$\times 10^4$ *(5'-AMP at 90°C), 0.14*$\times 10^4$ *(3'-AMP at 90°C), 0.60*$\times 10^4$ *(5'-AMP at 70°C), and 0.75* $\times 10^4$ *(3'-AMP at 70°C) (Saladino et al., 2006a).*

The degradation rates of the 3'-AMP were analyzed as a function of both temperature and formamide concentrations (Fig. 21, the analysis shown is at 90°C). The disappearance of the 3'-AMP (blue) in water (upper left panel) is matched by the appearance of the nucleoside (red), thus being primarily caused by the cleavage of the 3'-phosphoester bond. In water the β-glycosidic bond is cleaved with a slightly slower kinetics, and the corresponding product (adenine, green) appears with a slower kinetics. Eventually, all the nucleoside is cleaved into sugar (not detected) and adenine. The presence of low concentrations of formamide (starting at 3%) markedly protects the β-glycosidic bond (green line), allowing almost exclusively the cleavage of the phosphoester (red line). The same analysis was performed on 5'-AMP. The overall trend is similar to that of 3'-AMP, with the notable exception of the behavior in water. Under these conditions the β-glycosidic bond is cleaved faster than the phosphoester bond. Also, for the 5'-AMP the protective effect exerted by formamide is strong and starts at low concentration. The half-lives of both the 3'- and 5'-phosphoester bonds as a function of increasing formamide and at different temperatures are plotted in Fig. 22. Summarizing the numerous effects exerted by water/formamide and temperature on the stability of the β-glycosidic and the phosphate bonds in the nucleoside and in its 3'- and 5'-phosphate forms, we observe for the β-glycosidic bond: (i) high stability in water ($> 5 \times 10^5$ min) for the nucleoside, (ii) enhanced cleavage by low formamide/water ratios (< 33%) *versus* protection by higher ones (≥ 33%) (Fig. 20), and (iii) enhanced instability

exerted by the presence of a phosphate group, especially so in water (Fig. 20). This latter effect is contrary to what is observed in 5'-deoxyadenosine, where the

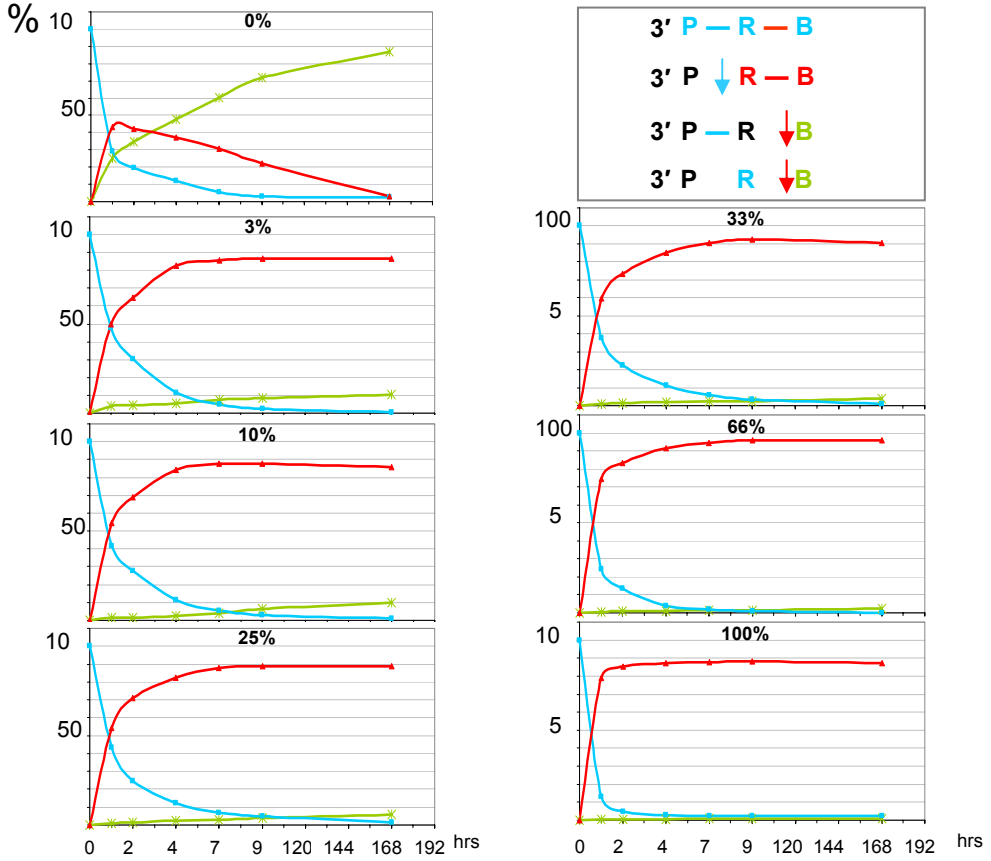


Fig. 21: HPLC analysis of the degradation kinetics of 3'-monophosphate adenosine. The complete 3'-AMP molecule is indicated in blue (Saladino et al., 2006a).

phosphate group exerts a strong protective effect on the β-glycosidic in water (Saladino et al., 2005c). For the phosphoester bonds we observed (Fig. 22) an overall instability (higher for the 3'- than for the 5'-phosphoester bond) and a substantial lack of effects by formamide, contrasting with the marked protection exerted by formamide on theβ-glycosidic bond (Saladino et al., 2006a).

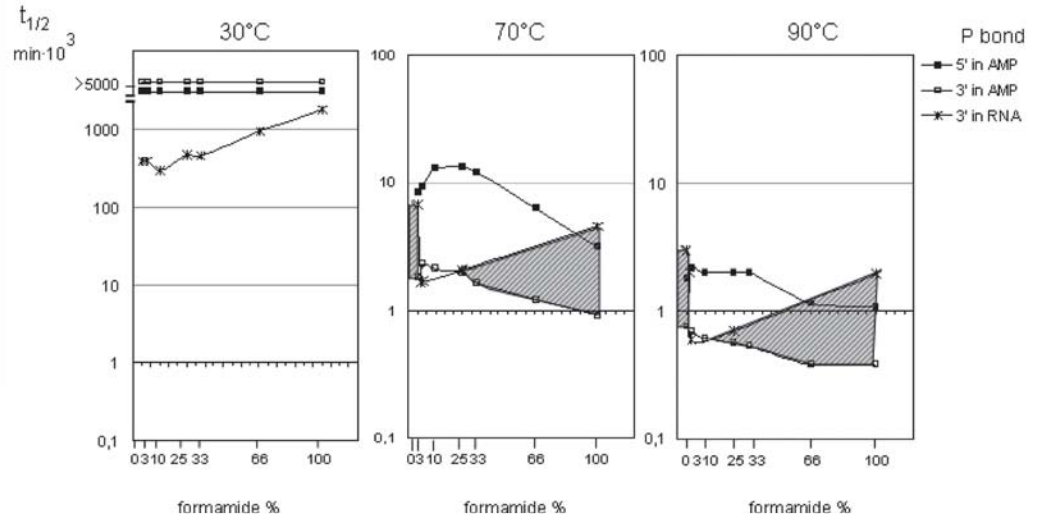


Fig. 22: Summary comparison of the stability of the phosphoester bonds in the polymer and in the monomers. The half-life (min×10^3) of the 3'-phosphoester bond in RNA is compared with that of the same bond in 3'-AMP and that of the phosphoester bond in 5'-AMP as a function of the formamide concentrations and of the temperatures reported. RNA data are from experiments similar to those reported in Fig. 19. For the meaning of the gray-shaded area, see Text.

The 5'-labeled 20-mer ribooligonucleotide described under "Methods" was treated at various temperatures for the indicated times (Fig. 19) in water or in the

presence of 1.5, 3, 25, or 100% formamide. Fig. 19 shows one example of the degradation progression observed. Fig. 19 shows that, as a general trend, increasing formamide concentration first increases and then protects RNA from the hydrolytic process. Fig. 22 shows the quantitative evaluation of this type of degradation experiment, performed at 30, 70, and 90°C (left, middle, and right panels, as indicated) for the appropriate range of times: lower temperatures required longer reaction times. Each panel shows the half-lives of the 3'-phosphoester bond determined from digestions similar to those reported in Fig. 19. The half-lives of the 5'- and 3'-phosphoester bonds, determined from the HPLC measurements described in Fig. 21, are also reported. The results in Fig. 22 show that in water and at high formamide concentrations at 70° and 90°C the average 3'-phosphoester bond in the oligonucleotide is more stable than the corresponding one in the nucleotide; the 5'-phosphoester in the oligo is so stable that its cleavage could not be measured. The 5'-phosphoester bond in the nucleotide is also always more stable than the 3'-one. The gray shade indicates the area of polymer-higher-than-monomer 3'-phosphoester bond stability (Saladino et al., 2006a). This scenario, however hypothetical, is chemically plausible and justifies the stability analyses reported here. The "*warm little pond*" imagined by Darwin (Darwin, 1888) as the original cradle would in this chemical system contain also formamide. Out of an origin-of-informational-polymers frame of interest the varying stabilities of the 3'- and the 5'-phosphoester bonds as a function of the molecular context are relevant *per se*.

COMPARISON OF BOND STABILITIES IN RIBOOLIGONUCLEOTIDES AND 2'-DEOXYRIBOOLIGONUCLEOTIDES

We compared (Fig. 23) the half-lives of the phosphodiester bonds: the 5'- and 3'-bonds in 2'-deoxyribooligonucleotides and the 3'-bond in riboligonucleotides. The ribo data are the same as those plotted in Fig. 22 and are here reported for the sake of comparison. The 2'-deoxyribo data are from Saladino et al., 2005c (for the experiment at 90°C) and from similar experiments performed with exactly the same materials, logic, and procedures performed at 70 and 30°C.The phosphoester bonds are in general more stable in RNA than in DNA. This is

especially true in water and at the higher temperatures studied under the same conditions in which the polymer is favored over the monomer. The gray shade indicates the area of the RNA-higher than- DNA 3'-phosphoester bond stability (Saladino et al., 2006a).

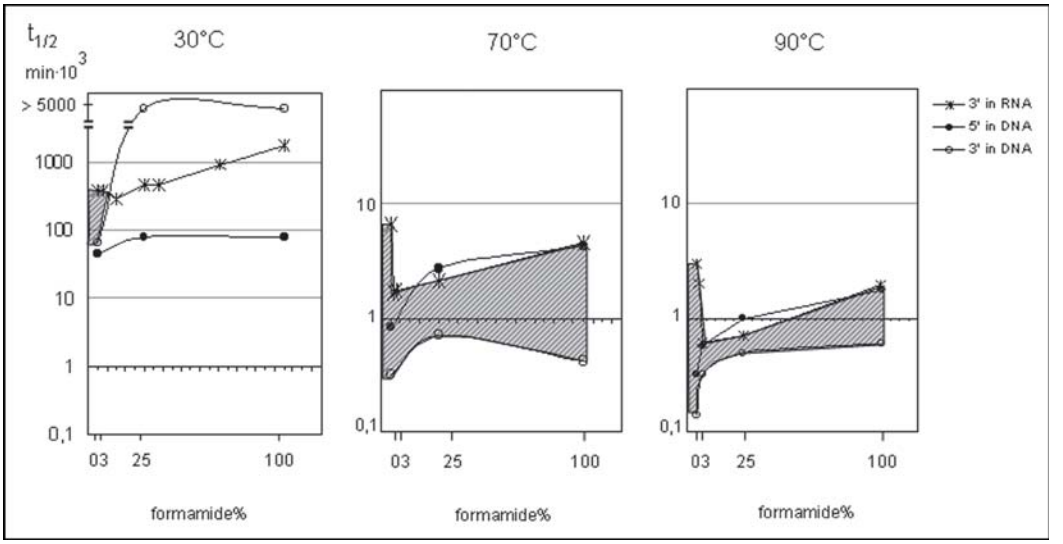


Fig. 23: Summary comparison of the stability of the phosphoester bonds in RNA and DNA. The half-life (min × 10^3) of the 3'-phosphoester bond in RNA (same data as in Fig. 22) is compared with the half-lives of the 3'- and 5'-phosphoester bonds in DNA, obtained at the temperatures and the formamide concentrations indicated. TheDNA data at 90 °C are from Saladino et al., 2005c. The data at 30 and 70°C are from experiments performed at the indicated temperature exactly as for the ones at 90°C. For the meaning of the grayshaded area, see text.

The “RNA world” (Gesteland et al., 1999) (Doudna and Cech, 2002) hypothesis is based on three important properties of RNA: its capacity to encode, express, and transcribe genetic information; the variety of tertiary structures, and hence of functions, it can assume; and its catalytic abilities. But DNA has cognate properties. A fourth property of the polymeric systems that might provide information on the origin of informational polymers and on the who came first problem is the ensemble of thermodynamic and kinetics considerations pertaining to polymerization process and polymers stability. Let us dub this property with a collective name: *persistence*. This topic is critically examined and brought to propositive conclusions in Halvorson and van Holde, 1980.

Starting from the consideration that the condensation reactions are not thermodynamically spontaneous in dilute aqueous solution or even at moderate water activities, two possible solutions have been proposed for their occurrence (Halvorson and van Holde, 1980). The first is the mechanism used by contemporary organisms, the activation of monomers by phosphorylation. The second is the evasion from the unfavorable Gibbs free-energy change ($\Delta G°$) by reducing the water activity. As for the first, the fact that phosphorylated monomers played a resolutory role in prebiotic polymerizations is highly unlikely on kinetic grounds. The second implies, in order to allow the formation of oligonucleotides (or for that matter of any other polymer) of appreciable chain length and in appreciable concentrations, both nearly pure monomers and highly dehydrating conditions. The idea of a primeval soup is therefore not thermodynamically sound. If syntheses of precursor monomers had occurred in an aqueous solution, the considerations reported above imply the necessity of a process involving dehydration and subsequent concentration. These thermodynamic considerations help in delineating the physico-chemical initial scenario. Small bodies of water (*i.e.* aqueous droplets trapped in crevices in rocks) subject to periodic evaporation and re-hydration were suggested (Halvorson and van Holde, 1980) as initial reaction milieu. This dynamic environment is compatible with the scenario into which our data may be adjusted with the specification that formamide must have also been present. This study on

the differential stabilities of the relevant bonds (monomers versus polymers) and (ribo *versus* deoxy) identify two thermodynamically permissive niches, which differ for DNA and RNA (Saladino et al., 2006a):

- >90°C and >80% H_2NCHO/H_2O for DNA
- >70–90°C, H_2O and/or various H_2NCHO/H_2O ratios for RNA.

Which of the niches provided the conditions that were actually used for kick-starting the replication-evolution process is not ascertained at present. However, this approach may bring the fourth property of the RNA world mentioned above (persistence) into an experimentally verifiable frame.

At the molecular level, in a prebiotic acellular context the founding phenotype of the developing system of informational molecules is stability. Along with the ability to reproduce, selection necessarily favored molecules able to keep for a longer time their macromolecular information.

MOLECULAR PHENOTYPES FAVOURING THE EVOLUTION OF COMPLEX RIBO POLYMERS

We have explored the stability of selected ribo oligomers in water and have determined the physical-chemical conditions in which the key 3' phosphoester bond is more stable when embedded in the polymer than when present in the monomer. A narrow pH range was identified in which complex sequences resist degradation markedly more than monotonous ones, thus potentially favouring the evolution of sequence-based genetic information. Based on this assumption we have analyzed the stability of ribo oligomers and compared it with that of the constituent monomers.

THE 3' PHOSPHOESTER BOND IN 3' AMP AND 3' CMP

The cleavage rate of the 3' phosphoester bond of 3' AMP and 3' CMP was determined by incubation in water at 90°C at the Tris HCl-buffered pH values of 3.37, 3.72, 4.31, 4.98, 5.26, 5.34, 5.60, 5.92, 6.48, 7.21, 7.69, 8.25, 8.57, 8.73,

9.02. The treatment lasted for 92 hrs and the products were analyzed by HPCL analysis at the time intervals.

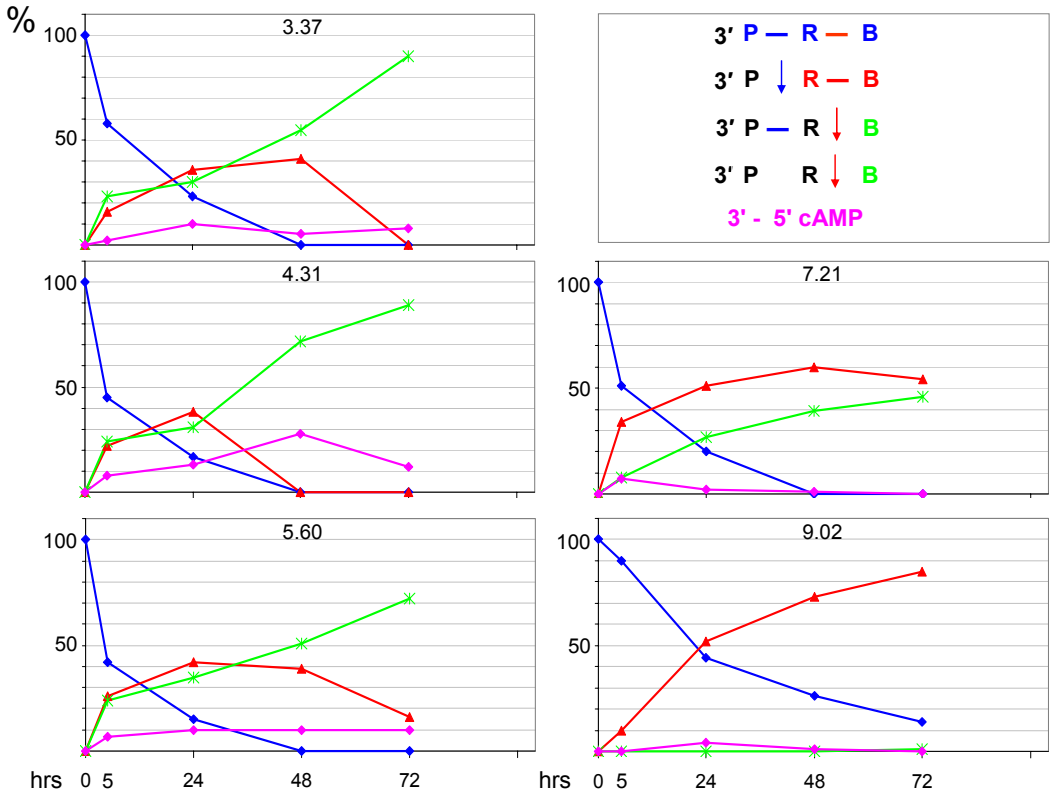


Fig. 24: Degradation kinetics of 3' AMP as a function of the pH indicated in each panel, HPLC analysis. Blue, nucleotide; red, nucleoside; green, base. The ribose and the ribose-phosphate moieties are not detected in the HPLC analysis and are indicated in black. See also Text. The line connecting the experimental points, here and in the following plots, has no mathematical meaning. It is given only to facilitate identification (Ciciriello et al., in press 2007).

This figure 24 (which shows selected examples of the large data) illustrates the degradation of 3' AMP (%, ordinate) as a function of time (abscissa) and of the pH (indicated in the central upper part of each panel). The upper panel on the right side provides the interpretation key. The results show that 3' AMP in water at 90°C has a t ½ ~ 0.3 x 10^3 min . This value is only marginally pH-dependent between pH < 3.37 and > 8.73, as shown in Fig. 24. The degradation of 3'AMP occurs by cleavage of the ß-glycosidic and/or the 3' phosphoester bonds. The first cleavage results in the production of adenine (green), the latter in the production of adenosine (red). Stability of adenosine was measured separately in a similar set of analyses (not detailed) showing that in the whole range of pH values analyzed adenosine was only marginally degraded. Thus, in the degradation of 3'AMP in water at 90°C adenine is mostly produced by depurination of the nucleotide form, not from the adenosine moiety deriving from its dephosphorylation. Following depurination, the phosphoester bond connecting the ribose and phosphate moieties (which in this analytical set up are not visible) is cleaved by a well characterized ß-elimination mechanism (Lindhal, 1993). Fig. 24 shows that adenosine depurination is faster between pH > 3.72 and < 4.98.
At pH values higher than 7.21 (Fig. 24) little adenine is formed, showing that in alkaline conditions the ß-glycosidic is protected and that degradation of the nucleotide mostly occurs by high pH-enhanced dephosphorylation. The increase of adenine after 48 hrs is given by the sum of the adenine formed previously plus the adenine being produced from adenosine till its consumption. Formation of a discrete amount of *bona-fide* 3'-5' cyclic AMP is also observed (magenta). The corresponding peak in the HPLC profile was assigned by comparison with real sample 3'-5' cAMP, while 2' AMP, 3' AMP and 2'-3' cAMP eluted in different positions. This matter is not directly relevant to the scope of this study and was not analyzed further.In conclusion, the degradation of 3' AMP is largely a pH-independent process between pH ~3.0 and ~7.0. In this range of values the contribution of depurination is higher between ~3.7 and ~5.0. The half-life of 3' AMP was also determined as a function of temperature at pH 5.5 in water, showing its steady temperature-dependent increase.

The cleavage rates of the 3'-phosphoester bond of 3' CMP were determined by a similar set of analyses for the same range of pH values. The results showed that the degradation of 3' CMP is an essentially pH-independent process and that the cleavage of the ß-glycosidic bond resulting in the production of cytosine is faster than the cleavage of the 3'-phosphoester bond yielding cytidine. In spite of this kinetic difference, the quantitative relevance of the two clevages is roughly equivalent. Both cytosine and cytidine are stable in these experimental conditions.

KINETICS OF RNA HYDROLYSIS

We performed the simple test of measuring the kinetics of degradation of a 5' labelled RNA in water at various temperatures on the three following oligos: $PolyA_{24}$, $PolyA_{12}C_{12}$, mixed-sequence oligo P1, respectively, treated at 90°C as a function of time.

Poly A_{24}

The pattern of hydrolytic degradation of the $PolyA_{24}$ at 90°C (Fig 25) shows that the 24-mer was regularly hit by hydrolytic events along its whole length, as shown by the appearance of the ladder-like degradation profiles. After about 6 hrs of permanence of the RNA in water, degradation started. The relatively high stability of the Poly A sequence in aqueous solution is well established (Smith and Allen, 1953; Lane and Butler, 1959; Kaukinen et al., 2002). The stability of RNA phosphodiester bonds has repeatedly been associated with the stacking interactions between adjacent bases (Li and Breaker, 1999: Bibillo et al., 1999; Kierzek, 1992). Nucleic acid base stacking is at present an essentially well understood phenomenon (Norberg and Nilsson, 1995a e b; 1996 a e b; 1998) (Luo et al., 1998) (Newcomb and Gellman, 1994) (Friedman and Honig, 1995) (Pohorille et al., 1984). The possibility that the initial stability of the PolyA oligo towards hydrolysis is a base stacking-related effect is discussed below. The upper panel shows the degradation observed in water at pH 5.5 (90°C). The bottom

panel shows the disappearance of full-length molecules of the same oligo at pH 6.2 (90°C).

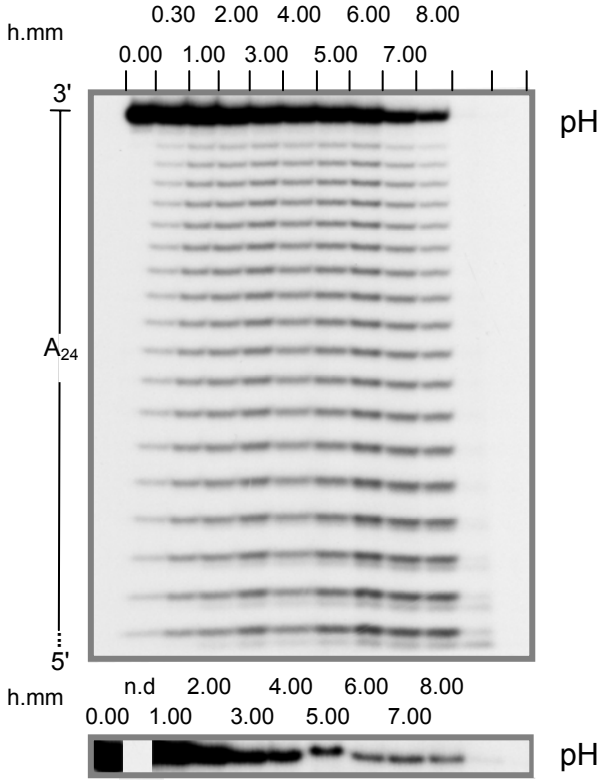


Fig. 25: Kinetics of degradation of Poly A_{24} in water at pH 5.5 and 6.2, 90°C. The upper panel shows both the full-length molecules and the degradation ladder of the sample treated in water at pH 5.5.After a lag period lasting for the first 6 hours, rapid degradation started (see points at 7 and 8 hours). Samples at 9 and 10 hours were more than 90% degraded. The lower panel shows only the full-length molecules of the sample treated in water at pH 6.2 (Ciciriello et al., in press 2007).

Poly $A_{12}C_{12}$

The cleavage profile of Poly$A_{12}C_{12}$ (Fig. 26) showed that the bipartite oligo molecule behaved towards degradation as two separate entities: the C-stretch and the A-stretch. The two sequence components did not appreciably affect each other.

In these experimental conditions (water pH 5.5, 90°C), the C-stretch component was rather unstable, its depolymerization already starting during the handling of the sample (i.e., see the T_0 sample, first lane). The cleavage pattern depicts the progressively decreasing size of the homogeneous sequence stretch and is compatible both with a multiple independent-hits kinetics and/or with a cooperative cleavage mechanism. Conformation and thermodynamic properties of oligocytidylic acids are known (Brahms et al., 1967). These oligomers possess a single-stranded stacked-base helical conformation at low temperatures and at neutral or alkaline pH (Brahms et al., 1967). The standard state free-energy change obtained at 0°C is only about 1 Kcal/mole in favour of stacking, thus allowing reversible formation of an ordered helical chain which may easily be disordered at higher temperatures or at constant temperature under influence of external factors.

Two functionally distinct sub-populations of molecules were observed (Fig. 26): one that was very sensitive to hydrolysis and was repeatedly cleaved to its consumption (indicated by the empty arrow in Fig. 26), the other that followed a slower kinetics (filled arrow). This behaviour is compatible with the following mechanism: all the molecules have similar conformation and accessibility but, once cleaved by a first-hit event, the hit molecule is rapidly cleaved to completion. This behaviour could be explained by faster unstacking in the early part of the kinetics in shorter segments and/or faster processive hydrolysis from the induced extremities. In spite of early reports (Brahms et al., 1967; Brahms et al., 1966; Van Holde et al., 1965; Kaukinen et al., 2003) describing that the loss of the helical structure with increase in temperature is not dependent on chain length, thus essentially being a non-cooperative process, evidence for cooperative stacking/unstacking effects was later reported (Kaukinen et al.,

2003). An alternative less likely interpretation of the cleavage pattern is that the CpC steps become more cleavable as they get closer to the A-stretch due to context effects. Ascertaining the mechanism(s) responsible for the higher sensitivity of the CpC steps closer to the A- stretch will require further analyses.
Which ever the mechanism leading to the rapid degradation of the polyC stretch after initial cleavages, the half-life of the bi-composite oligo analyzed here is determined by its first-hit kinetics. This is safely calculated from the disappearance of the full-length molecules. In these experimental conditions, the A-stretch component was more stable. The T_0 sample showed no signs of cleavages in this sequence portion (Fig.26, first lane). The degradation pattern of the first time point (T_0) showed that the cleavage kinetics of the two sequence stretches (A-stretch vs C-stretch) were largely independent, that the cleavage of the As was not progressive (in agreement with Leng, M. and Felsenfeld, G. 1966), that a "first-hit kinetics" condition applied. Values of 8 and 10 Kcal/mole in favour of stacking were reported (Brahms et al., 1966; and Leng and Felsenfeld 1966, respectively) indicating the higher tendency of the Poly A sequence to maintain an ordered stacked structure at least in the early moments of the treatment at 90°C. The poly$A_{12}C_{12}$ sequence is composed of eleven 5'ApA3', one 5'ApC3', eleven 5'CpC 3' steps. The 5'ApC3' step was cleaved less than both 5'CpC3'and 5'ApA3'.

Mixed-sequence RNA (5'-GGAAACGUAUCCUUUGGGAG-3')

This sequence (dubbed "P1", La neve et al., 2003) contains the highly scissible base step 5'UpA 3' and, out of the 16 possible combinations, only 5'GpC3' and 5'CpA3' (the other highly scissible step) are missing. This sequence was selected in order to have the highest possible number of sequence combinations and only one highly scissible site. The 5'GpC3' step was not introduced in order to avoid sequence combinations that would lead to stable intrastrand structures (not detailed). As it is, this sequence lacks noticeable stable intrastrand structures, as determined by the standard Zuker analysis (Zuker, 1989).

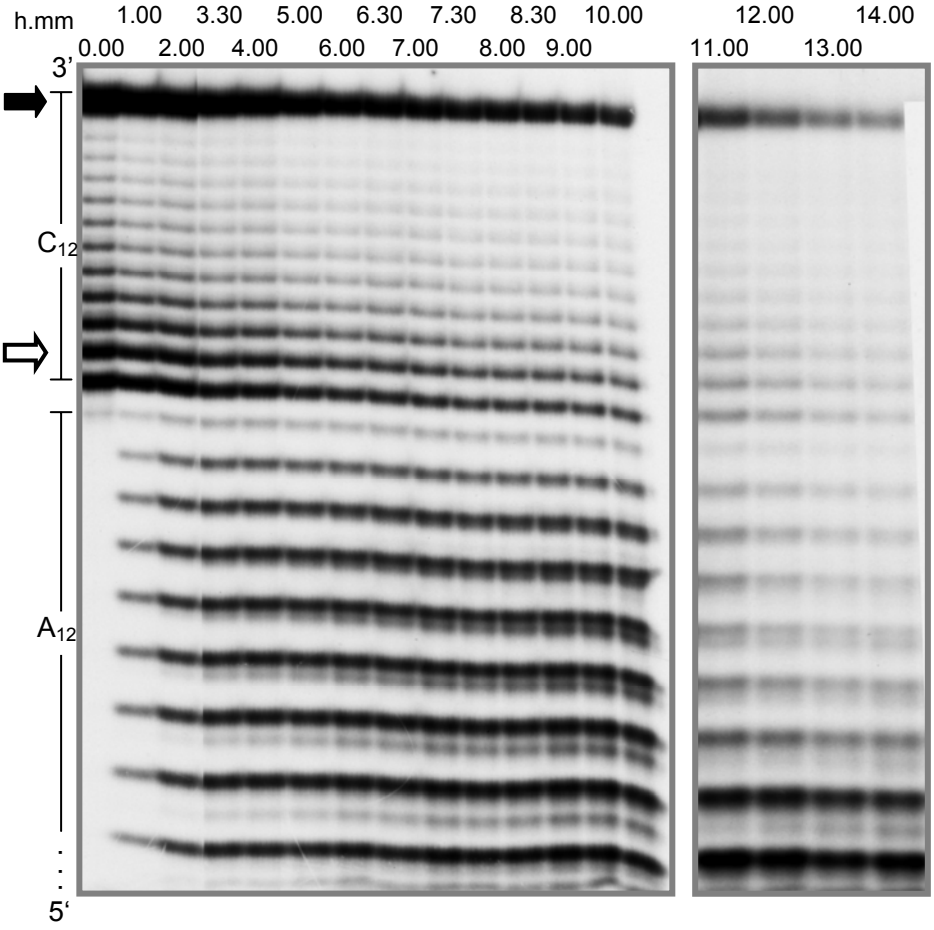


Fig. 26: Kinetics of degradation of Poly $A_{12}C_{12}$ in water, pH 5.5, 90°C. Experimental procedure and representation as for the Poly A_{24} analysis reported in Fig. 25. The C_{12} and A_{12} moieties of the oligo are indicated (left, upper panel) (Ciciriello et al., in press 2007).

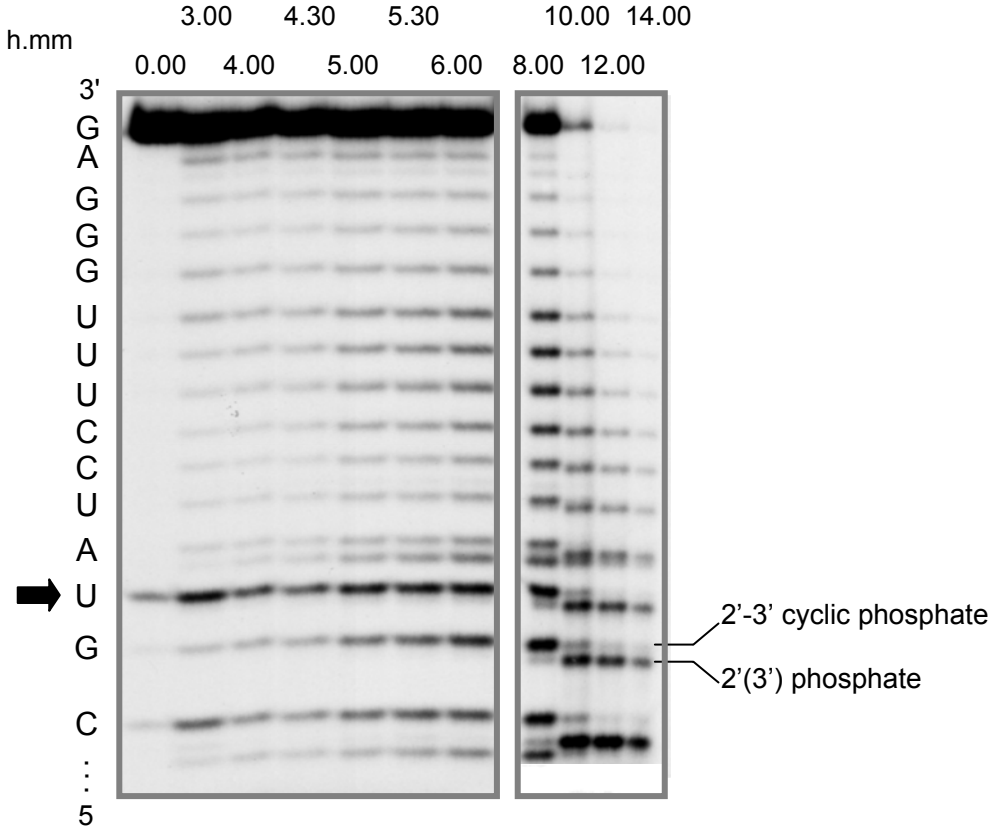


Fig. 27: Kinetics of degradation of P1 in water, pH 5.5, 90°C. The arrow (left) points to the 5' UpA 3' labile step. The bands representing the 2'-3' cyclic phosphate and the 2' or 3' free phosphate extremities are indicated (right, bottom) (Ciciriello et al., in press 2007).

With the exception of the cleavage of the 5'UpA 3' bond (indicated by an arrow on the left side of Fig. 27) the overall cleavage profile was rather homogeneous and only slight preference was shown for the centrally located cluster of pyrimidine steps 5'UpC3', 5'CpC3', 5'CpU3' (Fig.27). The double bands appearing in correspondence of cleavages at advanced stages of the degradation process (indicated on the right side of Fig.27) are due to successive steps of the hydrolytic process (Soukup and Breaker, 1999; Kuusela and Lönnberg, 1994). The cleavage of a 3' phosphoester bond first results in the production of a 3'-2' phosphate cyclic extremity (upper band in a 5' labeled oligomers), followed by the opening of the cyclic bond, yielding a non-resolved mixture of 2'- and 3'-monophosphate extremities (lower bands).

KINETICS ASPECTS. THE HYDROLYTIC DEGRADATION OF RNA IN WATER IS PRECEDED BY A LAG PERIOD

The average half-life of the 3' phosphoester bonds was calculated (see Methods) based on the disappearance of the full-length molecules, and on the principle that this is a measure of independent first-hit events. In homogenous-sequence molecules (as in Poly A_{24}) the calculated t½ value corresponds to the half-life of homogenously cleaved (see Fig. 25) 3' phosphoester bonds. The degradation kinetics of the Poly A_{24} oligo at pH 5.5 and pH 6.2 are shown. The lag period is only observed for pH 5.5. In spite of the relatively small acidity difference, at pH 6.2 the lag is lost. The two sequence blocks composing the Poly$A_{12}C_{12}$ oligo were cleaved with different kinetics, faster for the stretch of Cs, slower for the stretch of As (Fig. 26).

In this case the disappearance of the full-length molecules due to first-hit events were therefore mostly caused by cleavages of one of the relatively weaker 5'CpC3' steps.

For the heterogeneous sequence 5'-GGAAACGUAUCCUUUGGGAG-3' the cleavage pattern did not show strong sequence-related cleavage biases (Fig. 27). Such cleavage homogeneity is not *a priori* expected but is *a posteriori* justified

by the relevance of sequence-context effects (Kaukinen et al., 2002) averaging out potential local cleavage differences, as confirmed by the inspection of the relatively homogeneous cleavage pattern (Fig. 27). The kinetics of disappearance of the full-length molecules as a function of time at 90°C revealed a lag of about 6 hours before the onset of rapid degradation.

During this period the RNA oligo polymers remained as intact full-length molecules. Operationally, the lag period was defined as the period during which 90% of the molecules remain full-lengthed.

In conclusion, RNA in water at pH 5.5 (90°C) is resistant to hydrolysis for a defined period, after which it undergoes rapid degradation. This behaviour is largely sequence-independent.

Irreversibility: The lag resistance is not a reversible phenomenon. An RNA sample (conserved in water at -20°C) was defrozen and treated for 6 hrs at 90°C, then re-frozen at -20°C for 12 hrs. The sample was then re-heated at 90°C and the kinetics of hydrolysis was analyzed with the standard procedure.

The results showed that the molecules are in this case hydrolyzed with a rapid-start kinetics, not undergoing again to the pre-hydrolysis lag period. A dehydration step was introduced in a variant of this assay. After the 6 hrs treatment at 90°C, RNA was alcohol precipitated, then frozen and treated as in Also in this case the pre-hydrolysis lag was not observed. Hysteresis effects in RNA conformation have been previously observed and their basis analyzed (Revzin et al., 1973; Pinder et al., 1974). RNA meta-stability is based on acquired structures, from base stacking to complex foldings. In sequences which do not fold, as in our case, persistence of base stacking might contribute to stability for a certain period of time. However, the experiments described showed that stability, once lost, was not re-attained and is only explained by a persistent, conceivably covalent modification.

pH: The analysis of the pH-dependence of RNA stability was performed by measuring the residual amount of the full-length molecules (%, ordinate) after 4 hrs in water at 90°C at the indicated pH (abscissa). Fig. 28 compares the three

profiles. A well defined, often sharp dependence of the RNA oligo stability on pH was observed which locally varied as a function of the sequence composition.

THE HALF-LIFE OF THE 3' PHOSPHOESTER BOND: MONOMERS VERSUS POLYMERS. TEMPERATURE EFFECTS

The kinetics of hydrolysis in water was analyzed in detail as a function of temperature in the mixed sequence P1 oligo at 20, 30, 40, 50, 60, 70, 80, 90 °C.
The half-life values of the P1 RNA, calculated as described for the experiments reported in Fig. 27, are shown for the whole range of temperatures in Fig. 29. The Figure compares the half-life of the 3'-phosphoester bond in the 3'AMP monomer (filled squares) with the half-life of the same bond in the P1 oligo (filled dots).
The error bar for the 80°C temperature point is the average of 6 measurements, that for the 90°C point of 4 measurements. All the other points were measured once. The open dots indicate the duration of the lag period at each temperature. The comparison shows that at lower temperatures (up to 50°C) the 3'-phosphoester bond was more stable in the monomer, while above 60°C the stability of this bond was markedly higher when embedded in the polymer.
As evident, the difference is largely due to the lag period preceding the onset of hydrolytic degradation.

THE LAG PERIOD AND THE PH EFFECT

The kinetics of degradation of the three oligos tested is peculiar: a lag period during which the RNA backbone length does not change, which is similar for the three sequences, followed by rapid degradation. The lag lasts for several hours at 90°C and is lost at pH < 4 and > 6.

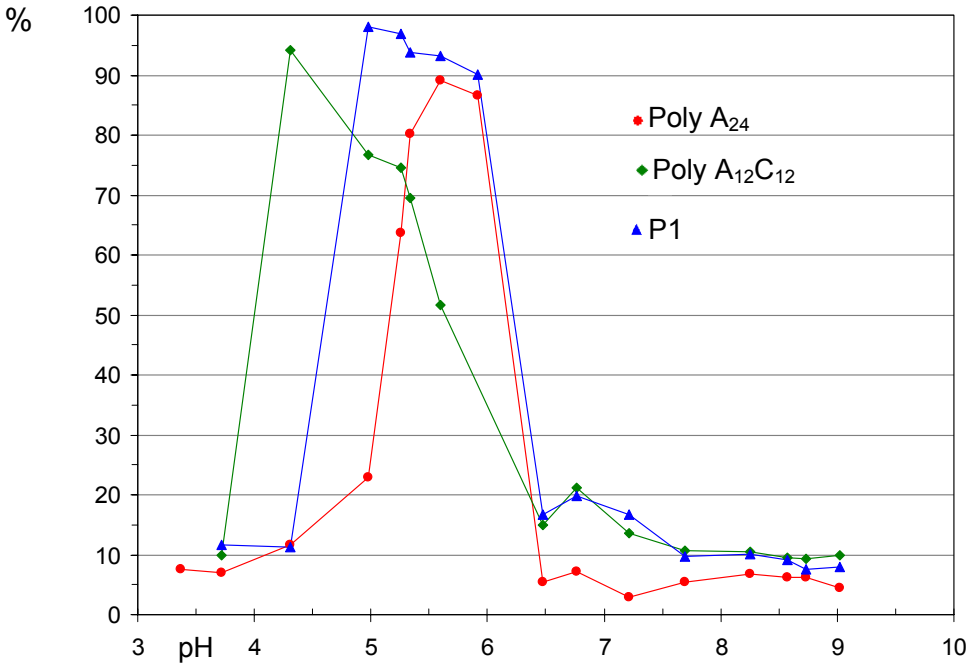


Fig. 28: Stability of different RNAs as a function of pH (Ciciriello et al., in press 2007).

Given the inconsistency of the occurrence of first-hit events at 90°C after several hours being due to the persistence of a protective stacked conformation, the existence and the characteristics of the lag period can be explained more mechanistically by default reasoning: the kinetics and mechanisms for the cleavage and isomerization of the phosphoester bonds of RNA by Brønsted acids and bases were thoroughly analyzed and reviewed (Oivanen et al., 1998).

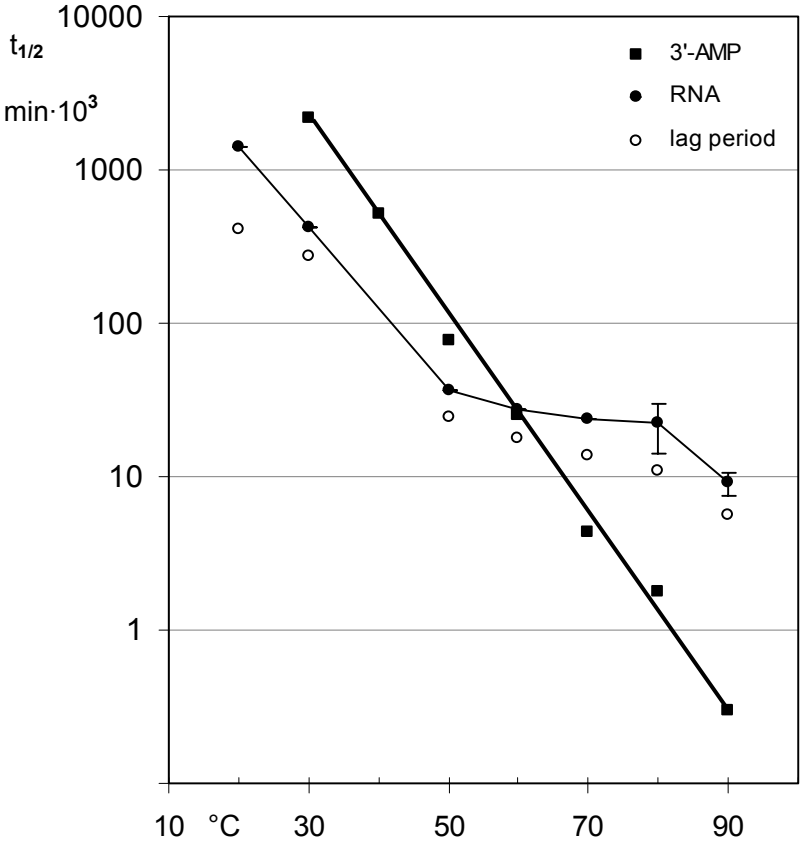


Fig. 29: The stability of the 3' phosphoester bond when present in 3'AMP (filled squares, and in the oligomer P1 (filled dots). In the oligomer data set, each point represents the half-life (ordinate) calculated in degradation analyses, performed at the indicated temperature (abscissa). Open dots indicate the duration of the lag period at the specified temperature (Ciciriello et al., in press 2007).

The pH-rate profiles for trans-esterifications have been determined over a wide range of acidity, extending from concentrated acid solutions to concentrated aqueous alkalines. Under neutral or alkaline pH conditions, the dominant pathway for RNA degradation is an internal phosphoester transfer reaction that is promoted by specific base catalysis (Li and and Breaker, 1999). In most instances the identities of the nucleotide bases that flank the target RNA linkage have a negligible effect on the p*K*a of the nucleophilic 2'-hydroxyl group, and only have a minor effect on the maximal rate constant for the transesterification reaction (as determined and reviewed in Li and Breaker, 1999). Alkaline conditions favour specific base catalysis, in which the 2' hydroxyl group is deprotonated by hydroxide to generate the more nucleophilic 2' oxyanion group. The ensuing reaction is the primary pathways for the uncatalyzed degradation of RNA polymerase under typical cellular conditions (Jarvinen et al., 1991).
Facile transesterification also occurs under strong acid conditions (Oivanen et al., 1998). With reaction conditions below pH 6, specific base catalysis becomes a minor mechanism relative to the competing mechanism of specific acid catalysis for RNA transesterification (Oivanen et al., 1998).
A bell-shaped pH-rate profile was reported, with the minimum reactivity centering at pH 5 (Oivanen et al., 1998). This shape was interpreted to indicate involvement of four kinetically distinct terms in the cleavage reaction, the monoanionic phosphodiester being largely predominant at pH 4 to 6. As pointed out (Li and Breaker, 1999), around these pH values both specific base catalysis and specific acid catalysis are near a minimum (Oivanen et al., 1998) and cleavage by a depurination/ß-elimination mechanism (Smith and Allen, 1953) becomes increasingly significant.
One is therefore left with a chemical environment encompassed between pH 4 and 6 in which the onset or the absence of depurination or of other reactions leading to base degradation or removal become relevant. In our experimental setup at 90°C depurination of 3' AMP levels off after 0.3 x 10^3 min (Fig. 24) being more prominent between pH 3.37 and 5.24. Loss of the pyrimidine base

cytosine or of the corresponding nucleoside cytidine from 3' CMP (data not shown) is not pH dependent and occurs in ~1 x 10^3 min.
The average duration of the lag period (Figs 25-27) corresponds to the time required for depurination and depyrimidination (Fig. 24 for depurination).
ß-elimination follows. Thus, by default a limited area of pH values is left in which nor specific base nor specific acid catalys occur and RNA resists degradation. On the acidic side, above pH 3.37 and below 5.24, depurination further limits pure-purine RNA stability, as seen in $PolyA_{24}$.
A pH-determined niche is thus defined in which RNA stability strictly depends on its sequence. The results showed that at well defined pH and temperature conditions, polymers are favored. We hypothesize that this property conferred to the polymer a sufficient Darwinian edge over its constituent monomers not only to allow its very survival in the polymeric form but also to provide a phenotype for evolution. Complex sequence resist more, in terms of time and in wider environmental conditions, thus being favoured.

NEXT STEP: POLYMERIZATION

The high temperature favouring the polymer over the monomer fits well with the scenario depicting biological evolutionary correlations: the forms of life arising at the temperatures characterizing early Earth were supposedly thermophilic or hyperthermo-phylic (Wiegel and Adams 1998; Forterre et al. 1995).
The evolutionary tree has 189 thermophilic roots (Schwartzman and Lineweaver 2004. Thus, it has been testing self-polymerization at high temperature. According to previous studies (Verlander et al., 1973), we have started with cyclic nucleotides: they have an energetic advantage and at the same time they are the principal chemical extremities producted in RNA degradation.
We have characterized self-polymerization of adenosine cyclic 2'-3' phosphate in our conditions (Fig. 30), providing the proof of principle for the plausibility of this general mechanism. If the $\Delta G°$ problem is the major obstacle for liquid-phase polymerizations in prebiotic conditions (Van Holde, 1980), we have found

that in formamide adenosine cyclic 2'-3' phosphate, also in the presence of mineral phosphate (previous data not shown), could spontaneously polymerize (Fig. 30). These analyses have been performed by HPLC.

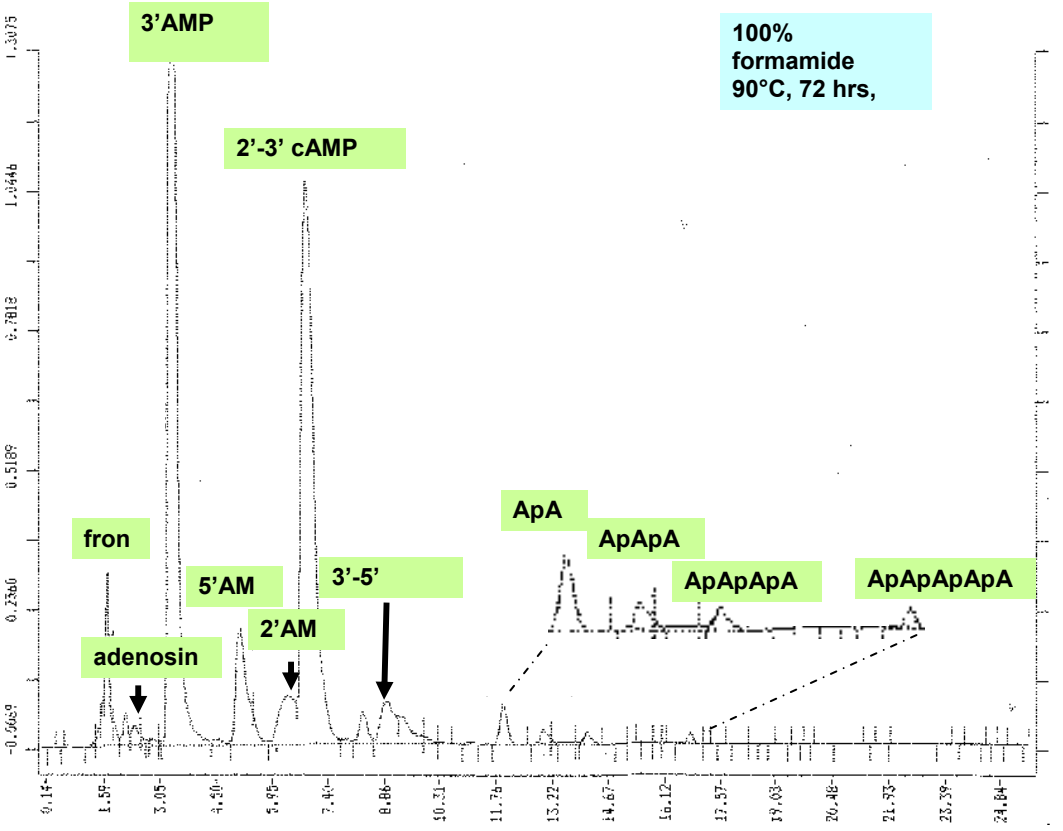


Fig. 30: HPLC chromatogram of adenosine cyclic 2'-3' phosphate, in formamide at 90°C for 72 hrs.

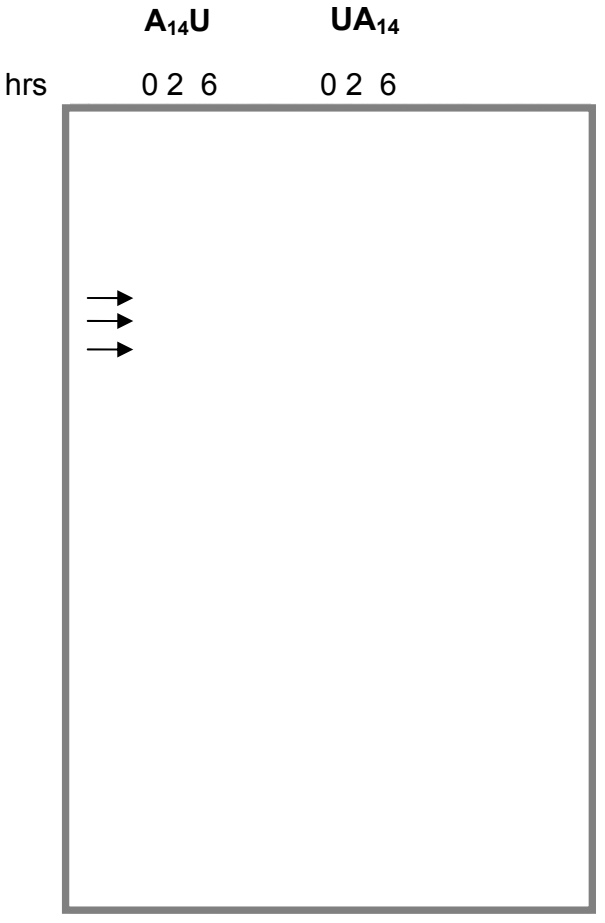


Fig. 31: Polymerization of 5'A_{14}U3' and 5'UA_{14}3' in the presence of 50mM carbodiimide at 37°C. The elongation phenomena are indicated (left, upper panel).

We obtained a consistent number of oligomers (2mer up to 6mer), we are not yet confident about the chemical nature of phosphate linkages but we are trying to understand it with another chemical approach. According to this, we have used a RNA A-rich oligomers to investigate elongations, in prebiotic conditions, at 37°C (Fig. 31) and higher temperature (data not shown, preliminary results). Carbodiimide, that we are able to obtain in our prebiotic synthesis, has improved our results.

We have observed elongation phenomena using an A-rich oligomer with a U at 3'-end, or an A-rich oligomer with a U at 5' -labelled end (Fig. 31). We have found that the first oligomer undergoes efficient polymerizations better than the second one, probably because the weaker linkage of 5'ApU3' than the linkage of 5'ApA3' (Norberg, and Nilsson, 1995), triggered the elongation phenomena (Fig31).

Conclusion

EVOLVING EQUILIBRIA: A CYCLIC SCENARIO FOR FORMAMIDE PREBIOTIC CHEMISTRY

The very fact that formamide yields, just by heating in the presence of catalysts, the full panel of nucleic bases shows the possibility of their accumulation at elevated temperature, and reveals that conditions exist allowing a positive balance between syntheses and degradations. In terms of the ability to form informational macromolecules, the intrinsic instability of the bases (Levy and Miller, 1998; Bada and Lazcano, 1982; Miller and Lazcano, 1995; Miyakawa et al., 2002; Miyakawa et al., 2002) and their prompt synthesis (Saladino et al., 2001) (Saladino et al., 2003) (Saladino et al., 2005b) (Saladino et al., 2004d), as reviewed in (Saladino et al., 2005a), are not only disadvantages. At the contrary, a synthesis/degradation/re-synthesis cycle offers the possibility to form a dynamically equilibrated pool of precursors, whose composition depends on the synthesis/degradation rate of each molecular species, and on the catalyst(s) present (Saladino et al., 2006c; 2006b). The advantage provided by a flexible and adjustable pool of precursors is an important evolutionary property. This so-called 'equilibration-of-the-pool' scheme (Saladino et al., 2005a) (Saladino et al., 2004c) is based on two simple considerations:

-The mechanism of degradation of purines (Saladino et al., 1996) and pyrimidines (Saladino et al., 1997) by formamide shows that formamide acts initially by nucleophilic addition, rapidly leading to base degradation. This process eventually re-affords formamide, and is markedly more efficient for purines than for pyrimidines. The order of base degradation is G≥A>C»T (Negri et al., 1996). In the absence of catalysts, all the bases are eventually degraded back to formamide.

-On the other hand, in the presence of the appropriate catalysts, formamide condenses into purines and pyrimidines, the yield of each base depending on the

catalyst present. Each catalyst affords a complex and specific panel of nucleic bases.

Without a way out towards polymers, and/or in the absence of protective mechanisms, conditions, or compounds, the cycle of syntheses/degradations is bound to remain pre-biologically futile (Ciciriello et al., 2007). The final composition of the pool depends on the equilibrium between syntheses and degradations of each base under the local, specific physico-chemical conditions. Let us dub this phenomenon ‘base-equilibration pool’. Another equilibration pool relates to phosphorylations. In the Results section, I reported that nucleosides are promptly phosphorylated in the presence of formamide and of inorganic phosphates, as originally reported by Schoffstall's and co-workers (Schoffstall, 1976; Schoffstall et al., 1982; Schoffstall and Laing, 1985; Schoffstall and Mahone, 1988). We have observed that comparable levels of nucleoside phosphorylation are obtained in the presence of formamide using a nucleoside monophosphate as phosphate donor. This phenomenon is caused by the formamide-mediated cleavage of the nucleotide phosphoester bond, followed by the transfer of the phosphate group to an acceptor site on the sugar moiety of an acceptor nucleoside.

The acceptor may be the same starting nucleoside or a different one, phosphorylation occurring at any of the possible 2'- , 3'- , 5'- , 2',3'-cyclic, and, presumably, also 3',5'-cyclic positions (Costanzo et al., 2007). In a composite pool of nucleosides, the final composition of the nucleotide pool will depend on the on/off rates of the phosphoester bond of each different nucleotide at each different position. The major factors affecting the final balance are 1) the higher stability of the cyclic phosphates relative to open forms, and 2) the preferential phosphorylation at the 5’-position, a phenomenon called ‘phosphorylation-equilibrated pool’.

Although the same type of pool-equilibration behavior has not been yet studied for the formation of nucleosides, there is no a priori reason to exclude its occurrence.

The way out from futile syntheses/degradation cycles of nucleobases and of their

phosphorylations could have been provided by polymerization itself. Thus, the comparison of the stability of the key bonds in the polymer relative to the stabilityof the same bonds in the monomers is a central point in understanding the basics of the thermodynamics and kinetics of polymerization. If conditions were eventually met in the pristine warm little pond imagined by Darwin (Darwin, F., 1888) conditions that favored polymerization, the sequence composition of the ensuing polymer necessarily reflects the composition of the ‘base-equilibration pool' or the ‘phosphorylation-equilibrated pool’. Preliminary experiments (see Results) have demostrated that, in our enviroment, elongation phenomena are possible but needs further investigations.
Given that the formation of a polymer subtracts from the pool, thus changing its composition and affecting the equilibria involved, polymerization itself could have been a relevant evolutionary factor .

Experimental Procedures

MATERIALS

Formamide(>99.5%,H_2O>0.1%) was from Fluka, adenine, ribose, ribose 5'-monophosphate, adenosine, adenosine 5'-monophosphate (5'-AMP), adenosine 3'-monophosphate (3'-AMP), 2'-deoxyribose, 2'-deoxyribose 5'-monophosphate, 2'-deoxyadenosine, 2'-deoxyadenosine 5'-monophosphate, and 2'-deoxyadenosine 3'-monophosphate and adenosine cyclic 2'-3' phosphate were from Sigma Aldrich, analytical grade.
Cytosine, cytidine, and their monophosphorylated forms (5'-CMP, 3'-CMP, 2'-CMP, 2':3'-cyclic CMP, and 3':5'-cyclic CMP) were also from Sigma Aldrich, analytical grade, and KH_2PO_4 was from Merck.
The deoxy-oligonucleotides used were:
Oli1, 5'-ACCTAACCGG(A)30CCGGTT-3'
Oli2, 5'-CCCGAACCGG(T)30CCGGTT-3'
Oli3: 5'-CCCGAACCGG[C]30CCGGTT-3'
Oli4: 5'-CCCGAACCGG[T]30CCGGTT-3'
These deoxyoligonucleotides were designed such as to be complementary. Upon annealing, 4-nucleotide-long 5'-protruding tails remain at both extremities that can be used for selective labeling at 3'.
The mixed sequence used was made of Oli5 (40 bases) and Oli6(44 bases) and was analyzed:
Oli5: 5'-GTAACTCGGTGTTAGAGCCTGTAACTCGGTGTTAGAGCCT-3'
Oli6: 5'-CCGAAGGCTCTAACACCGAGTTACAGGCTCTAACACCGAGTT
AC-3'
The degradation of RNA in water, in formamide, and in formamide containing aqueous solutions was studied on different RNAs. The P1 RNA has the sequence 5'-GGAAACGUAUCCUUUGGGAG-3', was purchased from RNATEC, and was kindly provided by E. Caffarelli. The others RNA was purchased from

Dharmacon and provided in the standard lyophilized form. They have the sequence:
Poly A_{24} : 5'-AAAAAAAAAAAAAAAAAAAAAAAA-3'
Poly $A_{12}C_{12}$: 5'-AAAAAAAAAAAACCCCCCCCCCCC-3'
$A_{14}U$: 5'-AAAAAAAAAAAAAU-3'
UA_{14}: 5'-UAAAAAAAAAAAAAA-3'
The phosphate minerals studied were:
Variscite $Al(PO_4)(H_2O)_2$,
Augelite $Al_2PO_4(H_2O)_3$,
Wavellite $Al_3(OH)_3(PO_4)_2(H_2O)_5$,
Hureaulite $Mn^{2+}_5(PO_3\{OH\}_2(PO_4)(H_2O)_4$,
Strengite $Fe^{3+}(PO_4)(H_2O)_2$,
Vivianite $Fe^{2+}_3(PO_4)_2(H_2O)_8$,
Ludlamite $Fe^{2+}_3(PO_4)_2(H_2O)_4$,
Libethenite $Cu^{2+}_2(PO_4)(OH)$,
Tarbuttite $Zn_2(PO_4)(OH)$,
Wardite $NaAl_3(OH)_4(PO_4)_2(H_2O)_2$,
Lazulite $Mg[Al(PO_4)(OH)]_2$,
Vauxite $Fe^{2+}Al_2(PO_4)_2(OH)_2(H_2O)_6$,
Cacoxenite $Fe^{3+}_{25}(PO_4)_{17}O_6(OH)_{12}(H_2O)_{75}$,
Purpurite $Mn^{3+}(PO_4)$,
Eosphorite $Fe^{2+}[Al(PO_4)(OH)_2(H_2O)]$,
Childrenite $Mn^{2+}[Al(PO_4)(OH)_2(H_2O)]$,
Laueite $Mn^{2+}[Fe^{3+}_2(PO_4)_2(OH)_2(H_2O)_2](H_2O)_4(H_2O)_2$,
Fluorapatite $Ca_5(PO_4)_3F$,
Hydroxylapatite $Ca_5(PO_4)_3OH$,
Anapaite $Ca_2[Fe^{2+}(PO_4)_2(H_2O)_4]$,
Scholzite $CaZn_2(PO_4)_2(H_2O)_2$,
Turquoise $Cu^{2+}Al_6(PO_4)_4(OH)_8(H_2O)_4$,
Pyromorphite $Pb_5(PO_4)_3Cl$,
Herderite $Ca[BePO_4F]$,

Calcioferrite $Ca_4MgFe_4(PO_4)_6(OH)_4(H_2O)_{13}$.
They were provided by Ezio Curti (ezio.curti@libero.it), former provider and consultant of the Collection of Minerals of the Department of Mineralogy (University La Sapienza, Rome). Hydroxylapatite was from Fluka "High Resolution" grade.
Na_3PO_4, $Na_4P_2O_7$, $Na_5P_3O_9$ were from Sigma Aldrich.
Montmorillonites K-10, KSF, Al-PILC, K-30 (Fluka) were used without further purification.

METHODS

GAS CHROMATOGRAPHY AND MASS SPECTROMETRY:
Was performed by the use of a HP5890II gas chromatograph and by a Shimadzu GC-MS QP5050A spectrometer equipped with an AlltechR AT-20 column (0.25 mm, 30 m). ^1H and ^{13}C NMR spectra were recorded on a Bruker (200 MHz) spectrometer, and chemical shifts are reported in ppm. Microanalyses were performed with a C. Erba 1106 analyzer. Chromatographic purifications were performed on columns packed with Merck silica gel, 230–400 mesh for flash technique. TLC was carried out by using Merck Platten Kieselgel 60 F254.

FORMAMIDE CONDENSATION:
Formamide (5.7 g, 5 mL, 0.12 mmol) was heated at 160°C for 48 hrs in the presence of the appropriate catalysts. The reaction mixture was allowed to cool, filtered to remove the catalyst and evaporated under high vacuum. Gas chromatography and mass spectrometry of the crude reaction mixture were performed by using an isothermal temperature profile of 100°C for the first 2 min, followed by a $10°Cmin^{-1}$ temperature gradient to 2808C and finally an isothermal period at 280°C for 40 min. The injector temperature was 280°C. Chromatography grade helium was used as carrier gas. The fragmentation patterns were compared with those of authentic samples. 6-methoxypurine was used as an internal standard. When necessary, the crude reaction was purified by

flash chromatography ($CHCl_3/CH_3OH$ 9:1), and the structures of isolated products were confirmed by spectroscopic techniques (^1H and ^{13}C NMR) and by comparison with authentic commercial samples.

PREPARATION OF UV-LASER ABLATED (UV-LA) OLIVINES:
CDAs were produced by fast condensation of vaporized target by high energy Nd:YAG laser ablation. Laser targets were oxide mixtures, (SiO_2, MgO, FeO). Each component was weighed in order to obtain the exact stoichiometric composition of olivines with different magnesium and iron content. The targets were prepared by pressing the oxide mixtures at 15 tons to produce pellets 13 mm in diameter and few millimeters in thickness. Surelite II laser (fundamental wavelength at 1064 nm) equipped with two crystals was used to obtain forth harmonics at UV wavelength of 266 nm. An optical set-up selects and focuses the UV laser beam on the target to give a power density of 10^9 Wcm^{-2} on a spot 2 mm in diameter. The target is mounted inside a vaporization chamber designed to work at different gas pressures. In this experiment, the O_2 atmosphere was 10 mbar.

PREPARATION OF PHOSPHATE MINERALS:
Pure crystals were isolated under the microscope, washed twice first with ethanol, then with analytical grade distilled water, air dried, manually ground in a ceramic mortar.

PHOSPHORYLATION PROCEDURE AND ANALYSIS:
Adenosine was dissolved in formamide and reacted at the concentration of 0.025 M in 1.5 ml Eppendorf tubes, final volume 1 ml. The reaction was carried out in formamide at 90°C in the presence of the indicated phosphate donor: KH_2PO_4 (final concentration 0.05 M) or 5' CMP (final concentration 0.05 M) or one of the indicated phosphate minerals (10 mg/ml, ground as indicated). Phosphates were added to adenosine from concentrated solution in formamide. Where indicated the mineral was pretreated at 130°C for 72 hrs in formamide.

After the indicated reaction time, the samples were analyzed by high pressure liquid chromatography (HPLC).

HPLC ANALYSIS OF PHOSPHORYLATION REACTIONS:
8µl aliquots of the reaction mixtures were diluted with an equal volume of water to a final concentration of 50% formamide and injected into a Supelcosil^{TM} LC-18-S 5-µm HPLC column (Supelco) 15 cm × 4.6 mm. Elution was performed at a flow rate of 2 ml/min at room temperature with methanol:30 mM ammonium phosphate, pH 5.3 (2.5:97.5), UV irradiation 254 nm, on a HPLC Beckman System Gold instrument. Identification of the peaks was performed by comparison with standards. In this HPLC system 3':5' cyclicAMP migrates very close to the large unreacted adenosine peak, thus preventing its precise evaluation. As for the cytosine system, four peaks are resolved, consisting of cytosine, cytidine, (5' CMP + 3' CMP + 2' CMP + 2':3' cyclic CMP), and 3':5' cyclic CMP. Thus, the meaning of the assays on cytidine phosphorylation is limited to the evaluation of the overall phosphorylation of the nucleoside.

MEASUREMENT OF PHOSPHATE RELEASE IN FORMAMIDE:
The crystal minerals were ground to a fine powder in a ceramic mortar. A suspension of the powder was diluted (1 mg/ml) in formamide, washed, and centrifuged four times. Samples were incubated at 130°C for the indicated times, and then 100 µl aliquots were evaporated in an oven at 250°C for 20 min, resuspended in 100 µl of ultrapure water, and analyzed for phosphate by the molybdenum blue-method, which detects only orthophosphate (PO_4^{3-}).

HPLC ANALYSIS OF MONOMERIC FORMS:
Samples were resuspended at a final concentration of 1 mg/ml in water or in the appropriate formamide reaction medium (usually in 0.5–1.0 ml).
Temperatures and incubation times are indicated. 10-µl aliquots of the reaction mixtures were diluted to a final concentration of 50% formamide in a final volume of 20 µl and injected into a Supelcosil^{TM} LC-18-S 5-µm HPLC column

(Supelco) 15cm×4.6mm. Elution was performed at a flow rate of 2 ml/min at room temperature with methanol: 30mM ammonium phosphate, pH 5.3 (2.5:97.5), UV 254 nm, pressure 1.5 atmosphere, on an HPLC Beckman System Gold instrument. Identification of the peaks was performed by comparison with real samples. Half-lives were determined by standard graphical procedures.

RNA/DNA PREPARATION AND LABELING:

5'-Labeling: 10 μmol RNA/DNA were labeled with [γ-^{32}P]ATP using polynucleotide kinase (Roche Applied Science). The oligo was then purified on a 16% denaturing acrylamide (19:1 acrylamide/bisacrylamide) gel. After elution, the residual polyacrylamide was removed by a NuncTrap Probe purification column (Stratagene). 2 pmol (typically 30,000 cpm) RNA were processed for each sample.

DNA; 3'-Labeling: 2 μg of each oligonucleotide were annealed with the same amount of the complementary oligo and labeled with [γ-^{32}P]dCTP. Labeling was performed using T7 Sequenase (USBC; Amersham Biosciences). The labeled oligo was purified on a 16% denaturing acrylamide (19:1 acrylamide/bisacrylamide) gel, and the polyacrylamide was removed by a NuncTrap Probe purification column (Stratagene). 2 pmol (typically 30,000 cpm) of DNA were processed for each sample.

DEOXYOLIGONUCLEOTIDE DEGRADATION PROTOCOLS AND ANALYSES:

3'- and 5'-labeled oligonucleotides were treated under the time, temperature, and solution conditions indicated where appropriate. To stop the reactions carried out in water (pH 5.5 at 90°C) the sample (typically 15μl) was diluted and precipitated with 3 vol of ethanol 96%, sodium acetate 0.3 M final concentration, pH 7.5, 20 μg glycogen. To stop the reaction carried out in formamide a solution of 5×10^{-4}M (final concentration) of tetrasodium pyrophosphate (Sigma) dissolved in water was added to a final volume of 40 μl.

The samples were vortexed for 1 min and then centrifuged at 13,000 rpm for 20 min. This procedure was performed twice. The wash was ethanol precipitated, resuspended in 5 μl of formamide buffer, heated for 2 min at 95 °C, and loaded on a 16% denaturing polyacrylamide gel (19:1 acrylamide/bisacrylamide).

DEGRADATIONS OF DEOXYOLIGONUCLEOTIDES BY FORMAMIDE AND IN THE PRESENCE OF DIFFERENT CATALYSTS:

Each oligonucleotide (2 mg) was annealed with the same amount of the complementary oligomer and labelled with [γ-^{32}P]dATP (Oli3 and Oli4) or with [γ-^{32}P]dCTP (Oli1 and Oli2). Labelling was performed by using the T7 Sequenase (USBC-Amersham Biosciences), the labelled oligomer was purified on a 16% denaturing acrylamide gel (acrylamide/bisacrylamide 19:1). The polyacrylamide was removed by a NuncTrap Probe Purification Column (Stratagene), 2 pmol (typically 30000 counts per minute) of DNA were processed for each sample. The DNA was precipitated with ethanol and resuspended in formamide (5 μL, Fluka). 97% formamide (10 μL) containing the indicated amounts of catalyst were added. After 20 min at 110°C, a solution of tetrasodium pyrophosphate (5×10^{-4}M, final concentration, Sigma) dissolved in water was added to a final volume of 40 μL. The samples were vortexed for 1 min, then centrifuged at 13000 rpm for 20 min. This procedure was performed twice. The wash was combined, precipitated with ethanol, resuspended in formamide buffer (5 μL), heated for 2 min at 95°C and loaded on a 16% denaturing polyacrylamide gel (acrylamide/bisacrylamide 19:1). For the analysis of the effect of formamide–catalyst on the heterogeneous sequence, the oligonucleotides indicated above were labelled with [γ-^{32}P]dCTP.

HALF-LIVES OF THE BONDS OF THE 3'- AND 5'-PHOSPHOESTER BONDS IN DEOXYOLIGONUCLEOTIDES:

For oligomers, the half-lives of the phosphodiester bonds were determined with standard procedure from plots. Lines were drawn tangential to the initial part of the curves, and the $t_{1/2}$ was graphically determined. For the 3'-bonds, the half-life

was calculated for the bonds located in three different positions from the label: the 7^{th}, 20^{th}, and 35^{th} bonds, counting from the labeled extremity. We assumed that each bond in the poly(dA) stretch is cleaved with the same efficiency as the other bonds, and we have not taken into consideration poly(dA) internal nearest neighbor, sequence context, and cleavage-induced extremity effects, for which we have not observed any evidence. The homogeneity of cleavage in the poly(dA) stretch contrasts with the sequence dependence of formamide-induced cleavage rates. The focus of the present analysis is the determination of the stability of the 3'- and 5'-phosphoester bonds related to adenine. Based on these considerations, we assumed that the actual half-life of, say, the 7^{th} bond is given by the experimentally determined value (that is, the % value of the band under consideration relative to the total signal of the lane) multiplied by 7 and divided by 2; that of, say, the 20^{th} bond by the observed value multiplied by 20 and divided by 2, etc. This procedure was adopted to average out experimental imprecision (deriving from gel electrophoresis, image scanning, background subtractions, tangential lane drawing, etc.) and to verify the linearity of the analytical range. When cleaving a terminally labeled oligomer, this type of kinetic analysis is meaningfully performed only in the less than one cut/molecule range (typically, cleaved molecules < 30%). Above this % value, multiple hits prevent reliable analysis. This procedure provides a quantitative evaluation of the linearity of the analyzed range: under ideal conditions the $t_{1/2}$ of the $7^{th} \times 7$ must correspond to that of the $20^{th} \times 20$ and to that of the $35^{th} \times 35$. To average out deviations from such correspondence, the $t_{1/2}$ value is calculated as the average of one central position and of two positions close to the two opposite extremities.

The data are reported "as the sample average value" $\mu \pm$ the "sample S.D. value" σ (standard deviation) of the $t_{1/2}$ values obtained by this procedure in the three different positions. The positions were selected as indicative of a centrally located bond (the 20^{th}) and of two oppositely located bonds proximal to the extremities (the 7^{th} to the 3'- and the 35^{th} to the 5'-extremity, respectively). For 5', the same procedure was followed, analyzing the positions 11^{th} and 25^{th} from the 5'-labeled extremity. The smearing of the upper part of the gel, typical for 5'-

labeled oligos, prevented analysis of more label-distal positions. For deoxymonomers, the $t_{1/2}$ values were graphically calculated.

RIBOOLIGONUCLEOTIDE DEGRADATION PROTOCOLS AND ANALYSES:
The 5'-labeled oligonucleotide was treated under the time, temperature, and solution conditions indicated where appropriate. To stop the reactions carried out in water (pH 5.5 at 90°C) the sample (typically 15µl) was diluted and precipitated with 3 vol of ethanol 96%, sodium acetate 0.3 M final concentration, pH 7.5, 20 µg glycogen. To stop the reaction carried out in formamide a solution of 5×10^{-4}M (final concentration) of tetrasodium pyrophosphate (Sigma) dissolved in water, pH 7.5, was added to a final volume of 40 µl. The samples were vortexed for 1 min and then centrifuged at 13000 rpm for 20 min. This procedure was performed twice. The supernatant was ethanol precipitated, resuspended in 5 µl of formamide buffer, heated for 2 min at 65°C, and loaded on a 16% denaturing polyacrylamide gel (19:1 acrylamide/bisacrylamide).

HALF-LIVES OF THE BONDS IN THE RIBOOLIGONUCLEOTIDE:
For oligonucleotides, the half-lives of the phosphoester bonds were determined from the rate of disappearance of the band representing the intact oligomer molecule. Cleavage of RNA normally requires participation of the 2'-OH group as an internal nucleophile by two "nucleophilic cleavage" events: the trans-esterification and hydrolysis reactions (Fig. 18). On the basis of this known mechanism two operative assumptions were made.
1) It was assumed that the cleavage of the 3'-phosphoester bond is largely more effective than the cleavage of the 5'-one and that for practical calculation purposes the 5'-phosphoester bond is not cleaved. In support, the cleavage of the 5' terminally labeled RNA molecule only shows the standard cleavage of the 3'-bond (see Fig. 19), whereas the double bands typically produced by 3'- and 5'-cleavages occurring in the same population of molecules were never detected.

This contrasts with DNA, where under several conditions both cleavages do occur, giving rise to the diagnostic double bands.
2) In the mixed oligonucleotide studied, the breakage of the phosphodiester chain is not sequence biased under the conditions used. This sequence may have, like many other RNAs, preferential breakage sites that require special defined conditions to be cleaved. Outside the special cleavage conditions this RNA is stable. The half-life of the oligonucleotide was determined with standard graphical procedure from plots of the % disappearance of the intact oligomer molecules. Given that one disappearing molecule represents one cleavage, and given the two assumptions reported (no 5'-cleavage, no sequence bias), the half-life of a 3'-phosphoester bond in the riboligonucleotide is given by the half-life of the oligonucleotide × [(*x*mer)-1] (for example: 19 is the number of 3'-phosphoester bonds in the 20-mer). For ribomonomers, the $t_{1/2}$ values were graphically calculated.

HALF-LIVES OF THE 3'-PHOSPHOESTER BOND IN 3'AMP:
The half-life of the 3' phosphoester bond in 3' AMP and 3'CMP was calculated at the pH and at the temperature conditions indicated.
Commercial distilled water was further purified by tridistillation-deionization with a MilliQ Advantage A10 or with a Sartorius ARIUM 611 VF apparatus. Pure or 10 mM TrisHCl-buffered water were pretreated for two hours at the temperature of the assay to be performed, a period of time sufficient to reach and maintain the temperature-specific pH. The temperature-stabilized pH values (determined on a Beckman Ø 40 pHmeter) are given throughout.
Samples were resuspended at the final concentration of 1 mg/ml in water or in the appropriate formamide reaction medium (usually in 0.5-1.0 ml) and incubated at various temperatures (20,30,50,60,70,80 or 90°C) at the indicated pH values for the appropriate periods of time. 10 µl aliquots were diluted to a final concentration of 50% formamide in a final volume of 20 µl and injected into a Supelcosil™ LC-18-S 5-µm HPLC column (Supelco) 15 cm x 4.6 mm. Elution was performed at a flow rate of 2 ml/min at room temperature with

methanol: 30 mM ammonium phosphate, pH 5.3 (2.5:97.5), UV 254 nm, pressure 1.5 atmosphere, on an HPLC Beckman System Gold instrument. Identification of the peaks was performed by comparison with real samples. Half-lives were determined by standard graphical procedures.

HPLC ANALYSIS OF POLYMERIZATION EVENTS:
Adenosine cyclic 2'-3' phosphate was resuspended at a final concentration of 1 mg/ml in water or in the appropriate formamide reaction medium (usually in 0.5–1.0 ml) or in the presence of 1 mg of phosphate mineral. 10-µl aliquots of the reaction mixtures were diluted to a final concentration of 50% formamide in a final volume of 20 µl and injected into a Supelcosil^{TM} LC-18-S 5-µm HPLC column (Supelco) 15cm×4.6mm. Elution was performed at a flow rate of 2 ml/min at room temperature with methanol: 10 mM TPCA, (20:80), UV 254 nm, pressure 1.5 atmosphere, on an HPLC Beckman System Gold instrument. Identification of the peaks was performed by comparison with real samples.

POLYMERIZATION EVENTS OF A PRE-EXISTENT RNA:
Oligonucleotides were incubated in water in the presence of 50 mM carbodiimide under the time indicated, at 37°C. To stop the reactions carried out in water (pH 5.5 at 90°C) the sample (typically 15µl) was diluted and precipitated with 3 vol of ethanol 96%, sodium acetate 0.3 M final concentration, pH 7.5, 20 µg glycogen. Loaded on a 16% denaturing polyacrylamide gel (19:1 acrylamide/bisacrylamide).

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La notte è divina. Non c'è una bava di vento, non un bisbiglio. Il silenzio è totale.
I galli non hanno ancora cominciato a cantare, e neppure gli uccelli. E come mai prima, mi sento sulla superficie tormentata, pietrosa, terrosa, verdeggiante, ma pur sempre blocco minerale e nulla più, d'un pianeta chiamato Terra dagli uomini, che è poi solo un granello di polvere gravitante nello spazio siderale, nell'infinito delle distanze, nell'infinito del tempo. La coscienza fisica del vuoto dello spazio mi prende alla gola. Assaporo, come non avevo assaporato prima, la violenza atroce delle parole di Pascal: «Il silenzio degli spazi mi sgomenta».
Il segreto del mondo è li, ed è spaventoso: mai un termine, mai un limite, mai una fine

Charles Duchaussois, Flash

Alla mia famiglia
Alla mia famiglia
Alla mia famiglia
Alla mia famiglia
Alla mia famiglia

E alle affinità intellettive per cui il passare del tempo non ha valore