POSSIBLE PREBIOTIC SIGNIFICANCE OF POLYAMINES IN THE CONDENSATION*, PROTECTION, ENCAPSULATION, AND BIOLOGICAL PROPERTIES OF DNA

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Abstract. Some properties of DNA condensed with spermidine have been compared with the properties of DNA condensed with $Co^{3+}(NH_3)_6$ to determine whether condensation of DNA with these trivalent cations protects DNA against the action of DNase I and increases transcription and encapsulation of DNA into liposomes. It was shown that DNA condensed with $Co^{3+}(NH_3)_6$ was resistant to the action of the endonuclease DNase I such as DNA condensed with spermidine was. However, DNA condensed with $Co^{3+}(NH_3)_6$ was significantly less active in transcription with the *E. coli* RNA polymerase than DNA-spermidine condensed forms. In addition, it was demonstrated that both compacted forms of DNA were more efficiently encapsulated into neutral liposomes; however, negatively, charged liposomes were scarcely formed in the presence of DNA condensed with $Co^{3+}(NH_3)_6$. These experiments and the well documented properties of polyamines increasing the resistance to radiations and hydrolysis of nucleic acids, as well as their biological activities, such as replication, transcription, and translation, together with the low concentration of Co^{3+} in the environment, lead us to propose spermidine as a plausible prebiotic DNA condensing agent rather than Co^{3+} and the basic proteins proposed by other authors. Then, we consider the possible role and relevance of the polyamine-nucleic acids complexes in the evolution of life.

1. Introduction

A critical process in the formation of primordial cells must have been the isolation of macromolecules away from the external environment by self-assembled membranes. Since amphiphilic lipids are the unique molecules that can self-assemble into closed and self-sealing membranes, called liposomes, these lipids have been regarded as molecules that could have formed primordial membranes in the Archean Earth (Hargreaves and Deamer, 1978; Deamer and Oró, 1980; Baeza *et al.*, 1987a).

* In this paper the term condensation is used to mean actually ionic condensation of DNA with Co^{3+}, or with polyamines, which is the terminology used in this specialized field, it does not mean covalent condensation as it would be in the case of chemical polymerization. Another term which is commonly used in this field instead of condensation is compaction or compacted forms, that we are using in some appropriate places.
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During the spontaneous self-assembly of liposomes, macromolecules can be encapsulated in their aqueous compartments (Szoka and Papahadjopoulos, 1978). Nucleic acids are macromolecules whose encapsulation into lipid membranes could be fundamental in origin of life studies.

DNA and RNA encapsulation into liposomes has been extensively studied in experiments of gene transport interest (Mannino and Gould, 1988); however, very low efficiency of nucleic acid incorporation in liposomes has been reported, due mainly to the long linear form and the polyanionic nature of DNA and RNA molecules. Therefore, charge neutralization and molecular condensation of DNA by polyamines and proteins have been used as a strategy to produce an increase in DNA encapsulation into liposomes (Tikchonenko *et al.*, 1988; Szelei and Duda, 1989). These procedures could also have occurred in the prebiotic encapsulation of nucleic acids in lipid membranes.

Jay and Gilbert (1987) proposed that in the prebiotic evolution, basic proteins might have mediated lipid vesicle formation around nucleic acids by neutralizing RNA or DNA charges and by condensing their long linear strands. Since the polyamine spermidine and $Co^{3+}(NH_3)_6$ are also DNA condensing agents (Gosule and Schellman, 1978; Widom and Baldwin, 1980) and they are structurally simpler than basic proteins, we believe that these simple agents could have played the condensing role in the prebiotic encapsulation of nucleic acids into lipid membranes, rather than basic proteins.

DNA compaction is explained in terms of the Manning's theory (Manning, 1978) of counterion condensation; counterions bound around DNA forming a cloud neutralizing part of the phosphate charge and condensing DNA. Wilson and Bloomfield (1979) defined the ionic conditions for compaction by the trivalent cation spermidine, and showed that 89–90% of the DNA charge must be neutralized for compaction to occur. Mono and divalent cations neutralize only 76 and 88% of the negative charge of DNA; therefore, they fail to produce DNA condensation. Then, DNA condensation in a aqueous environment only could be achieved with tri or more multivalent cations.

Polyamines are organic polycations with much simpler molecular structure than proteins; therefore, their prebiotic synthesis could have occurred before that of proteins. In keeping with this idea, we were able to obtain the polyamine putrescine by thermal decarboxylation of the amino acid ornithine under possible prebiotic conditions (Wong *et al.*, 1991); then, it might have been possible that in precellular conditions polyamines caused nucleic acid condensation before that of protein formation. In addition, nucleic acid-polyamine complexes have some important nucleic acid protective properties, and DNA-spermidine-complexes are transcriptionally more active than DNA without polyamine condensation (Bocian *et al.*, 1978; Johnson and Bach, 1966; Tikchonenko *et al.*, 1988; Tabor and Tabor, 1984; Baeza *et al.*, 1987b). Furthermore, an increase in the catalytic properties of RNA was shown in RNA-spermidine complexes (Guerrier *et al.*, 1983). Therefore, it is possible that in precellular conditions polyamines not only could have condensed

nucleic acids producing an increase in their encapsulation in liposomes, but they could also have given some nucleic acids protection that increased their primeval activities.

In this work we study some biological properties of DNA condensed with $Co^{3+}(NH_3)_6$ in comparison with the properties of DNA condensed with spermidine, and we conjecture whether these trivalent cations could have played some important roles in the encapsulation and the activity of DNA in prebiotic conditions.

Using electron microscopy and employing the Dubochet's method (Dubochet *et al.*, 1971) we were able to demonstrate more compaction of DNA with $Co^{3+}(NH_3)_6$ than with spermidine. Until now, DNA condensed with $Co^{3+}(NH_3)_6$ has been extensively studied only through physical-chemical analyses, but in this work some biological properties of this condensed DNA were studied. We found that DNA condensed with $Co^{3+}(NH_3)_6$ was also resistant to the action of the endonuclease DNase I such as DNA condensed with spermidine was (Baeza *et al.*, 1987b); however, DNA condensed with $Co^{3+}(NH_3)_6$ was significantly less active in transcription with the *E. coli* RNA polymerase than DNA compacted with spermidine. We also found that both compacted forms of DNA were more efficiently encapsulated into liposomes from egg-yolk phosphatidylcholine; however, liposomes containing egg-yolk phosphatidylserine were scarcely formed in the presence of DNA condensed with $Co^{3+}(NH_3)_6$ and a lot of amorphous lipid aggregates were formed. These results are serious objections in considering Co^{3+} as a prebiotic DNA condensing agent, therefore we propose that spermidine could have played the condensing role in the prebiotic encapsulation of DNA into lipid membranes rather than Co^{3+}. We also considered the possible prebiotic interaction of nucleic acids with polyamines before their interaction with proteins, and the role and relevance of these interactions in the evolution of life.

2. Methods

2.1. DNA PREPARATION

The pBR322 plasmid DNA was obtained from *E. coli* C600 by the gentle phenol method of Birnboinm and Doly (1979). Plasmid DNA dissolved in 50 mM tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 10 mM ethylenediamine-tetraacetic acid (EDTA), and 500 mM NaCl, pH 8, was fractionated in a Sepharose 4B column for elimination of bacterial RNA. Then, plasmid DNA was dialyzed against 20 mM Tris-HCl, 1 mM NaCl, pH 7, and kept at 5 °C until use. The $260/280_{nm}$ ratio of pBR322 DNA was 2.0.

Viral (^{3}H)SV4O DNA obtained from CV-1 cells infected at low multiplicity with wild type SV40 virus. The cells were cultured using Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and with 40 μCi of (6-^{3}H) thymidine (78 Ci/mmol). The viral DNA was extracted by the Hirt's method (Hirt, 1967) and purified by CsCl-ethidium bromide centrifugation. The specific activity of DNA

was an average of 35 000 cpm/ μg of DNA. The $260/280_{nm}$ ratio of SV40 DNA and $(^3H)SV40$ DNA was also 2.0.

2.2. DNA CONCENTRATION

DNA concentration was determined by Burton's method (Burton, 1956) and by titration against a standard on an ethidium bromide-agarose plate.

2.3. PREPARATION OF CONDENSED FORMS OF pBR322 DNA

$[Co^{3+}(NH_3)_6]Cl_3^-$, from Sigma Chemicals, was recrystallized by adding hot HCl to a saturated aqueous solution; the yellow precipitate obtained was collected, washed with ethanol and ether, dried, and stored in a vacuum desiccator.

Spermidine was purchased also from Sigma Chemical as the hydrochloride salt and was checked for purity by paper chromatography using an N-butanol/acetic acid/H_2O (50:25:25, v/v) solvent followed by ninhydrin spray. A single band was observed with an $R_f = 0.28$, corresponding to spermidine. The Sigma material was used without further purification.

Solutions of DNA and spermidine or recrystallized $[Co^{3+}(NH_3)_6]Cl_3^-$ were prepared in 10 mM Tris-HCl, 1 mM NaCl, pH 78, mixed and allowed to stand at 5 °C for 12 hr (Baeza et al., 1987b). The final DNA concentration was 33 μg mL^{-1} while spermidine and $Co^{3+}(NH_3)_6$ concentrations were 7.5, 11.5, and 16.5 mM, respectively.

For the preparation of condensed $(^3H)DNA$ an aliquot of 2 μg of $(^3H)SV40$ DNA (specific activity of 35 000 cpm/ μg) was added to the on-labeled SV40 DNA solution before mixing with the trivalent cation solutions. After 12 hr at 5 °C this mixture of radioactive and non-labeled DNA was totally sedimented in the compacted forms as demonstrated by electrophoresis in 1% agarose gels and by its radioactivity.

2.4. ELECTRON MICROSCOPY

To analyze the condensed forms of DNA we used Dubochet's method (Dubochet et al., 1971); 200 mesh copper grids previously shadowed with carbon were ionized with pentylamine, 20 μL samples were placed on freshly prepared grids, and compacted DNA was allowed to adsorb for 2 min. Grids were stained with a 2% aqueous solution of uranyl acetate and then shadowed with Pt-Pd (80 – 20%) for 6 sec at 5×10^{-6} T, 32 – 35 A, with an 8 ° inclination angle.

Uncondensed or extended fibrous DNA was observed by using the method described by Kleinschmidt (1968). The hyperphase (50 μL) contained 1 μg of DNA, 5 μg of cytochrome c, and 50% distilled formamide in 100 mM Tris-HCl, 10 mM EDTA, pH 8.5; the hypophase contained 16% distilled formamide in 10 mM Tris-HCl, 1 mM EDTA, pH 8.5. Grids of 200-mesh copper were covered with parlodium film and with a protein-nucleic acid monolayer, and then stained with 0.01 mM aqueous solution of uranyl acetate, treated with isopentane, and drained. Grids were shadowed as above, with a 6 ° inclination angle. A JEOL-100SX transmission electron microscope was used at 60 KV accelerating voltage for all studies.

2.5. CLEAVAGE OF CONDENSED FORMS OF pBR322 DNA BY PANCREATIC DNase I

To study the effect of DNase I on the condensed forms of DNA we used the method described in our laboratory (Baeza *et al.*, 1987b). Ten-microliter samples of DNA condensed with spermidine or with $Co^{3+}(NH_3)_6$ containing 2 µg of DNA and different concentrations of spermidine or $CO^{3+}(NH_3)_6$ were incubated with 2.5×10^{-3} Kunitz units of pancreatic DNase I in 10 mM Tris-HCl, 1 mM NaCl, 1 mM $MnCl_2$, pH 7, at 37 °C for various times. Different aliquots were used for each time period. Reaction was stopped by adding 5 µL of a solution containing 10 mM EDTA, 10 mM urea, 0.05% xylene cyanol, and 0.05% bromophenol blue. Reaction products were analyzed by electrophoresis in 2% agarose gels. Gels were stained with ethidium bromide and photographed by using a Polaroid MP-4 camera and a CHROMATOVUE transilluminator. As a control, a solution of 2 µg (10 µL) of DNA was incubated with 2.5×10^{-3} Kunitz units of DNase I, and the products were analyzed by electrophoresis in 2% agarose gels, as indicated above.

2.6. TRANSCIPTION OF CONDENSED FORMS OF pBR322 DNA

RNA polymerase was prepared from *E. Coli* SA1943 according to Burgess and Jendrisak (1975) and contained a full subunit component as determined by sodium dodecyl sulfate-polyacrilamide gel electrophoresis. The DNA was associated (12 hr at 5 °C) to different concentrations of spermidine or $Co^{3+}(NH_3)_6$. The transcription assays were carried out in a final volume of 300 µL, which contained 12 µg of pBR322 DNA or DNA condensed with spermidine or with $Co^{3+}(NH_3)_6$, 5 mM Tris-HCl, pH 7.9, 4 mM $MgCl_2$, 2.5 mM KCl, 1 mM dithiothreitol, 0.25 mM each of ATP, CTP, and GTP, 70 µM (^3H)UTP (specific activity 285 cpm/pmol), and 30 µg of DNA-dependents RNA polymerase from *E. coli*. The reaction was carried out at 37 °C for 0, 10, and 20 min. Aliquots were removed for (^3H)RNA determination (60 µL), electron microscopy (10 µL), and DNase I treatment (10 µL); the (^3H)UMP incorporation was determined as previously described (Gariglio *et al.*, 1979).

2.7. LIPOSOMES PREPARATION

Liposomes were prepared from egg-yolk phosphatidylcholine using the reverse-phase evaporation (REP) method (Szoka and Papahadjopoulos, 1978). This method was used without reduced pressure. Nine µmoles of phosphatidylcholine dissolved in chloroform were added to a 50 mL round-botton flask and the solvent was removed by bubbling a jet stream of nitrogen through the lipid solution using a rotary evaporator at 37 °C. The lipid film, was redissolved in 1 mL of diethyl ether, and 330 µL of 10 mM Tris-HCl, 1 mM NaCl, pH 7, containing 50 µg of (^3H)DNA or (^3H)DNA condensed with spermidine or with $Co^{3+}(NH_3)_6$, were added. The resultant two phase system was vortex-mixed and sonicated twice for 5 sec in a bath-type sonicator (Lab Supply G112SOI). The ether was also removed under nitrogen using rotary evaporation at 37 °C, thereby, producing the preparation of liposomes.

Liposomes containing (^{3}H)DNA or condensed (^{3}H)DNA were separated from unencapsulated material by centrifugation at 200 000 × g for 30 min. These liposomes were washed three times with 10 mM Tris-HCl, 1 mM NaCl, pH 7, buffer solution (Fraley *et al.*, 1980).

2.8. LIPOSOMES COMPOSITION AND MICROSCOPY

Lipid concentration in liposomes was calculated from quantitative determination of phospholipids (Bottoger *et al.*, 1961). In control liposomes cholesterol was also determined (Ferro and Ham, 1960). DNA encapsulation was determined by monitoring a 50 μL aliquot of liposomes containing (^{3}H)DNA or condensed (^{3}H)DNA for radioactivity in a triton/toluene/based scintillant (Fraley *et al.*, 1980). Typically 60 to 70% of the phospholipids were recovered in the liposomes.

Liposomes for negative-stain electron microscopy suspended in 10 mM Tris-HCl, 1 mM NaCl, pH 7, were diluted 1:1 with 2% aqueous uranyl acetate on carbon-formvar-coated grids. After 1 min, grids were drained on absorbent tissue and allowed to air dry. These liposomes preparations were observed in the electron microscope already indicated.

3. Results

3.1. ELECTRON MICROSCOPY OF LIPOSOMES AND CONDENSED FORMS OF pBR322 DNA

Since Dubochet's method (Dubochet *et al.*, 1971) does not affect ionic interactions it has been successfully employed to observe molecular complexes in which the main interactions are of ionic nature, such as, SV40 minichromosomes (Gariglio *et al.*, 1979) and DNA condensed with spermidine (Baeza *et al.*, 1987b). Using this method we demonstrated that spermidine and $Co^{3+}(NH_3)_6$ produce compacted forms of DNA similar to the doughnut-shaped toroids and elongated rods previously described (Gosule and Schellman, 1978; Widom and Baldwin, 1980; Baeza *et al.*, 1987b); in this paper we only describe the toroidal structures (Figure 1a, d). In no case we have observed extended structures simultaneously with toroidal forms suggesting that all pBR322 DNA molecules were condensed with the concentrations of spermidine or $Co^{3+}(NH_3)_6$ employed. Also it can be seen that the toroids obtained with both trivalent cations were approximately of the same size (0.3 μm of average perimeter); however, when toroids were obtained with $Co^{3+}(NH_3)_6$ they were thicker and the size of their hole became smaller suggesting that DNA molecules were more compacted with $Co^{3+}(NH_3)_6$ than with spermidine (Figure 1a, d). When 116 mM NaCl was added to the pBR322 DNA-trivalent cation toroids and these were incubated at room temperature for 30 min, electron microscopy showed no compact structures; only extended forms identifiable as pBR322 DNA molecules could be observed (Figure 1b, e), suggesting that DNA compaction by $Co^{3+}(NH_3)_6$ also can be reversed at this salt concentration as was demonstrated for DNA condensed with spermidine (Baeza *et al.*, 1987b). However, it can be seen that DNA dissociated
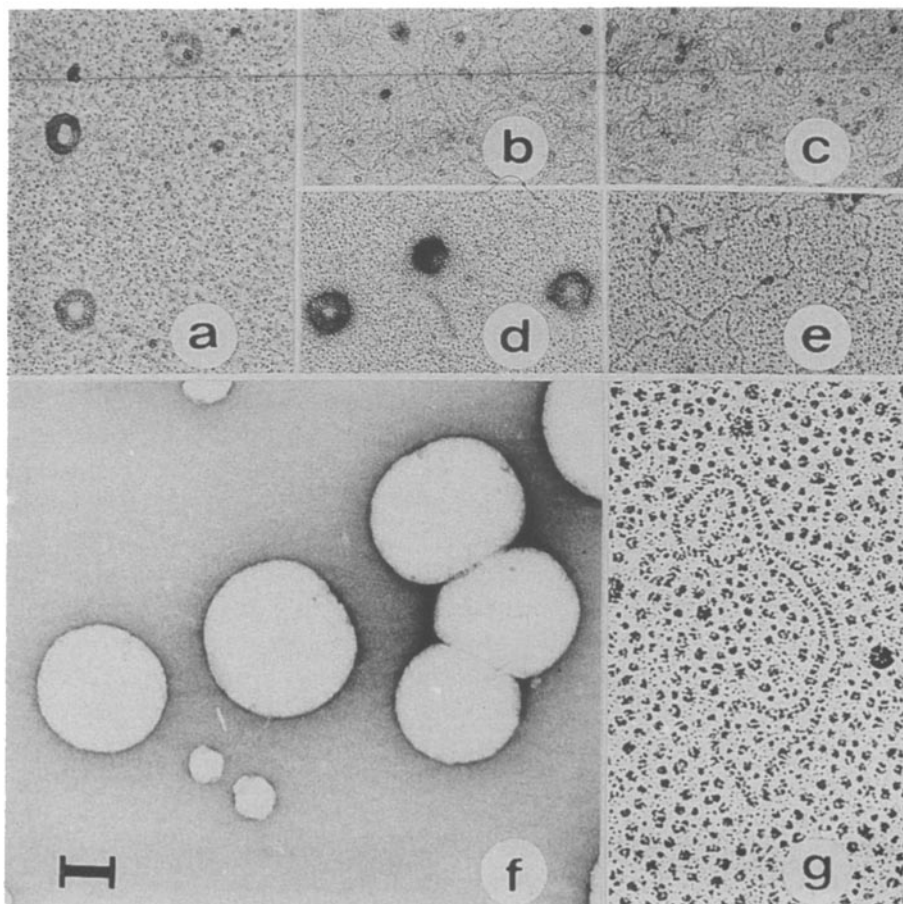
Fig. 1. Electron micrography of liposomes and condensed DNA. All DNA preparations were contrasted with uranyl acetate and shadowed linearly with Pt-Pd by Dubochet's method (Dubochet *et al.*, 1971). pBR322 DNA condensed with 33 μg mL^{-1} DNA and (a) 16.5 mM spermidine or (d) 16.5 mM Co^{3+}(NH$_3$)$_6$. (b) and (e) DNA condensed as in (a) and (d) but dissociated for 30 min with 116 mM NaCl. (c) pBR322 DNA without spermidine and Co^{3+}(NH$_3$)$_6$. (g) pBR322 DNA by Kleinschmidt's method (Kleinschmidt, 1968). (f) negative-stain of egg-yolk phosphatidylcholine liposomes. A solid line represents 0.1 μm.

from DNA-Co^{3+}(NH$_3$)$_6$ toroids was thicker than DNA dissociated from DNA-spermidine toroids, which suggest that some DNA association remains after the dissociation of DNA-Co^{3+}(NH$_3$)$_6$ toroids with 116 mM NACl. Electron microscopy by Dubochet's method also shows that when spermidine or Co^{3+}(NH$_3$)$_6$ were excluded, keeping other steps in the specimen preparation unaltered, only supercoiled pBR322 DNA molecules were obtained (Figure 1c). Electron microscopy by Kleinschmidt's method shows the classic image of naked and relaxed pBR322 DNA (Figure 1g), this methodology tend to extend the supercoiled DNA and the attachment of cytochrome c to DNA make the extended macromolecule thicker than DNA without cytochrome, giving a better electron microsopy image of DNA. With this

method we obtained an average perimeter of 2.8 μm for pBR322 DNA. Finally, negative-stain electron microscopy also shows the classic image of egg-yolk phosphatidylcholine liposomes, with 1.1. μm of average perimeter (Figure 1f). These perimeters suggest that DNA-trivalent cation toroids could be better encapsulated into liposomes than the supercoiled or the totally extended DNA.

3.2. RESISTANCE OF CONDENSED FORMS OF pBR322 DNA TO PANCREATIC DNase I

DNA condensed with polyamines shows DNA resistance to restriction endonucleases cleavage and to cleavage by a broader hydrolytic enzyme such as pancreatic DNase I. This DNA resistance to the enzymatic hydrolysis has been attributed to the compacted form of DNA because a possible polyamine inhibitory effect on the enzyme activity was discarded (Pingoud *et al.*, 1984; Kuosmanen and Poso, 1985; Baeza *et al.*, 1987b, 1988); trying to detect whether $Co^{3+}(NH_3)_6$ produce condensed forms of DNA with similar properties as polyamines, we analyzed the resistance of pBR322 DNA-$Co^{3+}(NH_3)_6$ condensed forms to the action of pancreatic DNase I.

Figure 2A shows the resistance of pBR322 DNA condensed with spermidine to the hydrolytic activity of DNase I. It can be seen that condensed pBR322 DNA forms containing 7, 115.5, and 16.5 mM spermidine remained practically undigested for any length of time up to 30 min (lanes a, b, c,). When pBR322 DNA was dissociated from these condensed forms with 116 mM NaCl it was really cleaved by DNase I (lanes d, e, f) because the high salt concentration produces the dissociation of DNA and spermidine, then pBR322 DNA is in a non-condensed molecular form (Figure 1b) susceptible to the hydrolytic activity of DNase I. These results also showed that free spermidine did not produce any inhibitory effect on DNase I as was previously demonstrated with 16.5 mM spermidine (Baeza *et al.*, 1987b).

Figure 2B shows that pBR322 DNA condensed with $Co^{3+}(NH_3)_6$ was also resistant to the hydrolytic activity of DNase I. It could be seen that condensed pBR322 DNA containing 7, 11.5, and 16.5 mM $Co^{3+}(NH_3)_6$ remained practically undigested for any length of time up to 30 min (lanes b, e, h). When DNA was dissociated from these compacted forms with 116 mM NaCl DNA cleavage took place rapidly by DNase I even at zero min of incubation (lanes f, i); however, when DNA was dissociated with 95 mM NaCl there was only partial cleavage at 30 min of incubation (lanes d, g, j). These results showed that free $Co^{3+}(NH_3)_6$ did not produce any inhibitory effect on DNase I activity; $Co^{3+}(NH_3)_6$ was present in the incubation system but the compacted forms of pBR322 DNA were not formed, due to the high salt concentration, as demonstrated by electron microsopy (Figure 1e); on the contrary, the presence of $Co^{3+}(NH_3)_6$ in the dissociated form appears to stimulate the activity of DNase I, since pBR322 DNa dissociated from these compacted forms by 116 mM NaCl was more rapidly digested by the enzyme than naked DNA (compare lanes f and i with lane a). These results also show that the resistance of pBR322 DNA-$Co^{3+}(NH_3)_6$ compacted forms to the activity of DNase I, can be attributed to the compacted form of pBR322 DNA induced by $Co^{3+}(NH_3)_6$.
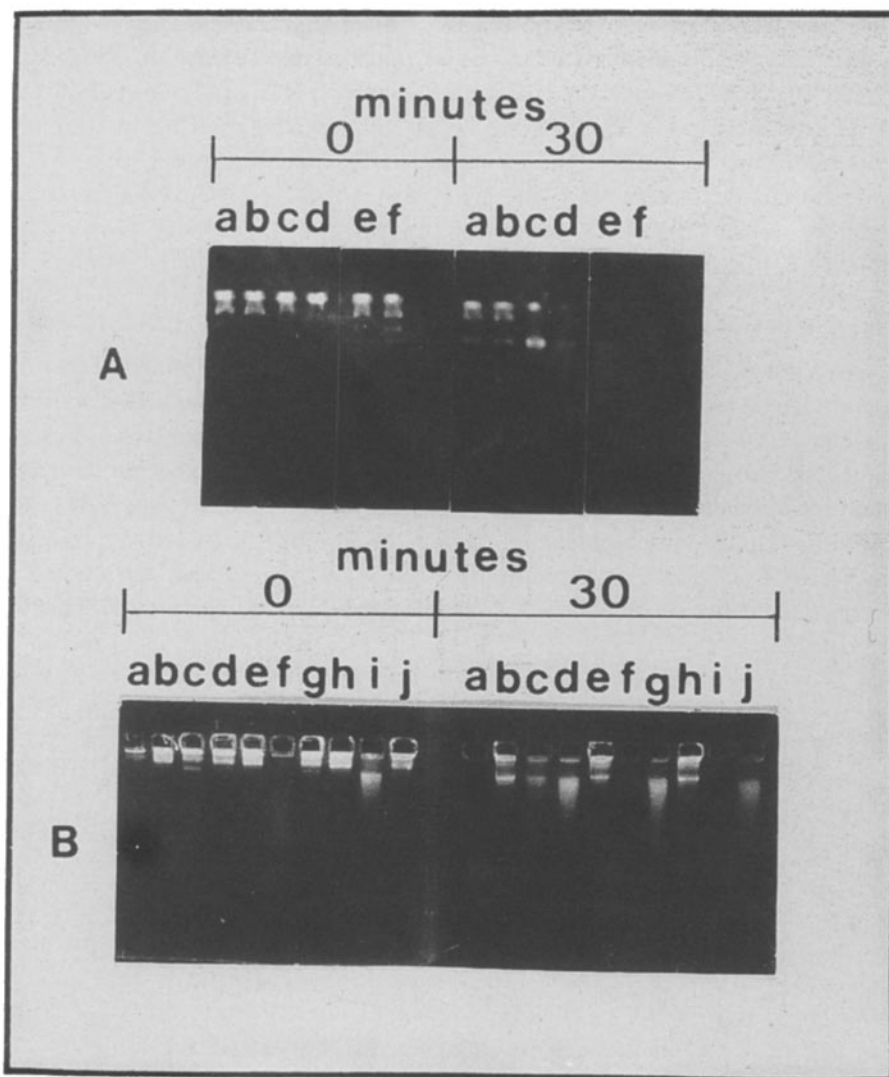
Fig. 2. Resistance of DNA condensed with trivalent cations to pancreatic DNase I. Compacted forms of pBR322 DNA were prepared with 33 µg mL^{-1} DNA as mentioned under Methods. Samples of 10 µg of condensed DNA containing 2 µg of DNA in 10 mM Tris-HCl, 1 mM NaCl and 1 mM MnCl$_2$, pH 7, were incubated at 37 °C with 2.5 × 10^{-3} Kunitz units of DNase I. (A). Lanes (a), (b), (c) pBR322 DNA condensed with 7, 11.5, and 16.5 mM spermidine; (d), (e), (f) DNA condensed as in (a), (b), and (c) but dissociated for 30 min with 116 mM NaCl before the addition of DNase I. (B). Lane (a) 1 µg of pBR322 DNA; (b), (c), (d) pBR322 DNA condensed with 7; (e), (f), (g) 11.5; and (h), (i), (j) 16.5 mM Co^{3+}(NH$_3$)$_6$; (c), (f), (i) pBR322 DNA condensed forms dissociated with 116 mM and (d), (g), (j) 95 mM NaCl, for 30 min before the addition of DNase I. Numbers were the incubation time.

The possibility that $Co^{3+}(NH_3)_6$ binds to DNase I and changes its cleavage specificity was ruled out in the same way as that described (Shishido, 1985; Baeza et al., 1987b). When DNase I was exposed for 12 hr to 7, 11.5, or 16.5 mM $Co^{3+}(NH_3)_6$ in 10 mM Tris-HCl, 1 mM NaCl, pH 7, at 5 °C and dialyzed against the same buffer, its enzymatic activity was as shown in Figure 2B (lanes a, b, e, h); i.e., plasmid DNA was completely digested, and pBR322 DNA condensed with $Co^{3+}(NH_3)_6$ was not digested. These results suggest that $Co^{3+}(NH_3)_6$ does not bind to DNase I.

3.3. IN VITRO TRANSCRIPTIONAL ACTIVITY OF CONDENSED FORMS OF pBR322 DNa

It is well known that polyamines stimulate the in vitro transcription catalyzed by bacterial and eukaryotic polymerases; probably the polyamines produce an activation of the enzymatic activity of polymerases increasing the RNA synthesis (Igarashi, 1988). In addition, we have demonstrated that DNA condensed with spermidine was transcriptionally more active in the presence of E. coli RNA polymerase than DNA without polyamine compaction (Baeza et al., 1987b). In order to compare the effect of $Co^{3+}(NH_3)_6$ and spermidine on DNA compaction, we studied the transcriptional activity of pBR322 DNA condensed with spermidine and with
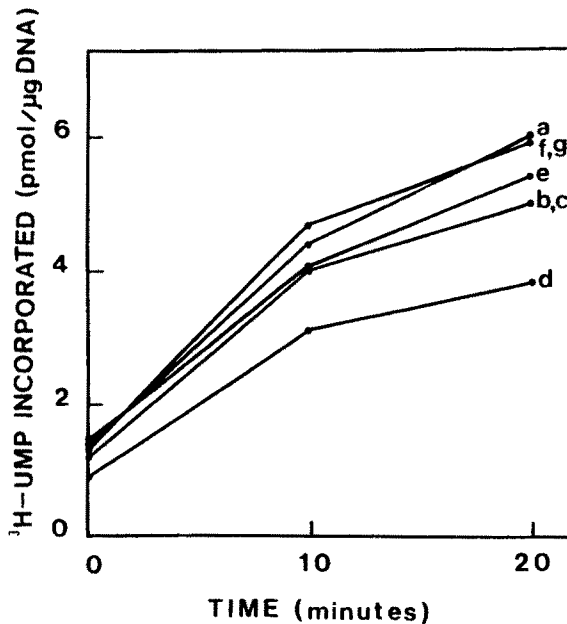

Fig. 3. Transcription kinetics of DNA condensed with trivalent cations. Samples of 300 μL of compacted forms of DNA containing 12 μg of pBR322 DNA in 5 mM Tris-HCl, pH 7.9, 2.5 mM KCl (low ionic strength) were prepared and incubated for RNA synthesis at 37 °C with the DNA-dependent E. coli RNA polymerase as described under Methods. (a) as a control, 12 μg of pBR322 DNA were incubated in the absence of trivalent cations; (b), (c), (d) pBR322 DNA condensed with 7, 11.5, and 16.5 mM spermidine; (e), (f), (g) pBR322 DNA condensed with 7, 11.5, and 16.5 mM $Co^{3+}(NH_3)_6$.

$Co^{3+}(NH_3)_6$ at low ionic strength (5 mM Tris-HCl, 2.5 mM KCl) to be sure that the condensed structures of pBR322 DNA remained in their compact form. Figure 3 shows the activity of *E. coli* DNA-dependent RNA polymerase on compacted forms of pBR322 DNA. It can be seen that pBR322 DNA condensed with 7 and 11.5 mM spermidine shows an increase between 70 and 125% in transcription, respectively; however, when pBR322 DNA condensed with 16.5 mM spermidine was used, which form more compact structures of DNA, RNA synthesis was practically the same as with naked pBR322 DNA. When we used pBR322 DNA-$Co^{3+}(NH_3)_6$ compacted forms obtained with the same trivalent cation concentrations used in the formation of DNA condensed with spermidine: i.e. 7, 11.5, and 16.5 mM, it could be seen that a decrease in RNA synthesis was obtained. Transcription of these compacted forms of DNA decrease with regard to transcription of naked pBR322 DNA when the $Co^{3+}(NH_3)_6$ concentration was increased. Parallel electron microscopy studies of compacted forms of DNA obtained with 7, 11,5 and 16.5
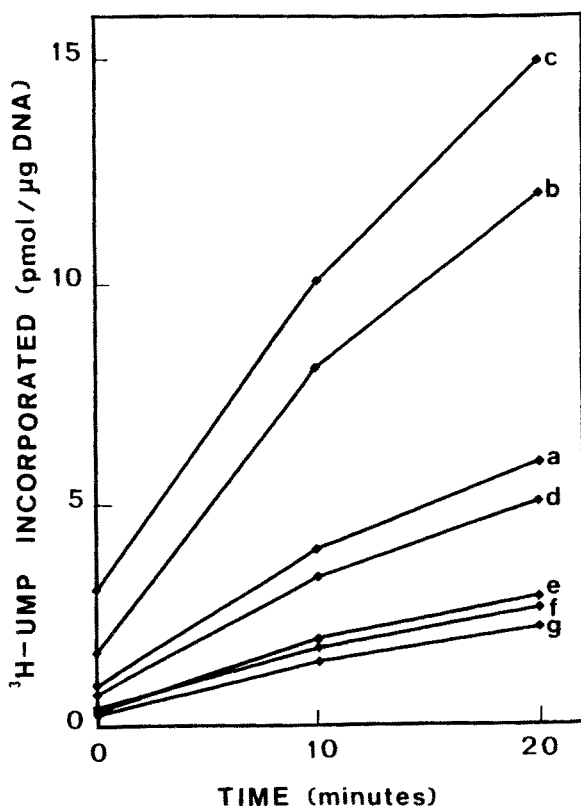

Fig. 4. Transcription kinetics of DNA condensed with trivalent cations at high ionic strength. RNA synthesis and DNA concentrations were as indicated in Figure 3; but reactions were carried out in 50 mM Tris-HCl, pH 7.9, 50 mM KCl. (a) as a control 12 µg of pBR322 DNA were incubated in the absence of trivalent cations; (b), (c), (d) pBR322 DNA condensed with 7, 11.5, and 16.5 mM spermidine; (e), (f), (g) pBR322 DNA condensed with 7, 11.5, and 16.5 mM $Co^{3+}(NH_3)_6$.

mM $Co^{3+}(NH_3)_6$ or spermidine showed the presence of toroids during transcription. These compacted forms were also resistant to the hydrolytic action fo DNase I (not shown).

When transcription was carried out at higher strength (50 mM Tris-HCl, 50 mM KCl) a decrease in transcription with the compacted forms of pBR322 DNA obtained with spermidine was observed (Figure 4b, c, d). However, when condensed pBR322 DNA obtained with $Co^{3+}(NH_3)_6$ was used the RNA synthesis was practically the same as with naked pBR322 DNA (Figure 4e, f, g). At this ionic strength, we found that both compacted forms of DNA were dissociated, as demonstrated by DNase I activity.

3.4. Encapsulation of condensed forms of pBR322 DNA

DNA condensed by polyamines and proteins has been used for efficient incorporation of long linear or circular forms of DNA into vesicles (Jay and Gilbert, 1987; Tikchonenko et al., 1988; Szelei and Duda, 1989) because compacted forms of DNA are more appropriate in size to fit into liposomes. In this study we compared the efficiency of incorporation of compacted forms of (^{3}H)SV40 DNA obtained with spermidine and $Co^{3+}(NH_3)_6$ into REP liposomes from egg-yolk phosphatidylcholine. Compacted forms of (^{3}H)SV40 obtained with both trivalent cation have an average perimeter (0.33 μm) similar to compacted forms of pBR322 DNA obtained with the same trivalent cations (Baeza et al., still in prep.). For comparison liposomes containing egg-yolk phosphatidylcholine, egg-yolk phosphatidylserine, and cholesterol (molar ratio 4:1:5) were formed, because it is well known that liposomes containing these lipids present the best efficiency in the encapsulation and delivery of DNA to cells (Fraley et al., 1980). Table I shows that SV40 DNA condensed with both trivalent cations, spermidine and $Co^{3+}(NH_3)_6$, had a higher efficiency of encapsulation into egg-yolk phosphatidylcholine liposomes than naked SV40 DNA. DNA and condensed DNA obtained with spermidine were even more

TABLE I

Efficiency of encapsulation of condensed and uncondensed SV40 DNA into REP liposomes[a]

Liposomes composition	Liposomes loaded with uncondensed SV40 DNA $\dfrac{\mu g\,DNA}{\mu mol\,lipid}$	Liposomes loaded with SV40 DNA condensed by spermidine $\dfrac{\mu g\,DNA}{\mu mol\,lipid}$	Liposomes loaded with SV40 DNA condensed by $Co^{3+}(NH_3)_6$ $\dfrac{\mu g\,DNA}{\mu mol\,lipid}$
PC	0.39 ± 0.07[b]	3.47 ± 0.28	$4.0\ \pm 0.30$
PC:PS:CHOL (4:1:5)[c]	0.50 ± 0.02	4.00 ± 0.20	0.01 ± 0.001

[a] The quantity of DNA incorporated into liposomes was determined as described in Methods.
[b] Standard deviation.
[c] Molar ratio.
(PC) phosphatidylcholine from egg-yolk; (PS) phosphatidylserine from egg-yolk; (CHOL) cholesterol.

efficiently encapsulated into liposomes containing egg-yolk phosphatidylcholine, egg-yolk phosphatidylserine and cholesterol, in agreement with Fraley *et al.* (1980); however, in the presence of condensed SV40 DNA obtained with $Co^{3+}(NH_3)_6$ these liposomes were scarcely formed and we observed a lot of amorphous aggregates probably composed by the condensed DNA and phospholipids. This effect was observed with the compacted forms of SV40 DNA obtained with 8.25 and 16.5 mM $Co^{3+}(NH_3)_6$.

4. Discussion

It is well known that nucleic acids are found in compact structures in native genomes. In viruses, nucleic acids are compacted by viral proteins and by polyamines (Laemmli 1975). In bacteria, DNA is compacted by non-histone proteins, polyamines, and RNA (Flink and Pettijhon, 1975). In eukaryotic cells, DNA is compacted into chromatin by histone and non-histone proteins, with polyamines being necessary for the organization of chromatin (Smirnov *et al.*, 1988). The condensation of nucleic acids is not only important in the packaging of these extremely long macromolecules, allowing them to fit inside cells or nuclei; but it also plays important roles in the regulation of the biological activity of nucleic acids (Smith, 1981; Duschak and Goldemberg, 1987).

Jay and Gilbert (1987) have theorized that in the origin of life, the first basic proteins could have helped package nucleic acids into lipid membranes. However, it is known that *in vitro*, tri or more multivalent cations such as the polyamines spermidine and spermin, and $Co^{3+}(NH_3)_6$ produce a compacted toroidal conformation of DNA (Gosule and Schellman, 1978; Widom and Baldwin, 1980) similar to the organization of DNA in some virus and bacteriophage heads (Allison *et al.*, 1981); consequently, it is plausible that in precellular conditions polyamines and $Co^{3+}(NH_3)_6$ could have helped package nucleic acids into lipid membranes.

Polyamines have an extremely simple molecular structure compared with proteins; therefore, their prebiotic synthesis could have occurred before that of proteins. Furthermore, polyamine complexes with DNA give some protective properties to DNA against X-ray and ultraviolet radiation damage, heat denaturation, and shearing (Bocian *et al.*, 1978; Johnson and Bach, 1966; Tabor and Tabor, 1984; Tikchonenko *et al.*, 1988) that probably could have been important on the primitive Earth. Besides, pBR322 DNA condensed with spermidine is transcriptionally more active than naked DNA (Baeza *et al.*, 1987b) suggesting that the enzymes acting on nucleic acids are designed to act on highly structured and compacted forms of nucleic acids. Then, we suppose that these compacted forms of DNA induced by polyamines may represent a primordial DNA genome, noy only with genetic properties, but also permitting a better encapsulation of DNA into precellular liposomes, in comparison with the longer and extended naked DNA. Since $Co^{3+}(NH_3)_6$ is a simpler DNA condensing agent than polyamines and proteins, in this study we compared some properties of the compacted forms of DNA induced

by both trivalent cations, $Co^{3+}(NH_3)_6$ and spermidine, since it is also probable that the interaction of $Co^{3+}(NH_3)_6$ with nucleic acids could have increased their genetic activities; then, the compacted forms of DNA induced by $Co^{3+}(NH_3)_6$ could also be condsidered as a primordial genome.

We demonstrated by Dubochet's method that more compaction of DNA was obtained with $Co^{3+}(NH_3)_6$ than with spermidine, because DNA-$Co^{3+}(NH_3)_6$ toroids were thicker and showed smaller holes than DNA-spermidine toroids. These results are in agreement with previous studies in which we demonstrated that an increase in spermidine concentration produces more compact DNA toroids with a concommitant reduction in the size of their holes (Baeza et al., 1987b). Furthermore, dissociation of DNA-$Co^{3+}(NH_3)_6$ toroids at higher ionic strength also produces non-condensed DNA, but with a thicker shape than DNA dissociated from DNA-spermidine toroids. These experiments suggest a higher interaction between $Co^{3+}(NH_3)_6$ and DNA than between the nucleic acid and spermidine. No irregular forms, suggesting precipitation of DNA by spermidine or $Co^{3+}(NH_3)_6$ were detected, indicating that DNA compaction by trivalent cations occurred without any nucleic acid losses.

DNA condensed with $Co^{3+}(NH_3)_6$ also showed resistance to the hydrolytic action of DNase I in a way similar to DNA condensed with spermidine (Baeza et al., 1987b); i.e., DNase I inactivity on compacted forms of DNA obtained with $Co^{3+}(NH_3)_6$ is due to the compaction of DNA by $Co^{3+}(NH_3)_6$ because free Co^{3+}, dissociated from the compacted forms of DNA by increasing the ionic strength, does not show any inhibitory effect on the enzymatic activity of DNase I; on the contrary, as spermidine, it exerts a stimulating effect on the enzyme. Furthermore, we confirmed the correlation between the presence of condensed forms of DNA and their insensitivity to DNase I as previously established with the DNA condensed by spermidine, which permit us to propose this simple biochemical method for the detection of DNA compaction induced by trivalent cations.

Some experiments have shown that DNA condensation by trivalent cations does not convert DNA into an inert molecule. Krasnow and Cozzarelli (1982) showed an increase in DNA ring catenation by DNA topoisomerases using ColE1 DNA condensed with spermidine or $Co^{3+}(NH_3)_6$ with regard to naked DNA. In addition, DNA condensed with spermidine is transcriptionally more active than naked DNA with the E. coli DNA-dependent RNA polymerase (Baeza et al., 1987b). We believe that in order to propose an agent as a possible prebiotic DNA condensing agent, the resultant compacted DNA must retain or increase its transcriptional properties; therefore, in this work we compared the transcriptional activity of compacted forms of DNA produced by spermidine or by $Co^{3+}(NH_3)_6$ with the E. coli DNA-dependent RNA polymerase. At the concentrations of trivalent cations that we used (7, 11.5, and 16.5) DNA condensed with spermidine was active in transcription; on the contrary, DNA condensed with $Co^{3+}(NH_3)_6$ does not show this property. An inhibitory effect of $Co^{3+}(NH_3)_6$ on RNA polymerase activity was discarded because at higher ionic strength [in which DNA is not compacted by $Co^{3+}(NH_3)_6$] the transcription

was similar to the one observed with naked DNA. Since pBR322 DNA remains condensed during transcription (monitored by electron microscopy) we supposed that the decrease in transcription was due to the higher DNA compaction detected in these condensed forms of DNA (Figure 1d). Furthermore, hexammine cobalt (III) is a trivalent cation with spherical symmetry, whereas spermidine has its three charges separated by propylene and butylene bridges; then, it is possible that they could interact with DNA in different ways thereby producing different DNA compaction. Probably, these distinct ways of DNA compaction could explain the low transcriptional activity of DNA condensed with $Co^{3+}(NH_3)_6$.

DNA compaction by spermidine or $Co^{3+}(NH_3)_6$ produces a reduction of 9 times on the pBR322 DNA or SV40 DNA perimeter; then, it is clear that the efficiency of encapsulation of condensed DNA increased 9 times over the encapsulation of naked DNA in liposomes formed from egg-yolk phosphatidylcholine. At the present time, we are using dipalmitoyl-phosphatidylcholine, which was synthesized under simulated prebiotic conditions (Rao et al., 1982), for the encapsulation of condensed DNA into liposomes. It is interesting that liposomes containing phosphatidylserine were scarcely formed in the presence of DNA condensed by $Co^{3+}(NH_3)_6$, suggesting that negatively charged liposomes in general will be formed with difficulty in the presence of DNA condensed with Co^{3+}.

The low transcriptional activity of DNA condensed by $Co^{3+}(NH_3)_6$ and its low encapsulation in negative liposomes, together with the low concentration of Co^{3+} in the environment, create serious objections in considering Co^{3+} as prebiotic DNA condensing agent and DNA condensed by $Co^{3+}(NH_3)_6$ as a possible primitive genome. Until now, condensed forms of DNA produced by spermidine have shown characteristics that lead us to consider them as a plausible prototype of primitive genomes in the origin of life, and that polyamines could have played the condensing role in the prebiotic encapsulation of nucleic acids into lipid membranes rather than Co^{3+} and basic proteins.

5. Prebiotic significance of polyamines

Polyamines are aliphatic amine metabolites occurring in millimolar concentrations in prokaryotic and eukaryotic cells. The most common are: 1, 3-diaminopropane $NH_2(CH_2)_3NH_2$; 1, 4-diaminobutane (putrescine) $NH_2(CH_2)_4NH_2$; 1, 5-diaminopentane (cadaverine) $NH_2(CH_2)_5NH_2$; spermidine $NH_2(CH_2)_3NH(CH_2)_4NH_2$ and spermine $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. These are small polycations at physiological pH that interact with nucleic acids and with other polyanions; such as, some proteins and some phospholipids (Tabor and Tabor, 1984).

Numerous studies have suggested an in vitro interaction between polyamines and nucleic acids. An increase in polyamines preceded the increase in DNA and RNA, suggesting the polyamines have a controlling or a stimulating effect on their biosynthesis (Boyton et al., 1980). Interruption of polyamine biosynthesis by mutations or inhibitors (Pegg, 1986) results in reduction or absence of cell growth,

with chromosome aberrations and alterations in nuclear morphology. Polyamines enhance DNA replication, and transcription, and RNA translation (Abraham and Pihl, 1981; Moussatché, 1985; Baeza *et al.*, 1987b) probably polyamines give a three-dimensional conformation to nucleic acids that produces an increase in their biological activities. Spermidine also enhances catalytic properties of RNA as was shown with the catalytic RNA subunit of *E. coli* RNase P (Guerrier *et al.*, 1983); this polyamine effect must be considered of primordial importance in origin of life studies, since RNA is considered the first informational molecule in the evolution of life (Bridosn and Orgel, 1980).

Polyamines also stabilize membranes and liposomes against lysis and they modulate the activity of several membranal enzymes, transbilayer transport mediate by lipids (Baeza *et al.*, 1990) and the bilayers fusion (Schuber, 1989). Furthermore, as liposomes containing RNA have been regarded as a possible model of precellular systems (Baeza *et al.*, 1987a), it seems plausible that polyamines could have been important in the stabilization and permeability properties of liposomes in precellular conditions.

The very simple molecular structure of polyamines, their roles affecting the conformation, packaging, encapsulation, and biological properties of nucleic acids; as well as their roles in the protection of DNA against X-ray, and ultraviolet radiations, heat denaturation, shearing, and nucleases activity; and their importance in the stabilization and permeability of liposomes; permit us to postulate that the interactions between polyamines and nucleic acids, and phospholipids could have arisen in precellular conditions. These precellular interactions could have played an important role in the evolution of life increasing nucleic acid activities, and stabilizing, and modulating precellular activities.

Polyamines could have arisen in prebiotic conditions from ornithine and arginine. Preliminary studies in our laboratory suggest that ornithine can be obtained by heating L-arginine at temperatures below 100 °C, and several laboratories are pursuing the prebiotic synthesis of basic amino acids including arginine, and in regard to another basic amino acid Shen *et al.* (1990) were able to demonstrate the prebiotic synthesis of histidine.

At the present time, we carried out the non-enzymatic synthesis of putrescine, the biological precursor of the polyamines spermidine and spermine, under possible primitive Earth conditions (Wong *et al.*, 1991) and we are trying to study the biological and catalytic properties of RNA-polyamine complexes, because RNA is considered to be a more ancient informational molecule than DNA.

Acknowledgments

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