PREBIOTIC SYNTHESIS OF NUCLEOTIDES

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Abstract. If an RNA-only world preceded more complex forms of life, then it is essential that the process whereby the first nucleotides were made be considered. Presumably there were no enzymes and no templates to facilitate the synthesis of the first nucleotides so another form of chemical evolution must have been involved. Answers to problems of this sort were sought vigorously in the 1960s and the early 1970s but many issues were left unresolved. Progress made in the last few years has added to this early work and brings us closer to a satisfactory solution. In this article key results, old and new, and some ideas as to how further progress is likely to be made are discussed. There are reasons for optimism. Substantial progress has been made on the synthesis of purines and ribose, phosphorylation and polyphosphorylation. The outstanding problems at this juncture relate to the synthesis of ribose to the exclusion of the other aldopentoses and to the problem of linking ribose to the purine bases.

Keywords: chemical evolution, formaldehyde, formose reaction, glycolaldehyde, hydrogen cyanide, nucleotides, phosphorylation, prebiotic synthesis, ribonucleic acid, ribose, trimetaphosphate

1. Introduction

The notion of an RNA-only world grew out of the discoveries made by Tom Cech (1981) and Sid Altman (1984) in the 1980s. Their observations demonstrated that RNA could form structures with enzyme activity. Prior to that time it was generally believed that only proteins possessed enzymic activity leading to the belief that nucleic acids and proteins must have coevolved. Skepticism over the RNA-only world hypothesis has arisen for a variety of reasons. Two major concerns are the difficulties in finding a feasible pathway to the first nucleotides and in finding a fitting way to replicate RNA. In this article the concern is with finding a feasible pathway to the first nucleotides. Such a pathway must satisfy the criteria for a prebiotic pathway. To be prebiotic, a pathway should employ chemicals and conditions that are likely to have been present around the time of the origin of life. Serious complications over this description have arisen because there is considerable disagreement on what the environment was like and how it varied form one locale to another. Our focus is on finding the simplest chemicals and the simplest conditions that could do the job in an anaerobic environment. The components that went into the first nucleotides may have originated from different regions of the prebiotic world. First, feasible reactions for synthesizing these components must be found.

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Then the means for assembling the components into fully formed nucleotides must be found. There are general concerns about the overall process that it is necessary to keep in mind. Most of these concerns relate to the chemical potential of the different components. For example, due to their greater stability the nitrogen bases were most likely to have been made first. They could linger without endangerment while conditions evolved under which ribose could be synthesized. Shortly after its synthesis ribose must be protected in some way from decomposition. At this juncture the question arises as to whether ribose became linked to one of the nitrogenous bases before or after phosphorylation. Our current view on this is that it was probably phosphorylated prior to becoming linked in such a way that the nucleoside formation step was bypassed in favor of nucleotide formation. Reasons for this view are given later.

There are other problems with respect to the location and timing of synthesis that are not dealt with in any great depth. The nitrogenous bases use HCN as their main precursor while ribose has a similar relationship to formaldehyde. These two building blocks (the nitrogenous bases and ribose) could not have been made at the same time and location because their precursor molecules, CH_2O and HCN, readily react with one another. This would have destroyed their potential to be useful in making the nucleotide building blocks although the products formed might have been useful in other ways such as the synthesis of amino acids.

Finally there is a concern over whether the first RNAs were chemically the same as contemporary RNAs. In considering alternatives it should be remembered that evolution proceeds in simple steps to increasingly complex assemblies. Abiding by this principle it seems unlikely that the first RNA contained all 4 bases found in contemporary RNAs. The notion that the first RNAs contained only two bases, adenine and hypoxanthine was originally suggested by F. Crick (1968); we have adopted this as a working hypothesis in our endeavors. The plausibility of a two base RNA is bolstered by A. Rich's observation (1958) that a stable helical duplex containing one polyadenylic acid chain and one polyinosinic acid chain can be made in aqueous solution. A complementary duplex containing adenylic acids and inosinic acids in both chains should be equally stable if not more so. These two purines can make a pair of hydrogen bonds with one another very similar to the two found between adenine and uracil in duplex RNAs. In spite of all this it has never been found that an all purine chain can template the syntheses of a complementary all purine chain under prebiotic conditions. Until this has been shown one must remain open to alternative possibilities.

The transition from a two purine RNA to a more conventional two purine-two pyrimidine RNA would have required many additional steps: First, a way to synthesize pyrimidines, second, a way to incorporate pyrimidines into nucleotides, third, a way to incorporate the pyrimidine nucleotides into polynucleotide chains, and closely related to this, a way to replicate the polypyrimidine chains. At the polymerization stage a polypurine chain could have served as the template for a complementary polypyrimidine chain. Transversions would have gradually redis-

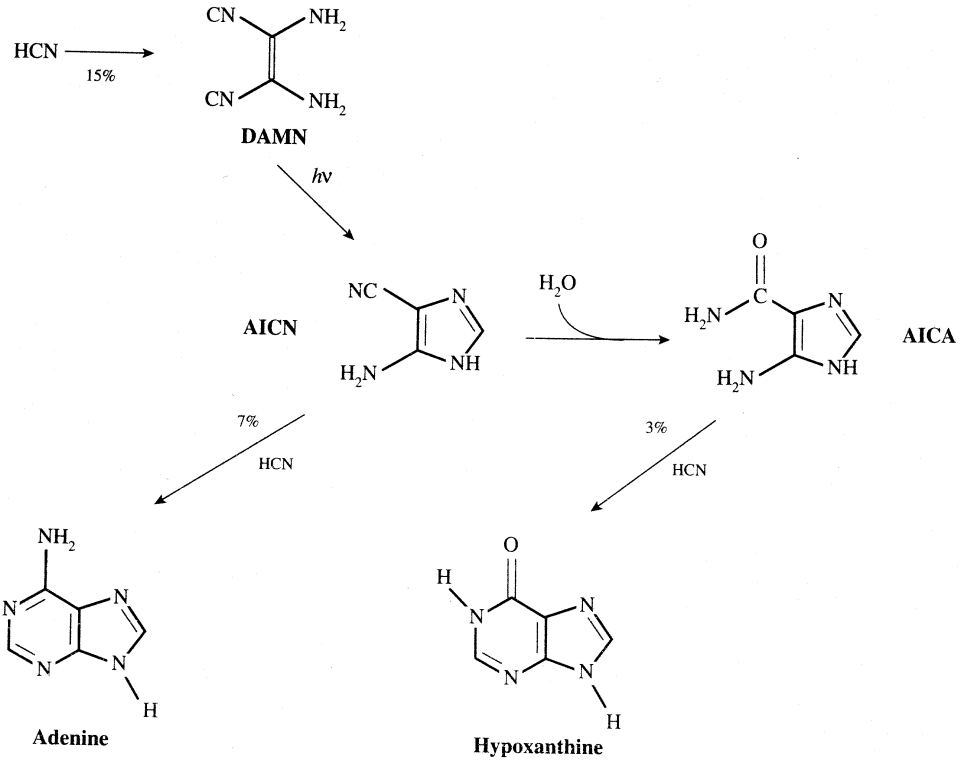

Figure 1. The steps in the prebiotic synthesis of adenine and hypoxanthine as they were formulated in 1989. Adapted from a review by Joyce (1989).

tributed the nitrogenous bases so that eventually both polynucleotide chains in a duplex contained approximately equal numbers of purines and pyrimidines.

With these boundary conditions in mind we turn to possible chemical routes for synthesizing the components and linking them together to form nucleotides.

2. A Prebiotic Pathway to the Purines

J. Oro (1961) discovered that when HCN was incubated in a sealed tube for prolonged periods of time adenine was formed. Considerable effort has been exerted since then to determine what happens at the various stages of this process and to find conditions for obtaining better yields. Figure 1 summarizes the progress made up to 1989 (Joyce, 1989). In this scenario, HCN synthesized in the atmosphere rained down to become concentrated in small bodies of fresh water by eutectic freezing. At subzero temperatures the concentrated solution of HCN slowly reacted to form diaminomaleonitrile (DAMN) in yields of about 15% based on the input HCN. Solutions of DAMN were exposed to solar radiation, which contained sufficient ultraviolet to rapidly and efficiently convert DAMN to aminoimidazole car-

bonitrile (AICN). By an unknown process partial hydrolysis of AICN to aminoimidazole carboxamide (AICA) took place and finally these two imidazoles served as the precursors for adenine and hypoxanthine, respectively. HCN was alleged to be a co-reactant in these two conversions. It is these final steps from AICN and AICA to adenine and hypoxanthine on which our attention was focused. This is because these conversions were inefficient in terms of yields despite the use of strenuous experimental conditions, which involved prolonged heating at elevated temperatures. The requirement for prolonged heating at elevated temperatures led us to suspect that HCN was probably modified before it could participate in the final steps to purine synthesis.

The late steps in the biochemical pathway to IMP suggested a more effective pathway from AICA to hypoxanthine. It was noticed that the eighth intermediate in the biochemical pathway (Figure 2) was none other than AICA linked to ribose-5′-phosphate (AICAR-5′-phosphate). In the ninth step this compound was formylated and in the tenth step the formylated intermediate was cyclized to inosine-5′-monophosphate (IMP), the end product of the pathway.

The late steps in the biosynthesis of IMP can be imitated by heating AICAR at 93 °C in a concentrated pool of ammonium formate HCOO-NH^{4+}. First the water evaporated, then the ammonium formate sublimed. The formate of the ammonium formate supplies the formyl group and the volatile reaction products pull the reaction making it thermodynamically favorable. After evaporation was completed the residue remaining was resuspended in water and analyzed by thin layer chromatography on cellulose using 0.5 M LiCl as the mobile phase. Following chromatography the ultraviolet absorbing components were detected on the dried thin layer paper with the help of an ultraviolet lamp (see Figure 3, cycle 1). The major product was inosine but considerable AICAR was still present. A small amount of hypoxanthine which probably resulted form hydrolysis was also seen. The yields could be improved by repeating the evaporation procedure (cycle 2). A second recycling (cycle 3) resulted in approximately 90% conversion to inosine. The same experiment was performed at 75 °C. At the lower temperature less than 50% conversion was observed after three cycles and a considerable amount of a faster moving component could be seen much more clearly (Figure 4). We suspect that this faster moving substance was the formylated intermediate since it is the only known stable intermediate in this conversion. The smaller extent of conversion seen at 75 °C indicates that this reaction has a high temperature coefficient for conversion.

The imidazoles AICN and AICA were efficiently converted into the two purines by the same procedure and the products were analyzed in the same way (Figures 5 and 6). The main difference was that more cycles were required to achieve the same efficiency of conversion that was observed in 4 cycles with AICAR. Another difference that was noted with AICA was that a more stable intermediate was produced. We cannot say if this intermediate has the same structure as the intermediate in the AICAR reaction because a different mobile phase was used

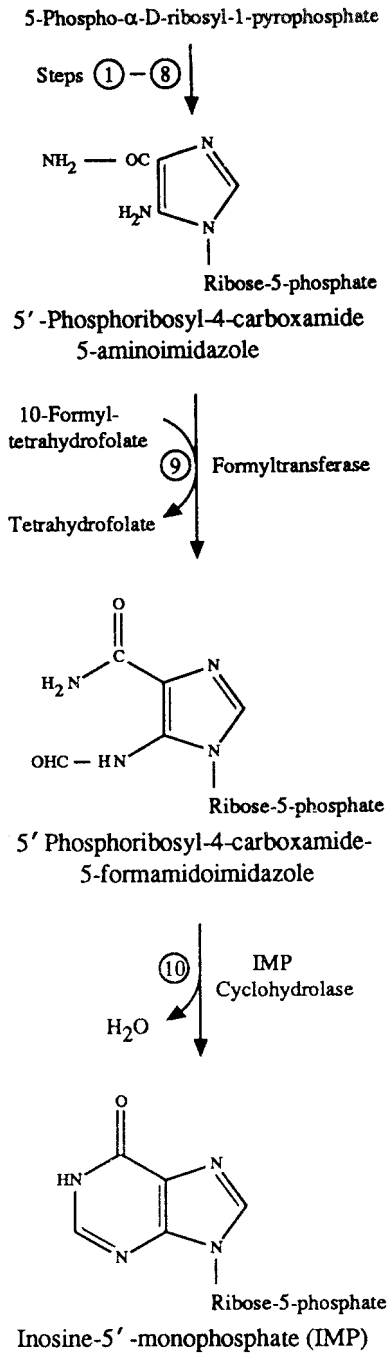

Figure 2. The last two steps in the *de novo* biochemical synthesis of inosine-5′-monophosphate in most cells.

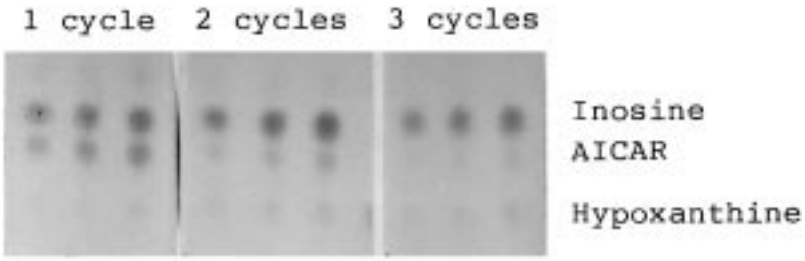

Figure 3. Chromatograms showing 3 cycle experiments resulting in the conversion of AICAR into inosine. In the first cycle increasing concentrations of AICAR (left to right, 0.5, 1.0 and 2.0 mg) were placed in 1 mL round bottom porcelain wells. The AICAR was dissolved in a solution containing 1 mL of water and 200 mg of ammonium formate. The solution was heated for 19 hr at 93 °C. During this time the water evaporated and the ammonium formate sublimed. The residue was resuspended in water and chromatographed on thin layer cellulose using 0.5 M LiCl as the mobile phase. The resulting chromatogram was blow dried and photographed with ultraviolet luminesence. It can be seen that the main product is inosine but there is still considerable AICAR present after the first cycle of heating. The system was recycled by adding the same amount of water and ammonium formate and carrying out the heating procedure as before. In the chromatogram labeled 3 cycles the recycling procedure was repeated again. After the third cycle it can be seen that almost all of the AICAR has been converted into inosine. A small amount of hypoxanthine that is seen must result from hydrolysis of the C-N bond linking ribose and hypoxanthine. In this and all other experiments leading to purine products the assignments have been confirmed by comigration studies with purine standards and ultra violet spectroscopy of eluted products in acidic, neutral and basic solutions.

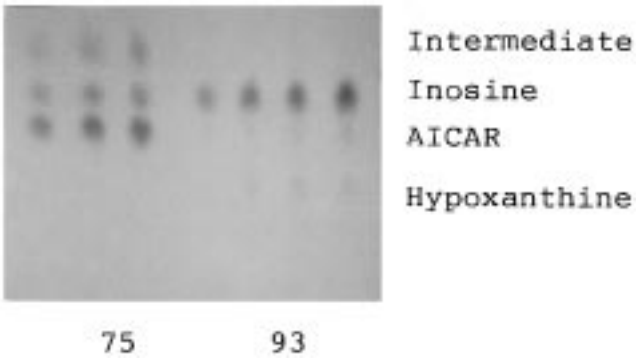

Figure 4. Chromatograms showing products of a three cycle experiment carried out at 75 and 93 °C. At lower temperature three different concentrations of AICAR were used (0.5, 1.0 and 2.0 mg). At 93 °C four concentrations were used (0.3, 0.5, 1.0 and 2.0 mg). It can be seen that the extent of conversion per cycle was less at the lower temperature. Also at the lower temperature an additional fast moving component can be seen. This was believed to be an intermediate in the conversion of AICAR to inosine as this component was eventually converted into inosine.

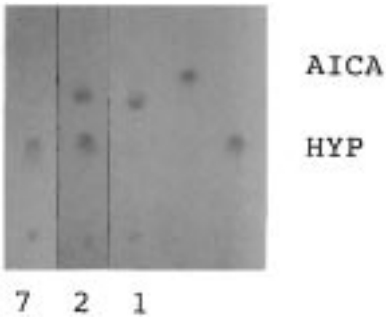

Figure 5. Chromatogram showing the first, second and seventh cycles of a seven cycle experiment in which AICA was converted into hypoxanthine. Also in the chromatogram to the right are shown columns containing pure AICA or pure hypoxanthine. The dried residues after incubation were resuspended in 0.5 mL of water and chromatographed on thin layer cellulose using a mixture of isopropanol, ammonia and water (7:1:2) as the mobile phase.

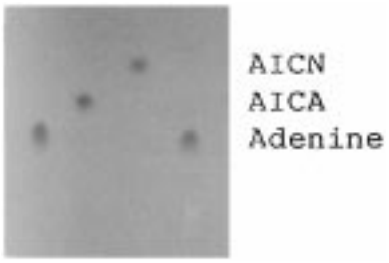

Figure 6. Chromatogram of the product of a four cycle experiment in which AICN was converted into adenine. Starting from the left, columns containing adenine, AICA and AICN and the product after four cycles of incubation of AICN are shown. Incubation conditions except for the number of cycles and post incubation treatment are the same as those described in Figure 5 for the AICA to hypoxanthine conversion.

in the chromatographic analysis (see legends for Figures 5 and 6). Infrared red spectroscopy revealed a strong absorption band at 1646 cm^{-1} in the intermediate that is not present in AICA. This is consistent with the presence of a unique formyl group in the intermediate as indicated in Figure 7.

The proposed steps in the conversions of AICA and AICN to the two purines are shown in Figures 7 and 8. The steps indicated for the conversion of AICA to hypoxanthine were identical to the steps observed in the biochemical conversion of AICAR-5′-phosphate to IMP (see Figure 2). The conversion of AICN to adenine was more complicated because of an additional ammonolysis reaction. In the AICN-to-adenine conversion formate and ammonium both were reactants. Remarkably both of these conversions were efficiently executed under the same conditions.

The question arises as to the origin of the ammonium formate used in these conversions. It seems likely that this arises from the hydrolysis of HCN. Whereas

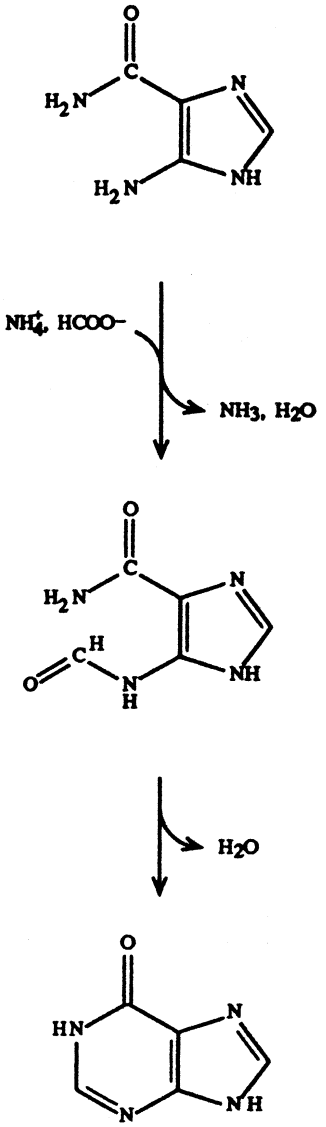

Figure 7. Proposed pathway for the conversion of AICA to hypoxanthine in our system.

HCN concentrates at subzero temperatures, in warm water HCN is susceptible to hydrolysis, first to formamide, and then to ammonium formate.

The re-cycling procedure which increases the overall efficiency of the conversions from AICA and AICN to hypoxanthine and adenine, respectively, might have taken place in small increments on a daily basis in a prebiotic world, daytime providing the hot-dry period of the cycle and night-time providing the cool moist part of the cycle.

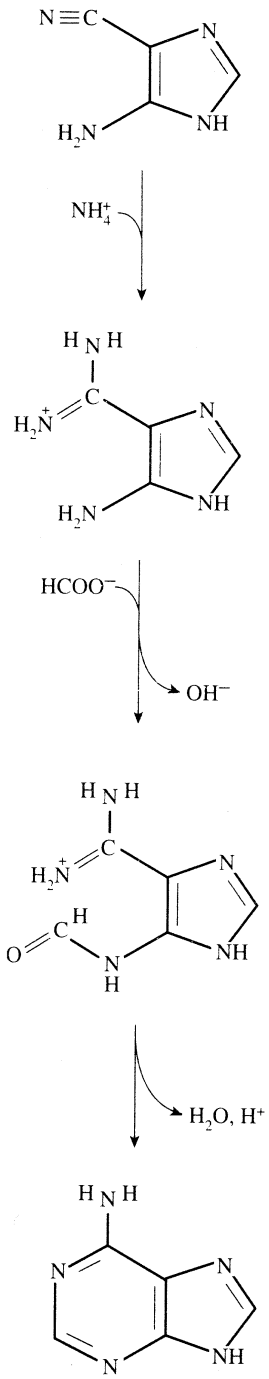

Figure 8. Proposed pathway for the conversion of AICN to adenine.

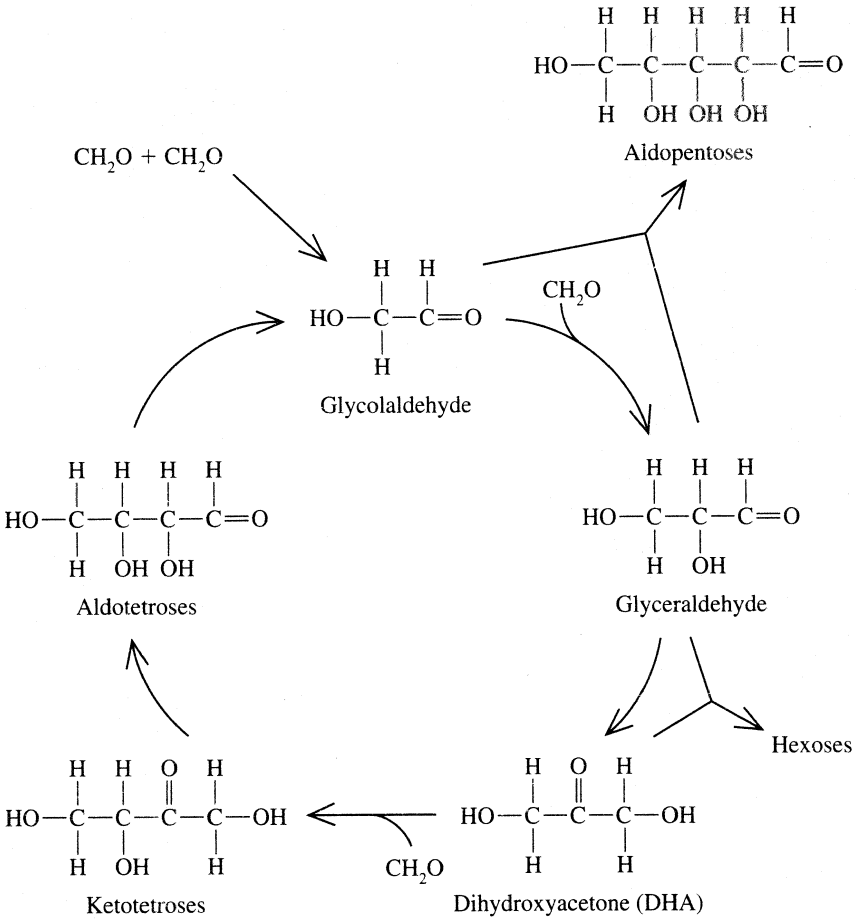

Figure 9. The glycolaldehyde cyclic pathway proposed by R. Breslow (1959).

If the overall pathway from HCN to the two purines is considered, seasonal fluctuations would constitute a more appropriate cycle. The winter season would be the best time for the conversion of HCN to DAMN as freezing is involved. The spring thaw with exposure to the ultraviolet rays of the Sun would be appropriate for the conversion of DAMN into AICN. Finally hot summer temperatures would be optimal for the partial hydrolysis of AICN to AICA and for the conversion of the two imidazoles into adenine and hypoxanthine, respectively.

3. Lead Salts Result in Increased Yields of Ribose in the Formose Reaction

The first observations relating to the prebiotic syntheses of ribose date back to 1861 when Butlerow showed that sugars could be made by mild heating of formaldehyde (Butlerow, 1861) in the presence of $Ca(OH)_2$ catalyst; this became known as the

formose reaction. Unfortunately the yields of ribose obtained in this way were very low. More recently it was found that an appreciable lag time which occurs before synthesis gets underway could be overcome by adding catalytic amounts of glycolaldehyde. Since glycolaldehyde was also believed to be an intermediate in the reaction this leads one to ask how a molecule that is an intermediate could also be a catalyst. Ron Breslow (1959) proposed a cyclic pathway to explain this (Figure 9). According to Breslow's scheme every glycolaldehyde that enters the cycle results in two glycolaldehydes being produced. This scheme received strong support from our finding that any of the five intermediates in the cycle showed the same catalytic effect as glycolaldehyde. Starting with glycolaldehyde the cycle displays an alternating pattern of aldol condensations and tautomerizations. The fifth and last reaction of the cycle is a reverse aldol condensation. Our analyses indicated that very few products outside of the cycle were synthesized until almost all of the formaldehyde had been taken up. There are many branchpoints from the cycle to other products. Only two of these branchpoints which are most relevant to our interests are shown. The diagram also suggests that two CH_2O molecules can react to make one glycolaldehyde. This reaction requires the combination of an organic catalyst and lead divalent cation (see below).

In most of our experiments 2.8% formaldehyde was used and incubations were done at 75 °C for varying lengths of time. The final products were analyzed by chromatography on thin layer cellulose paper with 88% phenol and 12% water as the mobile phase. After blow drying the thin layer chromatogram, residual phenol was removed by a very brief ethanol rinse. Finally the thin layer paper was stained with aniline hydrogen phthalate spray for sugar detection.

Despite the attractive features of the glycolaldehyde cycle, the reactions following the uptake of formaldehyde are numerous so that ribose constitutes only a minor product. The high pH (greater than 11.5) generated by a calcium hydroxide suspension permits too many unwanted reactions to take place. Because of this milder conditions for the synthesis of ribose were sought. To that end magnesium hydroxide suspensions which have lower pH were tried (unbuffered pH 10.3). At the lower pH generated by magnesium hydroxide, ribose once formed was much more stable. Unfortunately some of the reactions of the glycolaldehyde cycle were much slower at the lower pH. As a result, very small amounts of aldopentoses were formed from formaldehyde alone and only matching amounts were formed in the presence of glycolaldehyde. Thus glycolaldehyde did not function catalytically at the lower pH generated in a magnesium hydroxide suspension.

The frustration of this situation led to a search for additional catalytic agents. First reports that α-hydroxyacetophenone could catalyze sugar formation from formaldehyde were pursued (Mizuno and Weiss, 1974). When α-hydroxyacetophenone was added to a magnesium hydroxide suspension containing formaldehyde, it stimulated sugar synthesis but the yields were small and positive results required incubations of ten hours or more at elevated temperatures. Despite these limitations these were the first positive results observed at the reduced pH so a

TABLE I

The pKas for a number of divalent cation

Metal	pKa
Ca^{2+}	12.9
Mg^{2+}	11.4
Mn^{2+}	10.6
Co^{2+}	10.2
Cd^{2+}	9.6
Pb^{2+}	7.7

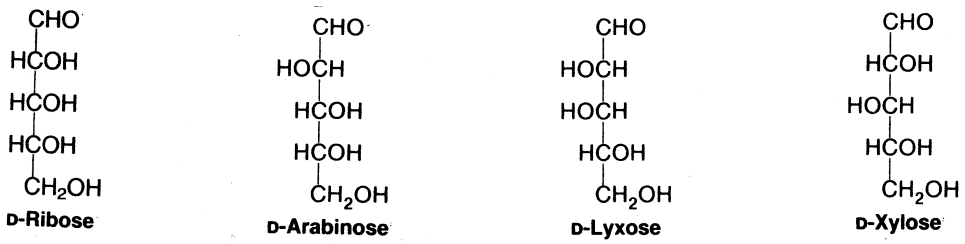

Figure 10. Fischer projections of the four aldopentoses shown in their straight chain form.

means for potentiating the catalytic action of α-hydroxyacetophenone was sought. An important clue came from a brief report by W. Langenbeck that lead hydroxide in conjunction with benzoin catalyzed the formation of glycolaldehyde from formaldehyde (Langenbeck, 1954). When lead nitrate (plumbous nitrate) was added together with α-hydroxyacetophenone the yields of aldopentoses from formaldehyde were increased by 20-fold over that found in the absence of lead. It was then possible to convert formaldehyde into aldopentoses with an efficiency of about 30%. Next it was found that α-hydroxyacetophenone could be replaced by any of the glycolaldehyde cycle intermediates. In fact most sugars could function as cocatalysts, and if one was willing to wait a few hours, no catalyst other than lead was needed.

Pb^{+2} is the only divalent cation that has been found with catalytic activity in the formose reaction. The low acid dissociation constant of Pb^{+2} may be a key factor in this unique characteristic of lead. Table I gives the pKas for some of the divalent metal cations that have been tried.

Starting from formaldehyde, ribose was the first aldopentose to appear in our synthetic system (see Figure 3 in Zubay, 1998). The question arose as to whether the other aldopentoses were derived form formaldehyde by *de novo* synthesis or as secondary products form ribose. Our opinion on this is that they may have arisen as

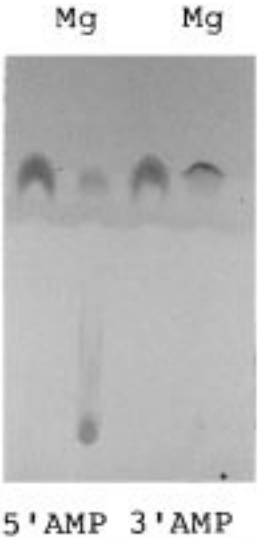

Figure 11. Chromatogram showing the products of a reaction involving sodium trimetaphosphate (0.5 mL of 0.2 M), a nucleotide (either the 5′- or 3′ nucleotide) of adenosine (10 mg), with or without magnesium chloride (0.2 mL of 1 M $MgCl_2$ or 0.2 mL of H_20) present. The solutions containing these components were allowed to evaporate at room temperature overnight. The dried residue was resuspended in water and chromatographed on polyethyleneimine thin layer cellulose with 0.2 M phosphate buffer, pH 3.4 as the mobile phase. In the absence of magnesium chloride the two nucleotides appear to be unaltered. However if magnesium chloride was present in the original solution the 5′ derivative was converted to a form which does not migrate. Additional studies indicated that this is the nucleoside-5′-tetraphosphate. In the case of the 3′-derivative where magnesium chloride was present the crescent shape peak observed is characteristic of the 2′,3′- cyclic derivative (see Riemann and Zubay, 1999).

secondary products. This is based on their kinetics of appearance and interconversion. Under our standard incubation conditions ribose was first detected at about 140 min, then arabinose at about 180 min, and finally, lyxose and xylose at about 240 min. This is the order that might be expected if arabinose, lyxose and xylose arise as secondary products. Thus arabinose which appeared shortly after ribose, could be made from ribose by a simple tautomerization involving the anomeric C1 carbon and the adjacent C2 carbon. But lyxose and xylose which appeared substantially later could only arise from ribose or arabinose by more complex changes involving the middle carbon C3 (the structures at the four straight-chain aldopentoses are shown in Figure 10). Further evidence documenting the presence of interconversions comes form the finding that when any one of the aldopentoses was subjected to standard incubation conditions for ribose synthesis, a pattern containing all four aldopentoses appears in the stained chromatogram (see Figure 4 in Zubay, 1998).

4. Scant Progress Has Been Made on the Synthesis of Nucleosides From Purines and Neutral Ribose

Limited success has been achieved in the synthesis of inosine from hypoxanthine and neutral ribose by dry heating in the presence of $MgCl_2$ (Fuller *et al.*, 1972). The situation for adenosine is much worse. In fact the major products formed on dry heating of adenine and ribose are adducts involving the C2 carbon of ribose and the amino group of adenine. For several years we have tried in vain to improve this system by making modifications in composition and in the manner of treatment. This has lead us to the belief that there is something inadequate with our starting materials or with the reaction conditions or both.

5. Attempts to Synthesize Nucleotides Directly From Purines and 5′-phosphoribosyl-1′-pyrophosphate

Since 5′-phosphoribosyl-1′-pyrophosphate (pRpp) is commonly used in the biosynthetic pathway for nucleotides, it is surprising that it does not appear to have been tried in prebiotic experiments.

A priori, pRpp has two obvious advantages over ribose as a coreactant: the pyrophosphate makes the C1 carbon of the sugar more attractive for nucleophilic attack and subsequent reaction; and the covalent linkage to phosphate takes the potential aldehyde function of ribose out of circulation.

In preliminary experiments we have been successful in condensing inosine and pRpp to form a product that comigrates with inosine 5′-monophosphate. The yields for this conversion are 10–20% based on the hypoxanthine input. Further experiments are necessary to find optimum conditions for the reaction and to verify the structural assignment.

6. Polyphosphorylation of Nucleotides Requires Trimetaphosphate

Finally turning to the phosphorylation problem, the chemistry for adding the first phosphate to ribose or a nucleoside, and then adding additional phosphates to make a polyphosphate, is quite different.

Lohrmann and Orgel (1971) have shown that a nucleoside in an aqueous solution containing urea and inorganic phosphate and then evaporated to dryness at elevated temperatures (70 to 100 °C) can add phosphates at the 5′ and 3′ locations. We have found that a wide range of organophosphate compounds can serve as phosphate donors (Riemann and Zubay, 1999). Some of these, especially those bearing high energy phosphates such as acetyl phosphate or phosphoenol pyruvate make effective donors at lower temperatures than does inorganic phosphate and some (e.g., 3′-phosphoglyceric acid) can function in the absence of urea. If the nucleotide synthesis experiments with pRpp described in the previous section prove to

be successful then we will be compelled to search for conditions that can be used in the phosphorylation of ribose to pRpp. Some of the phoshorylation experiments we are doing now may be quite helpful in this regard.

Lohrmann was the first to show that trimetaphosphate is an excellent reagent for making nucleoside-5′-polyphosphates (Lohrmann, 1977). We have confirmed and extended the findings of Lohrmann, and showing that it is not necessary to have precise humidity control during the dehydration phase of the experiment as long as there is a slow transition from the solution state to the dry film state. Our findings confirm Lohrmann's that nucleoside-5′-polyphosphates can be formed at room temperature from nucleoside-5′-monophosphates in evaporates containing sodium trimetaphosphate if Mg^{+2} is present. It was also found that nucleoside-3′-monophosphates form 2′,3′-cyclic compounds under the same conditions (Figure 11).

7. Concluding Remarks

Some skepticism about the RNA-only world hypothesis has arisen over the failure to find a feasible prebiotic pathway to the first nucleotides. In recent years substantial inroads on finding acceptable answers to the remaining problems in this pathway have been made. A feasible pathway to the purines exists. Effective conditions for phosphorylation and polyphosphorylation have been found although the availability of phosphate in the proper form for these reactions could be disputed. Substantial progress has been made on ribose synthesis. The one area where little progress has been made is on joining ribose to the nitrogenous bases.

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