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PROTOBIOCHEMISTRY: DEHYDRATION CONDENSATION IN AQUEOUS SOLUTION

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Special Thesis

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# PROTOBIOCHEMISTRY: DEHYDRATION CONDENSATION IN AQUEOUS SOLUTION

Gary David Steinman

(Ph. D. Thesis)

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1.
At moment X
the universe began.
It began at point X.
Since then,
through the Hole in a Nozzle,
stars have spewed. An
inexhaustible gush
populates the void forever.

2.
The universe was there
before time ran.
A grain
slipped in the glass:
the past began.
The Container
of the Stars expands;
the sand
of matter multiplies forever.

3.
From zero radius
to a certain span,
the universe, a Large Lung
specked with stars,
inhales time
until, turgent, it can
hold no more,
and collapses. Then
space breathes, and inhales again,
and breathes again: Forever.

– May Swenson, "Models of the Universe"
in Poetry in Crystal, Steuben Glass,
Spiral Press, New York, 1963.

## ABSTRACT

The results reported from many laboratories over the past decade make it clear that monomers necessary for biogenesis could have been formed under conditions believed to have existed on the primitive Earth. There remains the problem of finding reasonable mechanisms by which the biological monomers may have condensed in aqueous solution to produce more complex compounds, such as phosphates, acetates, pyrophosphates and peptides.

It was proposed and experimentally demonstrated here that dicyandiamide may have played a role in the types of condensations occurring on the primitive Earth. In dilute, aqueous, acidic solution, dicyandiamide is able to promote the synthesis of a variety of phosphate esters and phosphate anhydrides (see text for complete listing). The clay mineral, kaolin, was found to enhance dicyandiamide-mediated phosphate anhydride synthesis by surface catalysis. In addition, dicyandiamide successfully leads to the production of di- and tripeptides as well as the lipid-like compound, glycerol acetate.

In an attempt to find compounds which might act as more effective dehydrating agents than dicyandiamide, the possible use of dicyanamide was investigated. It was shown that the latter compound provides better yields in a number of syntheses of peptides and phosphates than the former reagent. That cyanamides could have been produced under primitive Earth conditions has been demonstrated.

An extensive study was made of the mechanism of dipeptide synthesis promoted by dicyanamide. In the case of glycine, it appears that reactivity increases with increasing length of the participating peptides. The yields of the reaction are proportional to the initial concentrations of the amino acid, dicyanamide, and HCl. The dipeptide bond formation is effected by the production of the corresponding urea of the condensing agent. Although the reaction goes best at low pH (1-2), due to the protonation of dicyanamide anion and the carboxylate group of the participating amino acid, it gives detectable yields under neutral conditions.

The synthesis of dipeptide is restricted from going to completion by the amine-catalyzed dimerization of the dicyanamide. The latter reaction apparently utilizes the amino addition compound, a substituted cyanoguanidine, formed from glycine and dicyanamide as its intermediate. Divalent cations fail to prevent this dimerization of dicyanamide, but the gradual addition of condensing agent to the reaction mixture permits higher yields of polypeptides. The appearance of an acylurea side product supports the suggestion that the reaction operates through an acylisourea intermediate, like the cartodiimides.

It is significant to note that the syntheses discussed here have been restricted to the conditions of simple, relatively stable reactants, moderate temperature, and dilute, aqueous solutions. In accordance with the hypothesis set up for this study, it is believed

that these circumstances reflect prebiotic events and demonstrate one means by which essential biochemicals could have been formed on the primitive Earth, thus providing materials needed for the origin of living systems.

# HISTORICAL REVIEW AND THEORETICAL CONSIDERATIONS

## Chemical Evolution

Once the primordial source of matter and energy has been postulated (see Appendix II), one is in a position to consider possible explanations for the ultimate appearance of life itself. In any consideration of the primordial origin of biological life, the means for the synthesis of biochemically significant compounds must first be established. Chemical evolution may be defined as that period in the Earth's history preceding the appearance of living systems in which the compounds necessary for the subsequent development of organisms were synthesized. In discussing possible solutions to this problem, the available sources of energy, the types of compounds probably at hand, and the general environmental conditions, such as atmosphere, temperature, and relative abundance of water, must be fully taken into account. In attempting to develop further insight experimentally into this problem, one must assume that primordial events can be re-enacted and recreated, with the limitation of less time, in the laboratory, i.e., that observations made in the test tube may indeed reflect prominent events that took place on the young Earth. Secondly, it is assumed that the course of primordial events went from the simple to the complex, that the first available reactants were simple in constitution. Thus, for example, proteins would not be expected to have appeared before appropriate reactions would have brought about the synthesis of amino acids from very simple nitrogen and carbon compounds.

Evolution of Earth

Although it was originally believed that the Earth originated as a fiery offshot from the Sun, the theory of the steady-state universe (8,8a) is now taken more seriously. This theory suggests that there is a continual dissipation and gravitationally-promoted reaggregation of stellar material. Such a concept could be used to explain the origin of the Earth. The dust of space consists of hydrogen (90%) plus small amounts of helium, neon, water, methane, ammonia, and various oxides, sulfides, carbides and silicates. The low gravitational attraction of the forming protoplanet allowed the lighter gases to gradually escape, giving rise to a secondary atmosphere later. Ultraviolet light from the Sun caused the photolysis of water into hydrogen and oxygen. The oxygen was probably picked up by reduced substances on the surface of the protoplanet giving oxidized minerals. Nitrides reacted with hydrogen and gave ammonia, sulfides gave $H_2S$, and carbides yielded various hydrocarbons. Therefore, a secondary atmosphere was formed consisting of greater amounts of methane, ammonia, and water in particular, as well as hydrogen.

The presence of carbon dioxide and water and other volatile gases but the absence of inert gases may be due to the fact that the substances listed first were chemically bound to the young planet (9). It has been suggested that later mild heating by radioactive sources such as uranium, thorium, and potassium, as well as gravitational compression, caused low melting materials to rise to the surface of the planet. The composition of volcanic gas, which may give some indica-

tion of the composition of the primitive atmosphere, is mainly water vapor and also has carbon dioxide, carbon monoxide, hydrogen chloride, hydrogen fluoride, hydrogen sulfide, nitrogen, ammonium chloride, and some methane. The acids in the vapor phase probably came into equilibrium with the aqueous phase on the Earth's surface and were thus drawn out of the atmosphere into solution.

The primordial abundance of hydrogen-containing compounds is also suggested by observations made of the atmospheres of other planets and the Sun. For example, the Sun is 87% hydrogen, 12.9% helium, 0.025% oxygen, 0.02% nitrogen, and 0.01% carbon (10). Methane and ammonia have been observed on other planets such as Jupiter, Saturn, Uranus, and Neptune (11). It has also been argued (11) that if much hydrogen were present in the primitive atmosphere, methane and ammonia would be thermodynamically favored. It is possible that carbon dioxide was removed from the early atmosphere by minerals which absorbed it and formed insoluble carbonates (11). The lack of krypton and xenon retention suggests that accumulation of material used in formation of the Earth took many years, thus suggesting a moderate temperature due to slow radiation of gravitational energy into space (12).

Several sources of energy were probably available to carry on reactions. First, and foremost, was ultraviolet light of wavelengths less than 250 mμ, which probably contributed as much as 570 calories per square centimeter per year (10). Electrical discharge may have given as much as 4 cal/cm$^2$/yr, radioactivity 0.8, and volcanoes maybe 0.13. As much as 5% of the Sun's emitted energy is in the ultraviolet (13).

This energy can cause hydrogen, methane, ammonia, and water vapor to give such reactive species as methyl, methylene, hydrogen and hydroxyl radicals. Ultimately, with the appearance of oxygen, ozone was produced and acted as a shield from UV irradiation. This ended simple evolution and started complex evolution.

Three general stages of the Earth's evolution can be outlined (14):

1) The first period began about $4.5 \times 10^9$ years ago and lasted $5 \times 10^8$ years (this figure is open to debate) after the Earth's formation. The atmosphere was highly reducing. Most chemical evolution occurred at this time. The absence of oxygen made ultraviolet radiation a significant force.

2) Next, the atmosphere became neither strongly reducing nor strongly oxidizing. Carbon dioxide replaced methane and nitrogen took the place of ammonia.

3) The final stage was the present oxidizing atmosphere. Oxidation states of uranium minerals suggest that the transition from the second to the third stage occurred about $1.8 \times 10^9$ years ago.

The fundamental modern theory of chemical evolution was first promulgated by A. I. Oparin in 1924 and was expanded in 1938 (15). Similar speculations were presented by J.B.S. Haldane in 1929 (16). Oparin's ideas followed from the theory of fiery origin of the Earth proposed by Jeans and thus considered high temperature reactions. For example, at elevated temperatures calcium carbide and water give hydrocarbons, methane yields acetylene and hydrogen, and calcium carbide

and nitrogen produce calcium cyanamide. Further reactions could give rise to alcohols, aldehydes, ketones, organic acids, and so forth. Oparin further suggested that the resultant organic suspension could lead to the formation of coacervates which would offer a delimitation from the surrounding environment. The main contributions of this theory were the suggestion of the reducing atmosphere, the possible role of the coacervate, and, most important, the idea that the course of events necessary for the ultimate appearance of biological systems could be explained by the laws of chemistry and physics and are subject to laboratory verification. However, it has been argued (17) from thermodynamics that the concentration of products formed under the conditions proposed by Oparin could never have reached a high enough level because energy sources would also favor destruction and small equilibrium constants. Thus, moderate-temperature chemistry would probably hold the key to elucidating the actual course of chemical evolution. Also, in considering this problem, one must be on guard not to transfer present systems unchanged backward to simpler levels in order to define earlier systems rather than to consider all pertinent factors and extrapolate along the line of evolution (18).

Amino Acids

A great deal of work has been done to determine how amino acids could have been synthesized during chemical evolution. One of the earliest experiments synthesized glycine by submitting a gaseous mixture of carbon monoxide, ammonia, and water to a silent electrical discharge (19). In the same year irradiation of an aqueous solution of potassium nitrite, ferric chloride, and carbon monoxide with ultraviolet light

gave a mixture of amino acids (20). Soon, a similar irradiation of an aqueous solution of formhydroxamic acid yielded amino acids and other organic compounds (21). Ultraviolet irradiation of glycol plus nitrate and titanium oxide produced amino acids (22). Serine, aspartic acid, valine, lysine, and other amino acids were synthesized when sunlight was directed at an aqueous solution of paraformaldehyde, potassium nitrate, and ferric chloride (23). With time of illumination, some amino acids disappeared and others appeared. Similarly, when a 500 watt electric bulb was used to irradiate an aqueous suspension of colloidal molybdenum oxide and paraformaldehyde for twenty-five days, amino acids were synthesized (24). Apparently nitrogen had been extracted from the air.

One of the most significant experiments, since it initiated great interest in the field of chemical evolution and directly tested some of Oparin's speculations, was carried out in order to synthesize amino acids under presumed primitive Earth conditions and involved passing an electrical discharge through a gaseous mixture of methane, ammonia, hydrogen, and water (25). Analysis by column and paper chromatography indicated that glycine, alanine, sarcosine, β-alanine, α-amhobutyric acid, formic acid, acetic acid, propionic acid, glycolic acid, and lactic acid had been produced. It was suggested that the amino acids were synthesized by a Strecker reaction since ammonia was available and the presence of aldehydes and cyanide was observed. The appearance of β-alanine was attri-

buted to a Michael addition of ammonia to acrylonitrile. To cooroborate the suggestion that the spark experiment operates by a Strecker mechanism, a mixture of hydrogen, ammonia, hydrogen cyanide, formaldehyde, acetaldehyde, and propionaldehyde was boiled for a week (26). In this case glycine, alanine, α-aminobutyric acid, glycolic acid, lactic acid, α-hydroxybutyric acid, iminodiacetic acid, and imino-acetic-propionic acid were identified as products, as before.

The possible significance of lightning as an important catalytic force during chemical evolution had been suggested earlier (27,28). The spark experiment was repeated (26). This time, additional analysis also indicated the production of N-methylalanine, aspartic acid, glutamic acid, iminodiacetic acid, and imino-acetic-propionic acid, as well as α-hydroxybutyric acid, succinic acid, urea, methylurea, and some undefined polymer. Evidence for a Strecker reaction was also noted by following the time course of appearance and disappearance of cyanide, aldehydes, ammonia, and amino acids during the sparking process. In the early stages of the synthesis cyanide, aldehydes, and amino acids increased while ammonia decreased. As the reaction proceeded, the ammonia concentration continued to decrease while the amount of aldehydes and of cyanide also began to decrease, even though amino acids continued to appear. When nitrogen replaced ammonia in the original reaction mixture, the same products were observed but in lower yield. With oxygen present, no products were noted. In these experiments, no purines or pyrimidines were identified.

The spark experiment was repeated using gaseous mixtures of

1) carbon dioxide, nitrogen, hydrogen, and water
2) carbon monoxide, nitrogen, hydrogen, and water
3) carbon dioxide, ammonia, hydrogen, and water
4) methane, ammonia, and water.

In each case, amino acids were synthesized (29). These results were corroborated, and were extended by adding hydrogen sulfide to a methane-ammonia-water mixture (30). Upon the application of electrical discharge, ammonium thiocyanate was observed among the products.

It has been argued that the reaction of methane, ammonia, and water to produce alanine involves a change in free energy of +62 kilocalories per mole (31). On the other hand, the synthesis of alanine from methane, carbon monoxide, and ammonia would involve a free energy change of -5.9 kilocalories per mole (31). Using the latter gaseous combination, alanine synthesis was demonstrated in the presence of electrical discharge, as well as the synthesis of glycine, β-alanine, α-aminobutyric acid, aspartic acid, and glutamic acid. This would also suggest that free hydrogen was apparently not necessary in the reaction.

The use of nitrogen sources other than ammonia or molecular nitrogen in amino acid production was investigated. Aspartic acid was synthesized when urea was heated with malic acid (32). Glutamic acid resulted when urea was heated with α-hydroxyglutamic acid (33). Also, heating glucose and urea together gave glycine.

The use of other forms of energy to promote amino acid synthesis has also been considered. For example, when dry ammonium carbonate was irradiated with γ-rays, ammonium formate, glycine, and alanine were produced (34). X-ray irradiation of various mixtures of methane, acetylene, hydrogen, water, nitrogen, and ammonia produced ninhydrin-positive material (35). When a mixture of methane, carbon monoxide, ammonia, and water was bombarded with the short wavelength energy from a hydrogen lamp, several amino acids were produced (36). The β irradiation of ammonium acetate in water led to the synthesis of glycine and aspartic acid (37). The β radiation was produced by a van de Graaff electron accelerator. An amide radical and a carboxymethyl radical were believed to be key intermediates. Ultraviolet irradiation of an aqueous solution of formaldehyde and ammonium nitrate produced serine, glycine, glutamic acid, alanine, valine, isoleucine, phenylalanine, and some basic amino acids (31). Similarly, merely refluxing an aqueous solution of formaldehyde and hydrazine led to the synthesis of glycine, valine, and lysine (38). The possible primordial origin of hydrazine has been demonstrated with electron bombardment of ammonia (39).

It is possible to cite several more examples of amino acid synthesis under possible primitive Earth conditions and such a survey is necessary since together these demonstrations form a pattern which helps to elucidate the probable prebiotic origin of these compounds. For example, amino acids were produced when hydroxy

acids, keto acids, and dicarboxylic acids were irradiated with ultraviolet light in the presence of ammonia or ammonium salts (40,41). This emphasizes the role of reduced nitrogen and also supports the contention that amino acid synthesis did not have to be restricted to a gaseous reaction. Similarly, when hydroxylamine was heated for fifty hours at 80-100° C with formaldehyde, amino acids were produced (42). Cyanide and ammonia were apparently important intermediates. The importance of cyanide in the whole scheme will be discussed further.

That hydrocarbons other than methane were possibly important in amino acid synthesis was shown by irradiating a mixture of ethane, ammonia, and water with short wavelength ultraviolet light (43). The products included glycine, alanine, α-aminobutyric acid, formic acid, acetic acid, propionic acid, and some liquid hydrocarbons. Methyl radicals were probably formed from the ethane. Most yields were increased if the reaction was mercury sensitized. This suggestion of methyl radical participation was corroborated by replacing methane in a methane-ammonia-water atmosphere with methanol (44). With electron discharge, the yield of amino acids was increased 50%. This is understandable since it takes 20% less energy to form methyl radicals from methanol than methane (45). A gaseous mixture of methane, ethane, ammonia, and water, upon electron discharge, yielded glycine, alanine, aspartic acid, asparagine, isoleucine, glycinamide (the significance of this compound will be discussed later), proline, an insoluble yellow polymer, and several

ultraviolet-absorbing compounds which had the spectral properties of purines and pyrimidines (46). Similar results were observed when a high energy electron linear accelerator was employed (47).

The fate of the methane was followed by using a $^{14}C$-labeled methane-ammonia-water mixture and bombarding it with β-particles from an electron beam linear accelerator (48). At the end of the experiment, carbon monoxide, carbon dioxide, ethane, ethylene, cyanide, urea, $C_3$-$C_7$ hydrocarbons, glycine, alanine, and lactic acid were identified. Urea synthesis was inhibited by phosphine. The production of sugars was suggested. The origin of β-radiation on the primordial Earth may have been $K^{40}$.

Irradiation of various compounds in aqueous solution apparently allows reactions which are prohibited under similar conditions in the gaseous phase. For example, by passing an electrical discharge into an aqueous solution of acetic acid, it was possible to synthesize glycine in the presence of air, which is 20% oxygen (49). The amino group was apparently synthesized from molecular nitrogen and water. Also, sparking an aqueous solution of glucose resulted in the production of glucosamine.

From the examples cited thus far it may be noted that since many different laboratory systems give essentially the same products, many answers are possible for defining plausible primordial conditions. However, certain characteristics may be concluded to be shared in common with most of these demonstrations. First of all,

any one of a number of energy sources could be used to promote the desired syntheses. Secondly, carbon and nitrogen in any one of several oxidation states could have served as reagent sources. Also, the required reactions couldhave taken place in the gaseous, aqueous, or even solid phase and probably involved all three. However, the most important point to be discerned is the central role of cyanide in these reactions. Recognizing this, certain experiments have been carried out which use cyanide as a reagent or as a known intermediate of other reactions.

Function of Cyanide

First of all, it has already been pointed out that during the electron irradiation of a methane-ammonia-water atmosphere much hydrogen cyanide is produced (26). Thus, when hydrogen cyanide was heated to 70° C for twenty-five days in 3 normal ammonium hydroxide, glycine, alanine, and aspartic acid were produced (50). When such a reagent mixture was heated at 90° C for eighteen hours, hydrolyzed, and analyzed more extensively, it was possible to also identify threonine, serine, glutamic acid, isoleucine, leucine, β-alanine, β-diaminopropionic acid, α-aminobutyric acid, guanidine, and urea (51). Seveal other compounds, both ninhydrin-positive and negative, remained unidentified. There was also some evidence for the presence of a polymer-like product.

Since it was known (52) that heating ammonia and methane over alumina or silica produces hydrogen cyanide, a gaseous mixture of methane, ammonia, and water as passed over silica heated to 900-1000° C and then trapped in ammonium hydroxide (53). Several amino acids were identified in the product mixture. The reaction apparently involved a cyanide intermediate.

Heterocyclic Bases

Heating aqueous solutions of ammonia and cyanide also led to the synthesis of adenine, guanine, and hypoxanthine (51,54,55). In the adenine synthesis, 4-aminoimidazole-5-carboxamide was also identified, thus giving a good idea of the mechanism of the reaction (55,56). (This compound, as well as glycine, acts as an intermediate in the biological synthesis of adenine.) Other products of this reaction were 4-aminoimidazole-5-carboxamidine, formamide, formamidine, glycinamide, and some ultraviolet-absorbing compounds. The reaction of guanidine with aqueous ammonia and 4-aminoimidazole-5-carboxamide (AICA) yielded guanine (57). The reaction of AICA with urea produced guanine and xanthine. Acrylonitrile, β-aminopropionitrile, and β-aminopropionamide are considered to be possible intermediates in the electron discharge synthesis of β-alanine described earlier. When any one of the first three compounds was heated with aqueous ammonia and urea at 130° C, uracil resulted (57).

By passing an electron beam through a gaseous atmosphere of methane, ammonia, and water, hydrogen cyanide was produced (58). Likewise, under these conditions, using $^{14}C$-labeled methane as one of the reagents, adenine was also identified (59).

With the ultraviolet irradiation of an aqueous solution of hydrogen cyanide for seven days, the final mixture contained 1% adenine, 0.5% guanine, and 10% urea (60). By promoting the reaction between malic acid and urea with polyphosphoric acid, uracil was synthesized (61). When an anhydrous mixture of two parts glutamic acid, two parts

aspartic acid, and one part an equimolar mixture of sixteen other natural amino acids was heated to 170° C, guanine was produced (62).

Thus it may be seen that means have been devised by which most of the biologically essential heterocyclic bases could have appeared on the primitive Earth. Again the role of cyanide has been emphasized. To ultimately elucidate the origin of such important compounds as DNA and RNA, as well as polysaccharides, the appearance of sugars must also be considered.

Sugars

It has been shown some time ago that formaldehyde could play a central role in the synthesis of sugars (63). When this reagent was placed in an alkaline solution, numerous carbohydrates resulted. When an aqueous solution of formaldehyde was irradiated with ultraviolet light, various hexoses and hydroxy acids were produced (64,65). When the product was heated to 100° C, a polymeric substance resulted which was probably furfurylalcohols or polyhydroxyphenols. Such compounds could conceivably have acted as precursors to aromatic amino acids.

By passing an electric spark through a gaseous mixture of methane, ammonia, and water, formaldehyde was produced (66). This type of experiment also produced ribose and 2-deoxyribose (67). Formaldehyde was also synthesized by sending an electrical discharge into an aqueous solution of methanol (68).

When an aqueous solution of formaldehyde was irradiated with ultraviolet light or $\gamma$-rays from a $Co^{60}$ source, various pentoses and hexoses were synthesized (67). With the UV experiment, ribose and

deoxyribose were produced, in particular. By heating to 50° C salt solutions containing formaldehyde and acetaldehyde, or glyceraldehyde and acetaldehyde, a 5% yield of 2-deoxyribose was observed (69)

These experiments therefore demonstrate that carbohydrates could be readily synthesized by conditions believed to have existed on the primitive Earth. They also show that formaldehyde probably played a central role in the synthesis of the sugars.

Nucleotides and Phosphorus

By irradiating an aqueous solution of adenine, ribose, and phosphate with ultraviolet light, it was possible to synthesize adenosine (70). Similarly, the ultraviolet irradiation of an aqueous solution of adenine and deoxyribose (both of whose possible primordial origin has already been discussed) and phosphate produced deoxyadenosine (71). The same compound was observed even if cyanide, magnesium ion, or cyanamide replaced the phosphate. By reacting adenine and ribose in the presence of ethylmetaphosphate and ultraviolet light, AMP, ADP, ATP, and A4P were reported to have been produced in addition to adenosine (72). The presence of a metapolyphosphate on the primitive Earth is questionable. The means by which such a polyphosphate compound could have originated, as well as resisted destruction by hydrolysis after only a short period of time under primitive Earth conditions even if it had been produced, has not been established. The polyphosphate used in the experiments described was synthesized from phosphorus pentoxide (72), itself a high energy compound which is readily hydrolyzed by water. Anhydrous heating of nucleosides and phosphate also produced nucleotides (73).

Pyrophosphate was successfully synthesized in aqueous solution by the reaction of potassium cyanate and the mineral, hydroxy apatite (75). When these two compounds were shaken together for twenty days at 35° C, consideraly more pyrophosphate was produced than when a soluble phosphate was used. Apparently the mineral surface allowed for a local concentration of phosphate and thus enhanced the kinetics of the reaction. Best yields were observed when the reaction was run around neutral pH. Carbamyl phosphate was believed to be an intermediate of the reaction. The use of cyanate had been suggested earlier (76) since it was known that cyanate and phosphate could react to give carbamyl phosphate (77). The simplicity of synthesis and structure of the compound made it a good candidate for an investigation concerned with chemical evolution.

A number of possible reasons have been cited as to why phosphoric acid, rather than other acids that were undoubtedly available to evolving systems, was chosen by nature as the primary means for distributing energy in biological organisms (78). First of all, the resonance energy of this acid is rather high. Secondly, the amides and anhydrides of phosphoric acid are kinetically more stable than the corresponding compounds of other acids. For example, acetic anhydride and pyrophosphoric acid are thermodynamically (with regard to heats of hydrolysis) similar, but in neutral solution in the absence of suitable catalysts the former hydrolyzes much faster than the latter. Simple phosphate esters do not release as much energy upon hydrolysis as to the pyrophosphates and mixed anhydrides. Thus, the elucidation

of the possible prebiotic origin of this bond is important as a supply of chemical energy needed by eobiotic systems which later appeared.

Organic Acids and Hydrocarbons

The electron bombardment of a methane-ammonia-water atmosphere leads to the production of several simple organic acids, as noted earlier (26). Long chain carboxylic acids of up to forty carbon atoms were synthesized by exposing a carbon dioxide and ethylene mixture to γ-radiation at room temperature (79). This demonstration suggested a very plausible means for the primordial origin of fatty acids needed for the subsequent appearance of lipids used in membranes.

The irradiation of a mixture of carbon dioxide and water with 40 Mev alpha particles produced formic acid and formaldehyde in small amounts (80). The importance of both compounds is evident from the discussion thus far. Oxalic acid and formaldehyde were produced with the alpha-irradiation of aqueous formic acid (81). Larger yields of oxalic acid were observed when calcium bicarbonate or ammonium bicarbonate was irradiated by the same means (82). The alpha bombardment of aqueous acetic acid led to the production of succinic acid, tricarballylic acid, malonic acid, malic acid, and cipric acid (83). The combination of carbon dioxide in aqueous solution and gamma rays from a $Co^{60}$ source yielded formaldehyde, acetaldehyde, formic acid, and what was believed to be oxalic acid (84). The best yields of oxalic acid with low dosage level were observed at very low or very high pH. When ferrous ion was present in the

reagent mixture, some carbon monoxide was produced. The yield of formaldehyde went down if oxygen were present.

Bombarding methane with low energy electrons produced hydrogen, ethane, ethylene, and acetylene, plus other lesser substances (85), whereas when high energy electrons were employed, the major products were hydrogen, ethane, ethylene, propane, and butane (86).

The effects of an electric spark and a semi-corona arc on methane have been compared (87). Saturated hydrocarbons predominated in the products formed using the latter type of energy source, whereas aromatics were the rule in the former case. Products were analyzed by mass spectrometry.

The possible origin of melanin was demonstrated with the ultraviolet illumination of phenylalanine and tyrosine (88). Under these conditions, polymeric melanin-like compounds were produced.

Thus far, a number of energy sources have been suggested. To complete the list, hypersonic organosynthesis has been investigated (89). Into a methane-ammonia-hydrogen atmosphere was fired a high speed bullet so as to momentarily set up a series of hypersonic shock waves. A number of organic compounds were observed but were not fully characterized.

Porphyrins

The role of porphyrins in the structure of hemoglobin, chlorophyll, the cytochromes, and otherimportant biological compounds is well known (90). Therefore, in order for efficient oxidation and reduction systems to have evolved to make better use of energy sources available, the appearance of porphyrins and porphyrin-utilizing compounds was important.

It hasbeen suggested (13) that prebiotic porphyrin synthesis followed, at least in its initial steps, the same general pathway used by biological systems, i.e., the condensation of glycine with acetate or succinate. This would then lead to the synthesis of $\delta$-aminolevulinic acid, followed by porphobilinogen, and ultimately yield porphyrins. Such a complete system has not been observed under primitive Earth conditions as yet.

It was demonstrated that the ultraviolet illumination of pyrrole and benzaldehyde in water produced porphine-like compounds (91). The exact requirement of the UV light was not determined.

Polymerizations

Thus, it may be seen that the possible means for the prebiotic origin of most essential monomers has been demonstrated. Much remains to be done but since no "eye-witnesses" are available to substantiate which experiments have truly recreated prebiotic events, tentative speculations must be made from the experimental evidence at hand. The role of cyanide and formaldehyde has been emphasized, for example. Therefore, assuming that all required monomers were, in one way or another, present, the means by which these units joined to form the macromolecules needed to define life itself must now be explored.

The possible origin of polypeptides has received a considerable amount of attention. Whereas biological systems now use a variety of complex enzymes to expedite the synthesis of these as well as other classes of macromolecules, a number of simple physical and chemical means have been examined to elucidate possible means for the prebiotic phenomenon of peptide bond formation which finally led to the appearance of proteins.

The polymerization of unsubstituted amino acids has been carried out using a number of different forms of energy such as heat (92), electrical discharge (93), ionizing radiation (94), and ultraviolet light (95). For example, by heating ammonium malate under anhydrous conditions for three hours at 200° C, an amino acidpolymer was formed which, upon hydrolysis, was shown to consist of aspartic acid and alanine (33). By heating to 160-200° C an anhydrous mixture composed of two parts aspartic acid, two parts glutamic acid, and one part an equimolar assemblage of sixteen other amino acids, a high molecular compound, termed a "proteinoid", was synthesized (92). Addition of various phosphates, such as orthophosphate, ATP, polyphosphoric acid, and calcium phosphate to the reaction mixture allowed the temperature necessary to bring about the desired reaction to be lowered to 65° C (96,97,98). (When polyphosphoric acid was used, it was also employed as the solvent for the system.) The reaction did not appear to involve simple acid catalysis since sulfuric acid was unable to promote the desired synthesis. Polyphosphoric acid has also been used in the production of diketopiperazines (99). The "proteinoid" formed by the heating of the anhydrous amino acid mixture gave positive biuret, xanthoproteic, and Hopkins-Cole reactions (100). Its infrared spectrum was much like that of a true protein and it exhibited similar solubility properties of proteins in that it could be salted in and then salted out of solution. The proteinoid contained both D and L amino acids and did not bear antigenic properties. Its molecular weight ranged from 3,600 to 8,600, depending on the temperature of synthesis. It was susceptible to the action of proteolytic en-

zymes (101). The proteinoid appeared to have nutritive value for bacteria (102).

However, it must be pointed out that a number of objections could be raised for invoking this type of synthesis as the primary source of polypeptides under primitive Earth circumstances. First of all, as was already pointed out, the temperature of the young Earth was believed to have been relatively low, somewhat comparable to what it is today. Secondly, the extent of the contribution of volcanic action to chemical evolution is open to debate (103). Finally, it has been pointed out that the mean life of alanine is $10^{11}$ years at 25° C but is only 30 years at 150° C since it tends to decarboxylate easily at this temperature to give methylamine and carbon dioxide (104). The presence of water in the primordial environment must also been considered.

The ultraviolet irradiation of an aqueous solution of glycine and alanine was used to synthesize small amounts of glycyl-glycine and glycyl-alanine (105). By heating to 140° C for nineteen hours glycine in 2 normal ammonium hydroxide, polyglycine of up to eighteen units was produced (106). The product gave a positive biuret test, yielded glycine upon hydrolysis, and exhibited an infrared spectrum similar to that of polyglycine II.

Another approach to the formation of polypeptides was with the use of aminoacetonitrile (107,108). By heating this compound to 135° C in the presence of kaolinite, diglycine and triglycine were produced. Aminoacetonitrile itself can be made from ammonia, formaldehyde and hydrogen cyanide. Assuming thatpolyglycine was available, formalde-

hyde was reacted with polyglycine in the presence of water and kaolin to yield 2-3% seryl residues. Likewise, by replacing formaldehyde by acetaldehyde in this reaction, 1.5% of the glycyl residues were converted to threonine. This also emphasized the possible role of surface catalysts suggested earlier (108a).

By heating anhydrous glycinamide to 100° C, it was possible to synthesize polyglycine (109). As was noted earlier, glycinamide can be made by passing an electric spark through an atmosphere of methane, ethane, ammonia, and water (46). Similarly, by warming a solution of glycinamide in 2 normal ammonium hydroxide to 100° C, polyglycines of thirty to forty units were produced. The nature of the product was substantiated by infrared spectrometry. The product also exhibited a positive biuret test. Upon acid hydrolysis, glycine was recovered.

When a suspension of ferrous sulfide in aqueous ammonium chloride, through which methane was being bubbled, was irradiated with ultraviolet light from a mercury lamp, a small peptide was produced (110). Upon hydrolysis, phenylalanine, methionine, and valine were tentatively identified.

By repeating the experiment in which an electric spark was passed through a methane-ammonia-water atmosphere, it was possible to also identify threonine, serine, isoleucine, leucine, and lysine (111). In addition, peptide-like products were observed. For example, upon hydrolysis, one such polymer yielded glycine and isoleucine in a 2 to 1 ratio.

Thus it may be seen that a number of approaches have been tested to explain the prebiotic origin of polypeptides. The most significant were probably those carried out at moderate temperatures in the presence of water since these appear to consider the most generally accepted conditions of the primitive Earth rather than invoking bizare circumstances, whose occurrence was either questionable or was limited to a very small segment of the Earth's surface. The success of a given laboratory demonstration, no matter how efficient, is only significant in terms of chemical evolution as long as a realistic approach has been used to recreate primitive conditions.

The origin of polysaccharides has also been considered. By heating glucose in 0.08 normal hydrochloric acid to 98° C for ten hours, gentiobiose, isomaltose, cellobiose, maltose, sophorose, and trehalose were produced (112). A melt of glucose in phosphoric acid heated to 140°-170° C yielded a non-dialyzable polymer (113). Glucose was the only product recovered after acid hydrolysis of the polymer.

At this juncture it is important to consider the role of pH during chemical evolution. Evidently this is an unsettled point. On the one hand, the experiments just discussed indicate that desirable polymers could be synthesized if the pH were on the acid side of the scale (112,113). Also, by proposing that the composition of gases found in present-day volcanoes reflects the nature of the primordial atmosphere, a strong argument could be made for the abundance of acids on the primitive Earth (9). On the other hand, if a large amount of

ammonia were present in the early atmosphere (11), a corresponding prevalence of ammonium hydroxide in the primordial ocean would also be expected. Similarly, the presence of deposits of mineral carbonates (116) would also argue for an alkaline environment. Of all the questions posed by the recreation of chemical evolution, the problem of pH is one of the most difficult and uncertain to settle.

In the field of polynucleotides, polycytidylic acid was synthesized by heating cytidine monophosphate and polyphosphoric acid together under anhydrous conditions at 65°C for two hours (117). The polymer produced appeared to be ribonuclease-sensitive. Ethyl metaphosphate, described earlier (72), has also been used in a number of dehydration condensations (118). For example, it was used to condense ribose and adenine to give adenosine and 2'3'-diphosphoadenosine. With this polyphosphate, deoxyribose and adenine gave deoxyadenosine, amino acids yielded polypeptides, and nucleotides produced polynucleotides. Using the metaphosphate it was also possible to synthesize polysaccharides. However, as discussed earlier, the presence of such a phosphate compound in any significant quantity under primitive Earth conditions is quite questionable.

Discussion

It is evident that the prebiotic origin of biopolymers has not been satisfactorily explained. The object of the present disseration is to further investigate this particular question. Since it may be assumed that a large percentage of the surface of the young Earth was covered by water, the role of solution chemistry

must certainly be taken into consideration. Since most polymerizations of biological interest involve the removal of molecules of water, the most pressing problem would be to find a means for preferentially taking up this aqueous byproduct while the entire system is dissolved in water. Also, the synthetic procedure must be carried out under such conditions that the rate of polymer production is greater than the opposing rate of hydrolysis or monomer destruction. For example, at elevated temperatures amino acids tend to decarboxylate at a relatively rapid rate (104). Thus, for appreciable amounts of polypeptides to have accumulated, the entire system would have had to be in an environment of moderate temperature.

If specific compounds, in addition to the monomers waiting for polymerization, were to be employed in promoting the dehydration condensation process, such chemicals would have to be simple enough to have been synthesized under primitive Earth conditions and stable enough to have resisted destruction under these conditions for a significant period of time. Since chemical evolution is taken to be that period preceding the appearance of living biological units in the course of the Earth's history, such compounds as enzymes cannot be invoked as candidates for dehydrating agents or catalysts, at least in the earlier, more simple stages of development, since enzymes themselves are polymers of amino acids formed by dehydration condensation. Also, compounds such as polyphosphoric acid which are easily hydrolyzed on their own would be unlikely participants in the desired polymerization of monomers in aqueous solution. As indicated throughout the discussion thus far, cyanide and its relatives appear to have been important agents during chemical evolution. Therefore, the possible role

of this class of compounds in promoting dehydration condensation as well as worthy of further consideration. Since polymerization of nearly all biological monomers involves the same process of water removal, it would be desirable to find a compound ableto promote this reaction in most, if not all, of the pertinent cases.

It may be assumed for the moment that suitable means have now been uncovered to elucidate the origin of biologically essential monomeric units such as amino acids, fatty acids, sugars, heterocyclic bases, and the like. Without a plausible means for explaining the appearance of polymers, a critical link in the chain of evolutionary events would remain lacking so that the course of events after polymer production would be difficult to reveal. This is so since the expanse or limitation of conditions which polymer production would require would be undetermined so that speculation as to the most likely state of the primitive Earth would also remain unclear. Although one can never be certain of these conditions, reasonable speculations can be made based upon the types of experiments that successfully produce the desired compounds under possible primitive Earth conditions. For example, the success of the methane-ammonia-water experiments (25) corroborates the suggestion of the reducing atmosphere (15). Thus, the importance of determining the most likely course of polymerization under primitive Earth conditions is evident.

# Part I:

## USE OF CYANAMIDE AND RELATED COMPOUNDS TO EFFECT DEHYDRATION CONDENSATION

### General Introduction and Background

As was extensively discussed in the previous section, the need exists for the demonstration of a means by which biopolymers could have evolved in aqueous solution during chemical evolution. In general, the usual means by which biomonomers condense into polymers of biological interest is for molecules of water to be chemically removed. For example, the joining of two amino acids to form a dipeptide, as has been known for a long time, involves the elimination of a molecule of water (119), as shown in Figure 1.

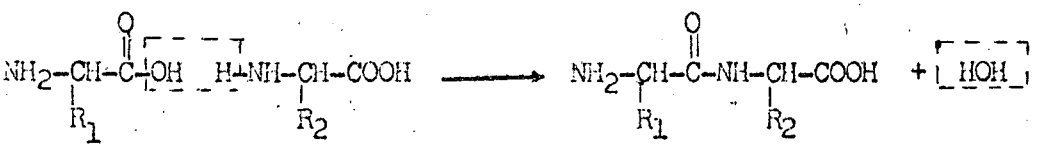


Fig. 1. Dimerization of amino acids

Similarly, phosphorylations of various compounds require the elimination of water. An example of this would be the addition of a phosphate group to adenosine to form AMP (119) (Figure 2).

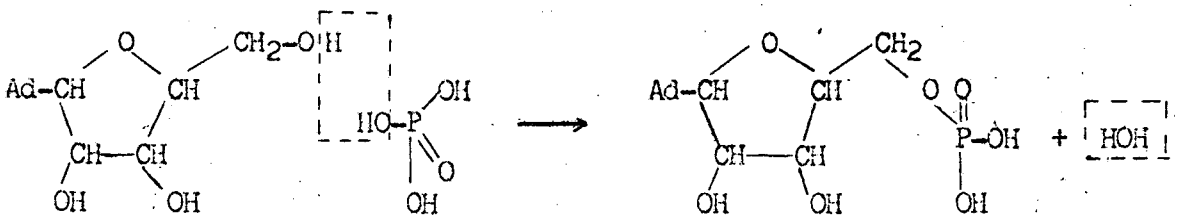


Fig. 2. Phosphorylation of adenosine (Ad = adenine)

The problem encountered in carrying out such a process in the presence of water is evident. The use of polyphosphates and high temperatures, to promote such as process, as discussed earlier, is probably unrealistic in terms of chemical evolution, as is the need to suggest anhydrous regions on the surface of a young planet which undoubtedly experienced endless shifts and relocations of aqueous bodies. Therefore, it is necessary to look for a chemical process that may have existed on the primitive Earth which could have carried out such a reaction. It seems that a more likely approach to the problem of biopolymer synthesis would be to find conditions that would result in dehydration condensations while the reactants are dissolved in aqueous media.

The important role of hydrogen cyanide during chemical evolution has already been indicated. By the experimental evidence cited it is reasonable to assume that appreciable amounts of the compound were present.

That hydrogen cyanide may have acted as an agent for promoting dehydration condensation has been proposed (119). As indicated in Figure 3, the suggested ability of this compound to induce peptide formation would yield formamide as a side product by which the unwanted water molecule would be removed from the appropriate sites on the reacting molecules.

A similar type of reaction might be suggested by the known application of various carbodiimides in promoting polymerizations (120). Dialkylcarbodiimides have been used to synthesize phosphate esters of nucleosides, polynucleotides (phosphodiesters), polypep-

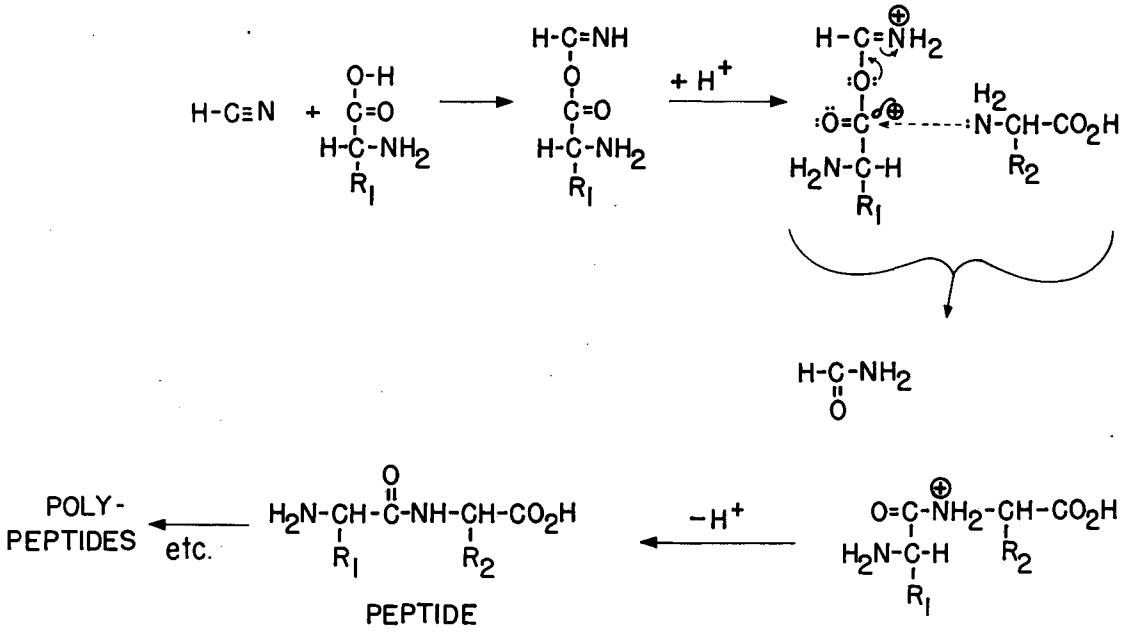



Fig. 3. Suggested mechanism for peptide formation using HCN as the dehydrating agent (from ref. 119).

tides, phosphoramidates, cyclic phosphates, pyrophosphates, and mixed anhydrides (120). These reactions are usually carried out under nonaqueous conditions. It has also been demonstrated thatpeptide synthesis using substituted carbodiimides can be done in the presence of water (121,122). The production of polyglycine with dialkylcarbodiimides gives the best yields at low pH (121). Similarly, phosphate esters have been synthesized in the presence of water using carbodiimides (120). In some of these phosphorylations, partially aqueous environments were actually advantageous since they enhanced the solubility of the reactants. This might also suggest that phosphate is more reactive with the proposed intermediate of the reaction than water. Using the carbodiimides it is also possible to form lactones and carboxylic esters (120).

However, from the standpoint of chemical evolution, there is little reason to suppose that any significant quantity of dialkylcarbodiimides was present on the primitive Earth. A more likely candiate is cyanamide, a compound which hydrolyzes relatively slowly, that was quite likely present on the primitive Earth, and that is a tautomer of carbodiimite.

$$NH_2\text{-}C{\equiv}N \rightleftharpoons NH{=}C{=}NH$$

Fig. 4. Tautomerism of cyanamide and carbodiimide

Cyanamide has been successfully employed in the synthesis of nucleoside pyrophosphates (123). By heating for 12 hours at 75° C a

mixture of adenosine-5'-phosphate, uridine-5'-phosphate, and cyanamide in the presence of a small amount of water, it was possible to produce $P^1P^2$-diadenosine-5'-pyrophosphate, $P^1$-adenosine-5'-$P^2$-uridine-5'-pyrophosphate, and $P^1P^2$-diuridine-5'-pyrophosphate. Similarly, dimers of acylated amino acids were formed in nonaqueous media by heating to 80-100° C in the presence of cyanamide (124,124a).

Cyanamide is made in the laboratory by reacting cyanogen bromide with ammonia (125). By gradually adding an aqueous solution of lead acetate to thiourea in 12% aqueous potassium hydroxide, it is also possible to produce cyanamide (126). The disodium salt of cyanamide can be prepared by heating sodamide with carbon, sodium cyanide, or carbon dioxide (127,128). Cyanamide reacts with glycine to give glycocyamine and sarcosine to give creatine at elevated temperatures (129).

Silver ion can be used to precipitate cyanamide (130). On condensation with various dialkylcyanoacetic acids, cyanamide produces pyrimidine derivatives.

The structure of cyanamide is still open to debate. It has been suggested that both the diimine and cyanoamine forms (see Figure 4) exist in equilibrium in aqueous solution (131,132). Cyanamide is a base with a dissociation constant of the order of $10^{-11}$ (129). With acid hydrolysis the compound yields urea. The reaction is of the first order with respect to cyanamide (133).

Slightly acid solutions of cyanamide are stable for several months (134). However, upon standing or heating, both solid cyanamide and solutions of the compound tend to polymerize to give dicyandia-

mide (135).

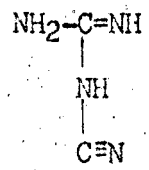


Fig. 6. Dicyandiamide

The dimerization is accelerated by both acid and base. The optimum rate is found at pH 9.6 (136). By heating cyanamide to 200° C, melamine is produced (137). A similar heating of dicyandiamide also produces the cyclic trimer, melamine (129).

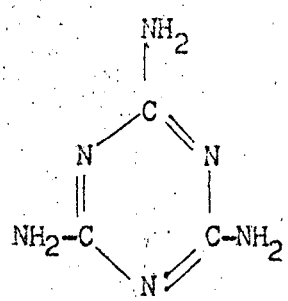


Fig. 7. Melamine

Aqueous solutions of dicyandiamide are neutral (133) and decompose very slowly with time (129). The hydrolysis of this compound, also called cyanoguanidine, yields guanylurea (133), a strong base (138).

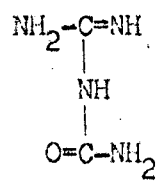


Fig. 8. Guanylurea

It has been argued that because of the electron-withdrawing capacity of the nitrile group of dicyandiamide, the hydrogen of the secondary amine would, in fact, be quite labile in aqueous solution (139). This hydrogen would then be available to satisfy the basicity of the amidinium group. Therefore, the most probable structure of dicyandiamide would be that one which contains two amino groups. In addition, since dicyandiamide displays a high melting point, salt-like character, low solubility in nonpolar solvents, and high temperature coefficients of solubility in water and alcohol, the compound may exist to some degree as a zwitterion (140).

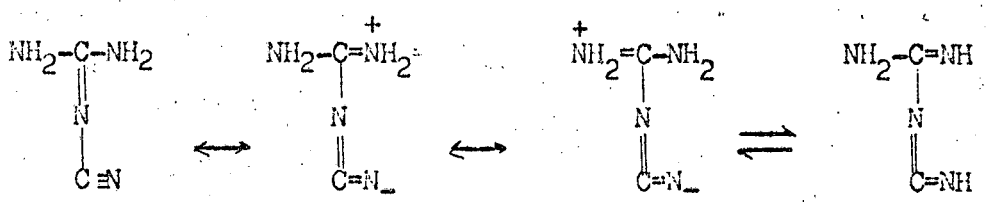


Fig. 9. Proposed structure of dicyandiamide in aqueous solution

Such structures have been suggested from X-ray diffraction studies of the solid as well (141). It is important to note for reference later in this discussion that Figure 9 suggests the existence of the carbodiimide group within dicyandiamide. Similarly, the diimide form was argued for on the basis of the relatively low toxicity of the compound which tends to suggest the absence of C≡N groups (142). For some time it was believed that dicyandiamide (DCDA) may even bear a four-membered ring containing pairs of alternating carbon and nitrogen atoms with

amino groups (or imino groups, depending on the tautomeric form considered) attached to the carbons of the ring (143,144). The ring was thought to exist in neutral and alkaline solution as well as in the solid, whereas the open form was found in acidic solution. This was suggested from the types of products found after hydrolysis. However, Raman spectral studies (145) supported the proposal of the open diamine form indicated in Figure 9. The suggestion of the open form is further supported by the fact that hydrogenation of dicyandiamide yields guanidine and methylamine (140). Thus it may be seen that the exact nature of the structure of DCDA in solution remains uncertain.

Compounds able to supply an active hydrogen can add across the nitrile group of dicyandiamide (140). For example, hydrazine reacts with cyanoguanidine to give aminobiguanide (147). Another potentially important set of reactions of dicyandiamide, from the standpoint of chemical evolution, is that with esters of acetoacetic acid, disubstituted malonic acid, or cyanoacetic acid, various pyrimidines and barbituric acids can be synthesized (138,148,149). Using copper acetate as a catalyst it is possible to form 1-amidino-O-alkylureas from dicyandiamide and alcohols (150).

Another significant relative of cyanamide is dicyanamide, the dinitrile of iminodicarboxylic acid (151). This compound, as the sodium salt, can be prepared in the laboratory from the reaction of cyanogen bromide and the disodium salt of cyanamide (152). The crystalline hydrate of dicyanamide can be isolated, but is somewhat

unstable (153). If, when dicyanamide is hydrolyzed, one mole of water is taken up, cyanurea is formed, whereas with two moles of water, biuret is produced (152,154). Hydrolysis of cyanurea similarly gives biuret.

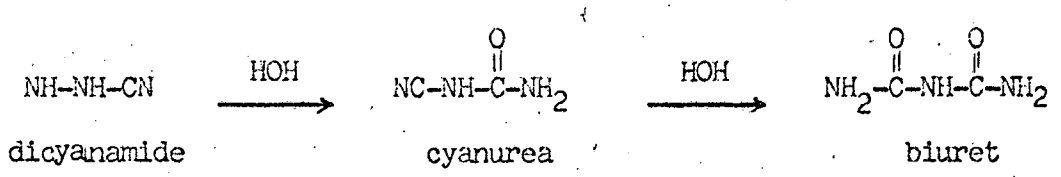



Fig. 10. Hydrolysis scheme of dicyanamide

Free dicyanamide is difficult to isolate since it tends to polymerize (152). However, it is reasonably stable in solution, and, by conductivity measurements as a function of pH, exhibits properties of a strong acid (152). By heating sodium dicyanamide to dull redness, the compound is converted to the cyclic trimer, sodium tricyanomelamine, which is melamine (see Figure 7) with a nitrile group replacing one hydrogen of each amino group (130).

Experimental Methods - General

The general method employed in this series of experiments first required a reaction period of arbitrary length in which the condensing agent was dissolved in aqueous solution with the compounds to be joined. Usually at least one of the reactants was labeled with a radioactie atom such as $^{14}C$ or $^{32}P$.

To the product solution was added a detectable amount of the compound suspected of being one of the products of the reaction. Enough of the standard was added to permit location by an established method, such as spraying or ultraviolet photography, with respect

to whichever was applicable in each case, and the resultant mixture was spotted on a sheet of Whatman No. 4 chromatography paper. The general chromatographic method followed has been described in detail elsewhere (156). Two-dimensional resolution was carried out.

After the chromatography process was completed and the sheet was dried, radioactive ink spots were placed as positional references at selected points on the paper and the chromatogram was folded to the size of standard X-ray film.

In those experiments where the added standard was UV-absorbing, advantage was taken of this property to permit detection of the final location of the carrier. In the dark room, positive contact photographic paper was placed under the chromatogram, and a Hanovia 125 watt Type 16200 mercury ultraviolet lamp placed about two feet above the paper illuminated it for fifteen seconds. The photographic paper was developed by standard techniques. UV-absorbing carriers and the ink spots appeared white on a black background.

Advantage was taken of the fact that carbon-14 has a half-life of 5770 years and emits a beta particle of 0.156 Mev (157), making it detectable with standard X-ray film (156). A sheet of blue-sensitive X-ray film was placed in juxtaposition with the chromatography paper and together both were fitted into a light-tight envelope. After sufficient exposure time, the film was withdrawn in the dark and developed by standard techniques. The dried film was then placed on top of the contact print made earlier. Alignment of the two was achieved by adjusting their positions so that the radioactive reference spots appearing as black marks on

the X-ray film were directly over the corresponding white marks on the contact print. In this manner it was possible to ascertain if one of the radioactive products appearing on the film, whose original source of radioactivity had been one of the reactants, corresponded both in position and shape to the standard outline on the UV contact print (shadowgram) of the same chromatogram.

Those carriers which responded to chemical sprays, such as ninhydrin for amino acids and benzidine-periodate for glycerol, were located and compared to the radioactive products in a corresponding manner.

NOTE:

In the experiments to follow, the general outline is arranged in terms of the types of bonds formed. First, nucleosides are discussed, followed by phosphate esters, acid anhydrides, peptides, carboxylic esters, and finally, glycosides. Each set of data is introduced with a statement of the particular relevance of the compound under study to the general problem of biogenesis. Each experiment follows the general analytical outline just noted. Particular aspects of the experiment are listed, such as concentrations, reaction times, chromatographic solvents, carrier detection, and resultant data. Finally, a short discussion is presented of the results and their relevance to the general problem at hand.

## Experimental Data and Discussion

### A. Nucleosides

Nucleotides are compounds containing an organic heterocyclic base and a sugar (90). They make up, with phosphate, the essential structural and functional elements of deoxyribonucleic acid (DNA) and ribonucleic acid. As discussed earlier, means for the possible primordial origin of ribose and 2-deoxyribose, as well as some of the purines and pyrimidines found in nucleic acids, have been experimentally demonstrated. Therefore, the next problem would be to establish how these subunits joined to provide the nucleosides and nucleotides needed in macromolecular construction. The linking of a purine or pyrimidine to a pentose moiety involves overall the removal of a molecular of water. It has already been postulated in this discussion that cyanamide's dimer, dicyandiamide (DCDA), might operate in a manner similar to that of dialkylcarbodiimides as a dehydrating agent. Therefore, in order to investigate one possible function of dicyandiamide, the synthesis of adenosine from adenine and ribose was examined.

To be sure of the identity of the dicyandiamide being used, a number of tests were made on the compound. First of all, the molecular weight (Rast) of the compound closely matched the theoretical value of dicyandiamide. Elemental analysis and melting point determination also supported the identification of the reagent.

Since it is known that the hydrolysis of dicyandiamide is an acid-catalyzed reaction (130), an attempt was made to utilize this reaction in the desired synthesis of adenosine. A one milliliter

aqueous solution was prepared containing chromatographically purified $^{14}C$-labeled adenine ($0.80 \times 10^{-3}$ M), unlabeled ribose ($0.7 \times 10^{-2}$ M), hydrochloric acid ($10^{-2}$ M), and dicyandiamide ($10^{-2}$ M). The solution remained in the dark for forty hours and was then brought to a volume of about 0.1 milliliter by evaporation under reduced pressure. To the solution was then added a small amount of known adenosine, and together they were applied to a sheet of Whatman No. 4 paper. The organic phase of a n-butanol:acetic acid:water (4:1:5 by volume)mixture (158) was used as solvent in the first dimension. The solvent system employed in the second dimension in this experiment was an isopropanol:water (4:1) mixture (159). After the paper was dried, the adenosine carrier was located by the shadowgram method and the labeled products were located by X-ray film. No coincidence was observed so as to indicate that the combination of dicyandiamide and hydrochloric acid did not promote the condensation of adenine and ribose to give adenosine.

As mentioned, adenine is an ultraviolet-absorbing compound. Similarly, dicyandiamide also absorbs UV light. (This point will be discussed further.) Therefore, with the possibility thatultraviolet light might act to excite both compounds and thus promote the synthesis of adenosine, the following experiment was carried out: A three milliliter aqueous solution was prepared containing $^{14}C$-labeled adenine ($0.3 \times 10^{-3}$ M), ribose ($3 \times 10^{-3}$ M), and dicyandiamide ($2 \times 10^{-2}$ M) and then was irradiated with a high pressure General Electric Type A-H6 mercury lamp at a distance of 7.5 centimeters for 4.5 hours.

Throughout the illumination period the sample, contained in a sealed fused silica cuvette, was cooled by a steady air stream blown across the surface of the vessel. The possible role of acidic conditions has already been noted. Therefore, a similar solution, which also contained hydrochloric acid ($10^{-2}$ N), was prepared and irradiated in the same manner. Both product solutions were analyzed by the chromatographic method just outlined. Neither sample exhibited adenosine as a product.

The role of phosphate in enhancing various dehydration condensations was illustrated in a number of cases already cited. Therefore, in the expectation thatphosphate might assist dicyandiamide in synthesizing adenosine, the following experiment was examined: A one milliliter aqueous solution was prepared containing $^{14}C$-labeled adenine ($0.8 \times 10^{-3}$ M), phosphoric acid ($10^{-2}$ M; final pH 2.1), ribose ($0.7 \times 10^{-2}$ M), and dicyandiamide ($10^{-2}$ M). This solution remained in the dark for 40 hours at room temperature and was then analyzed in the manner already described. No adenosine was found in the product.

Finally, it was reported that the ultraviolet irradiation of adenine and ribose in the presence of phosphate produces adenosine (70). This synthesis was repeated using a three milliliter aqueous solution of $^{14}C$-labeled adenine ($10^{-4}$ M), phosphoric acid ($10^{-2}$ M), and ribose ($0.3 \times 10^{-2}$ M). A similar solution was prepared which also contained dicyandiamide ($10^{-2}$ M). Both solutions were irradiated with ultraviolet light for 4.5 hours and analyzed in the same fashion as with the previous samples. Adenosine was observed in both

cases, and no significant quantitative difference was noted between the two. The results of these six experiments have been summarized in Table I. DCDA apparently did not promote nucleoside synthesis.

Table I. Results of Experiments to Synthesize Adenosine

| Expt.* | Acid** | UV*** | DCDA**** | Adenosine Product |
|---|---|---|---|---|
| 1 | HCl | - | + | - |
| 2 | - | + | + | - |
| 3 | HCl | + | + | - |
| 4 | $H_3PO_4$ | - | + | - |
| 5 | $H_3PO_4$ | + | + | + |
| 6 | $H_3PO_4$ | + | - | + |

* All contain adenine and ribose

** All acids used at $10^{-2}$ M

*** 4.5 hour exposure from mercury lamp

**** Dicyandiamide

B. Phosphate Esters

1) Adenosine monophosphate

Phosphate esters of nucleosides (nucleotides) are important biologically in that they form the units of nucleic acids and are involved in various energetic and synthetic functions in living systems (90). For example, the triphosphate of adenosine (ATP) appears to be the universal biological conveyance of metabolic energy. Thus an important link in elucidating the course of chemical evolution would be the synthesis of nucleotides under possible primitive Earth conditions.

As indicated in Figure 2, the phosphorylation of nucleosides is a dehydration condensation reaction. Such a synthesis, as discussed

earlier, has been successfully carried out with the dialkylcarbodiimides. Therefore, a reasonable test of the possible role of dicyandiamide as a condensing agent would be the production of nucleoside phosphates.

The synthesis of adenosine monophosphate (AMP) was attempted using ultraviolet irradiation of a solution containing phosphoric acid ($1.3 \times 10^{-2}$ M), DCDA ($1.7 \times 10^{-2}$ M), and $^{14}$C-labeled adenosine ($0.38 \times 10^{-3}$ M). The use of ultraviolet light to excite both the adenosine and the DCDA was logical since the former compound is known to absorb strongly around 260 mμ(160), whereas the cyanamide dimer in water, as demonstrated in the Cary Model 14 spectrophotometer, exhibits a strong absorption at 214 mμ. After the sample was irradiated for 4.5 hours with ultraviolet light (in exactly the same manner as the corresponding experiment discussed under nucleoside synthesis), the volume of the sample was decreased by evaporation under reduced pressure. Carrier adenosine-5'-monophosphate was added to the solution and together they were applied to Whatman No. 4 chromatography paper. Then n-butanol:acetic acid:water (4:1:5-organic phase) was employed as the solvent in the first (long) dimension. Isopropanol:water (4:1) was used as solvent in the second (short) dimension. A shadowgram was made of the carrier AMP. After sufficient exposure to the chromatogram, a sheet of X-ray film was then developed and by comparison with the shadowgram indicated that AMP had indeed been synthesized.

As a result of this observation, several questions remained to be answered. First of all, only the 5'-monophosphate had been used

as a carrier. Although the 5'-hydroxyl of ribose is the only primary hydroxyl and would thus appear sterically most susceptible to attack in contradistinction to other positions, both the 2' and 3' hydroxyls, even though bonded to secondary carbons, might also be susceptible to phosphorylation. In other words, after disregarding the small steric differences of the available hydroxyls of adenosine, one might expect from the apparent nonspecificity of the reaction that all three hydroxyls would be amenable to phosphorylation. The synthesis was repeated as before. The product, after irradiation, was divided into three parts. Adenosine-2'-monophosphate was added to one fraction, while the 3'-phosphate was added to the second, and the 5' to the third. Each irradiated sample was analyzed for the appropriate monophosphate by the paper chromatography method previously described. The results appeared to indicate thatonly the 5'-monophosphate was produced.

It was noted that when a solution containing adenine, ribose, and phosphate was irradiated with UV light, adenosine but no AMP was produced (70). Therefore, this synthesis was repeated but DCDA was included. AMP was observed to be a product of the reaction.

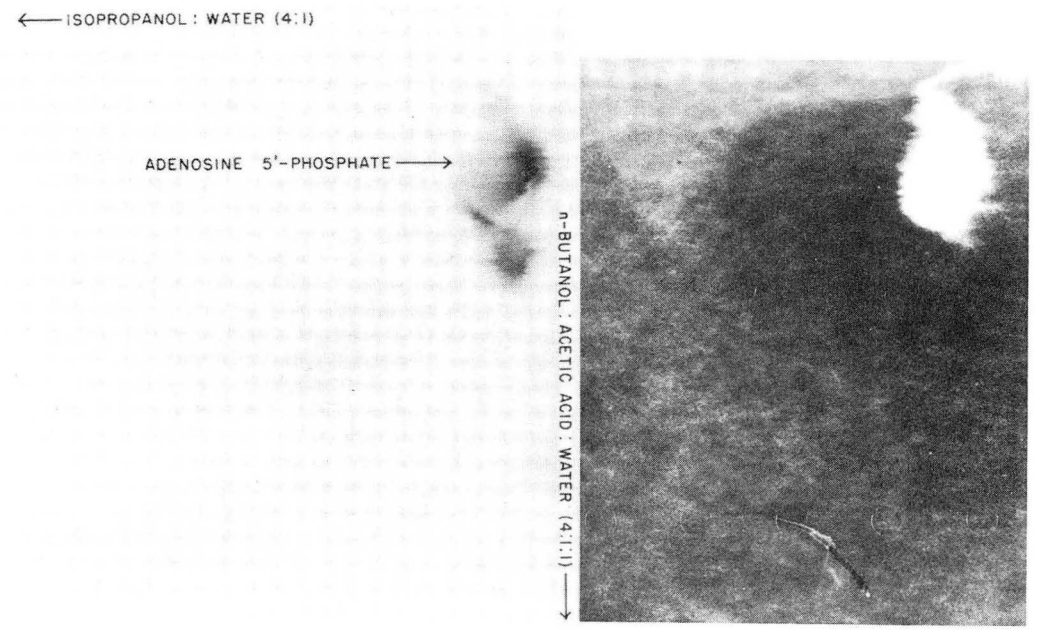
At this point the exact function or need for the ultraviolet light in the phosphorylation step was uncertain. Therefore, the synthesis of AMP was repeated. A solution was prepared containing adenosine ($0.4 \times 10^{-3}$ M) and DCDA ($1.7 \times 10^{-2}$ M). A 3 ml aliquot was irradiated with ultraviolet light for 4.5 hours whereas another 3 ml aliquot remained in the dark for the same period of time. Both samples were analyzed by paper chromatography using the same solvents as before. AMP was identified in both cases. The prdoucts were eluted and

rechromatographed with the same solvents. AMP was again observed (see Figure 11). Thus, the phosphorylation of adenosine appeared not to require the UV light and was actually a dark reaction.

It may be recalled that phosphoric acid was used in every phosphorylation already discussed and it was intended to make use of the acid-catalyzed hydrolysis of DCDA. Whether DCDA might operate at neutral pH remained to be ascertained. To determine the effect of pH on the phosphorylation of adenosine, a solution was prepared containing $^{14}C$-labeled adenosine ($0.3 \times 10^{-3}$ M), DCDA (0.02 M), and phosphate (0.01 M), and was adjusted to pH 7. An aliquot of the solution was irradiated with ultraviolet light for 4.5 hours. Another aliquot remained in the dark. Both solutions upon chromatographic analysis exhibited no AMP as a product. Thus, the role of acidic conditions was emphasized.

Table Ia. Results of Experiments to Synthesize AMP

| Expt. | Adenosine | UV | $H_3PO_4$ | DCDA | phosphate (pH 7) | AMP product |
|---|---|---|---|---|---|---|
| 1 | + | + | + | + | - | + |
| 2 | + | + | + | + | - | + |
| 3 | + | - | + | + | - | + |
| 4 | + | + | - | + | + | - |
| 5 | + | - | - | + | + | - |
| 6 | + | + | + | - | - | - |




ZN-5299

Fig. 11. Autoradiogram and shadowgram of chromatogram showing the synthesis of adenosine-5'-monophosphate from $^{14}C$-labeled adenosine and phosphoric acid using dicyandiamide.

2) Ribose-5-phosphate

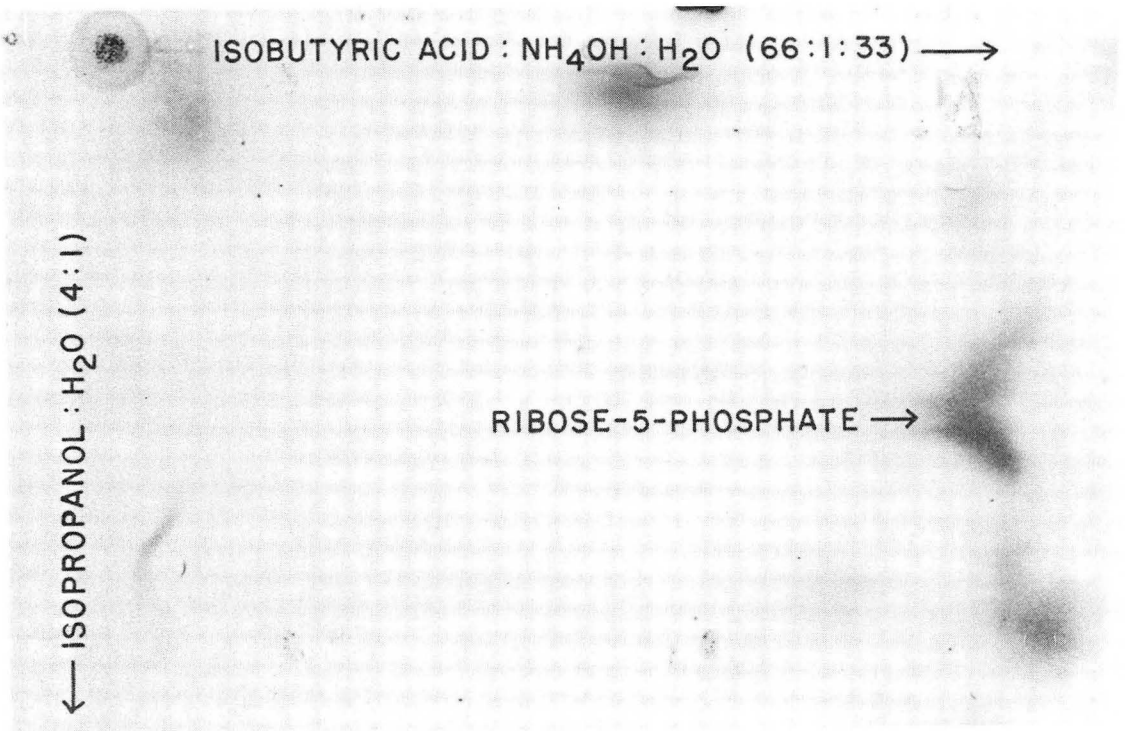
The successful phosphorylation of the ribose moiety of adenosine would suggest that DCDA might also act with phosphoric acid to phosphorylate unbound sugars, such as ribose. The role of the sugar phosphates in metabolism is quite significant. For example, ribose-5-phosphate is a key compound in the phosphogluconate oxidative pathway, or "pentose shunt" (90). Other sugar phosphates are important intermediates in the photosynthetic process. Similarly, glucose-6-phosphate plays a prominent part in glycolysis and as a source of material for lipogenesis. The need for such compounds to be available to protometabolic systems appearing later on the primitive Earth is readily apparent. Assuming the first metabolizing systems were heterotrophic, as will be discussed later, the means for the prebiotic appearance under probable primitive Earth circumstances of these essential phosphate compounds must be considered.

An aqueous solution was prepared containing chromatographically purified $^{14}C$-labeled ribose (9 mM), DCDA (10 mM), and phosphoric acid (10 mM). The mixture remained in the dark at room temperature for 22 hours. The product solution was spotted on Whatman No. 4 paper adjacent to an aliquot of standard ribose-5-phosphate and resolved with the organic phase of a n-butanol:acetic acid:water (4:1:5) mixture as solvent. After it was dried, the half of the chromatogram containing the standard was sprayed with the usual molybdate reagent for phosphates (161). (A one liter volume of the spray contained 50 ml of 60% perchloric acid, 10 ml of concentrated hydrochloric acid, and

10 grams of ammonium heptamolybdatetetrahydrate.) After the chromatogram was sprayed, the standard was exposed to ultraviolet light and the characteristic blue color of the phosphate compound appeared. By exposing the other half of the chromatogram to X-ray film, the autoradiogram indicated that one of the labeled products exhibited a similar $R_f$ value to the ribose-5-phosphate standard. This fraction was cut out and eluted with water. Fresh standard ribose-5-phosphate was added to this aample and together they were applied to Whatman No. 4 paper. Chromatography was carried out with isobutyric acid: conc.ammonium hydroxide:water (66:1:33) as a solvent (160) in the first dimension and isopropanol:water (4:1) in the second. The standard was located with molybdate spray and X-ray film indicated that the radioactive product coincided in both position and shape to the ribose-5-phosphate carrier (see Figure 12). This supported the suggestion that DCDA in the presence of phosphoric acid might act as a means for phosphorylating sugars in aqueous solution.

3) Glucose-6-phosphate

To investigate the possibility of synthesizing glucose-6-phosphate with DCDA, a solution was prepared containing chromatographically purified $^{14}C$-labeled glucose (0.64 mM), phosphoric acid (0.01 M), and DCDA (0.01 M). The solution was allowed to stand for 19 hours at room temperature (159). Carrier glucose-6-phosphate was then added, and the entire solution was chromatographed on Whatman No. 4 paper using as the first solvent system (162) methanol: conc. $NH_4OH$:water (6:1:3) an methanol:ethanol:water (9:9:2) as the solvent system in the second di-




ZN-5302

Fig. 12. Autoradiogram of chromatogram demonstrating the production of ribose-5-phosphate from phosphoric acid and ribose using dicyandiamide.

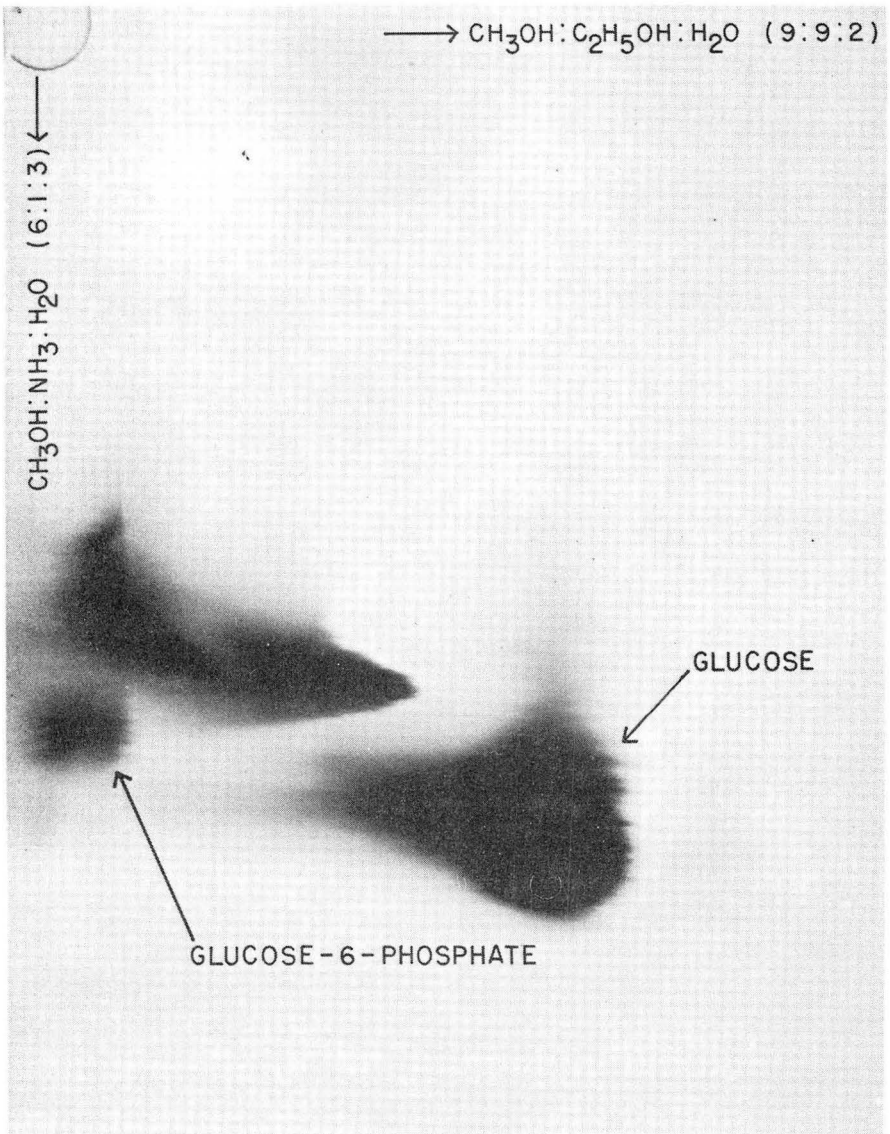
rection (163). The sugar phosphate standards were located by means of a benzidine spray. (This reagent contains 0.1 gm of benzidine-HCl and 3 gm of TCA in 8 ml of 8.5 N acetic acid and reacts with reducing sugars upon heating to give brown spots on the chromatogram (164)). An identical experiment was performed using freshly prepared cyanamide monomer (0.01 M) in place of the dimer.

The results, recorded in Table II, demonstrated that cyanamide can induce the formation of the phosphomonoester of glucose (see Figure 13). A standard chromatogram of the original labeled glucose indicated that no glucose-6-phosphate was detectable in the reagent. The yields were obtained by measuring the amount of radioactivity exhibited by each spot on the chromatogram with a Baird-Atomic Geiger-Müller Scaler Model 123. The dimer of cyanamide appears to be about twice as effective as the monomer in accomplishing this reaction.

Table II. Formation of Glucose-6-Phosphate

| Glucose | Cyanamide | DCDA | $H_3PO_4$ | Glucose-6-Phosphate Product |
|---|---|---|---|---|
| + | + | - | + | + |
| + | - | + | + | + |
| - | - | - | + | - |

Later studies (to be discussed) indicated that phosphoric acid and DCDA can react to yield pyrophosphate. It might therefore be suggested that it was this pyrophosphate product which acted to phos-




ZN-5303

Fig. 13. Autoradiogram of chromatogram demonstrating the production of glucose-6-phosphate from glucose and phosphoric acid using dicyandiamide.

phorylate the glucose. To settle this point an aqueous solution was prepared containing labeled glucose (0.05 mM), phosphoric acid (0.01 M), and pyrophosphate (0.005 M). No glucose-6-phosphate was detected in the product analyzed by paper chromatography.

To determine the effect of pH on the phosphorylation of glucose using DCDA, the following group of experiments was performed: A stock aqueous solution was prepared containing $^{14}C$-labeled glucose (0.04 mM), DCDA (0.01 M), and phosphate (0.01 M). The solution was divided into five equal parts and the pH of each was adjusted to 1.0, 2.0, 3.5, 4.2, or 6.8. Each sample remained in the dark at room temperature for 23 hours and was then analyzed by paper chromatography using the same solvent system as before. Since carriers had been added, benzidine spray located the glucose-6-phosphate and indicated which radioactive spot was the desired product. The amount of radioactivity of the compound and the total material on each chromatogram was measured with the Geiger-Müller counter. These determinations indicated that no glucose-6-phosphate appeared to have been synthesized in those cases where the pH of the reaction solution had been 3.5 or higher but was quite evident at pH 2.0 or lower.

In an attempt to find compounds which might act as more effective dehydrating agents than DCDA, the possible use of dicyanamide (DCA) was investigated in the case of these phosphorylations (165). An aqueous solution was prepared containing $^{14}C$-labeled glucose (0.08 M), phosphoric acid (0.08 M), and the sodium salt of dicyanamide (0.1 M). A similar solution was prepared containing DCDA in place

of sodium DCA. After 40 hours at room temperature in the dark, each solution was applied to Whatman No. 4 paper and chromatographed adjacent to a standard glucose-6-phosphate sample using 95% ethanol: water:formic acid (70:29:1) as solvent (166). The standard was located with benzidine spray. The results as measured with the Geiger-Müller counter indicated that DCA is about three times as effective in the production of glucose-6-phosphate as is DCDA, with the yield using DCA being 1.9%.

4) O-phosphoserine

In the biosynthetic pathway for the production of serine, phosphoserine is the precursor to it (90). Although it has not been demonstrated as yet, reversal of the steps of this reaction sequence could conceivably serve as a source of 3-phosphoglyceric acid and, ultimately, glycogen. Such a process bears significance as a possible key to protometabolic systems. Also, some important proteins, such as casein, contain as much as 0.9% phosphorus mainly in the form of phosphate bound to the hydroxyl groups of serine (167). It was once believed that phosphodiester bonds might also contribute to the secondary structure of proteins (168,169) but this point is presently under debate. Thus, the synthesis of O-phosphoserine is significant not only from the standpoint of the phosphorylation in aqueous solution of a hydroxyl-containing compound other than a sugar but also because of the biological significance of the compound itself.

An aliquot of $^{14}C$-labeled serine was chromatographically purified. An aqueous solution was prepared containing this serine (8.3 mM) as well as phosphoric acid (0.01 M) and dicyandiamide (0.01 M). The solution was kept in the dark at room temperature for 15 hours.

A one ml aliquot of the solution was spotted on Whatman No. 4 paper adjacent to a standard sample of O-phosphoserine. Themixtures were resolved with the organic phase of a n-butanol:acetic acid:water (4:1:5) mixture as solvent. Molybdate spray located the standard and X-ray film showed that two products might be the desired compound. Each was eluted, added to the phosphoserine carrier, and rechromatographed using the n-butanol:acetic acid:water solvent in the first dimension and phenol:water (9:1) in the second. X-ray film remained in juxtaposition with each dried chromatogram a sufficient period of time and, after development, located the radioactive compound. The corresponding location on the chromatogram was divided in half. One-half was cut out and sprayed with the molybdate mixture for phosphate; the other half was sprayed with ninhydrin for the amino groups. The cut portion was rejoined to the chromatogram and the standard was located by both the positive ninhydrin and positive molybdate reactions. One of the radioactive spots matched the shape and position of the carrier and was thus identified as O-phosphoserine. The other product may have been the phosphoramidate but this was not definitely established.

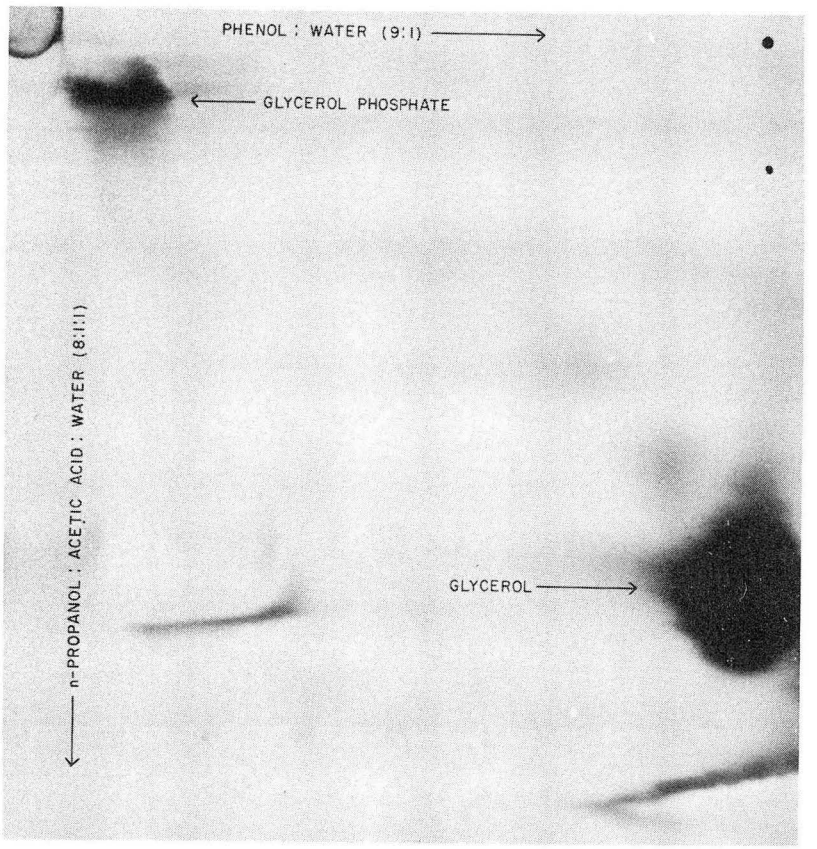
5) Glycerol-1-phosphate

The central role of glycerol-1-phosphate in glycolysis is well known (90). With suitable catalysis it can serve as a source of material for carbohydrate synthesis or lipogenesis. Its importance is thus evident. A prebiotic source of the compound would be very functional as a vital source of supply for metabolizing systems once they appeared and would also promote the initial appearance of such

systems.

In order to explore the possibility of synthesizing glycerol-1-phosphate, an aqueous solution was prepared containing chromatographically purified $^{14}C$-labeled glycerol ($2 \times 10^{-3}$ M), phosphoric acid (0.01 M), and DCDA (0.01 M). Another solution contained glycerol (0.01 M), $^{32}P$-labeled phosphoric acid (0.01 M), and DCDA (0.01 M). Both mixtures remained at room temperature in the dark for 17 hours. They were then chromatographed parallel to one another on Whatman No. 4 paper using a mixture (170) of ethanol and water (8:1) as solvent. Products with very similar $R_f$ values were n oted in the two cases. The product where labeled glycerol had been used was eluted and rechromatographed adjacent to a glycerol phosphate standard using ethyl acetate:acetic acid:water (3:3:1) as solvent (171). The standard was located with molybdate spray and indicated that the labeled product observed with X-ray film had similar chromatographic properties.

The experiment using labeled glycerol was repeated exactly as before. Carrier glycerol-1-phosphate was added to the product solution and together they were spotted on Whatman No. 4 paper. In the first dimension, n-propanol:acetic acid:water (8:8:1) was employed as solvent (170), and phenol:water (9:1) was used in the second direction. Molybdate spray located the carrier and showed that one of the radioactive products noted on the autoradiogram of the chromatogram matched the standard compound in both position and shape (see Figure 14).




ZN-5300

Fig. 14. Autoradiogram of chromatogram exhibiting the formation of glycerol-1-phosphate from glycerol and phosphoric acid using dicyandiamide.

6) Phosphodiester

a) d-ApAp

The linkage of two nucleotides in a natural polynucleotide consists of a phosphate group bonded as an ester to the 3' hydroxyl of one of the pentoses and the 5' hydroxyl of the other member of the pair (90). Such a bond appears to be the primary linkage between the units comprising ribonucleic acids, as well as deoxyribonucleic acids.

Since dimerization by a diester bond in the case of deoxynucleotides (5') immediately eliminates the possibility of a 2'-5' linkage, $^{14}C$-labeled d-AMP was chosen as the reagent for the study. A number of experiments were carried out, each utilizing a method which had previously proved successful in other phosphorylations. The set-ups are summarized in Table III.

Table III. Summary of Experiments to Synthesize the Phosphodiester of Deoxyadenosine-5'-monophosphate

| Expt. | d-AMP* | HCl** | NaDCA*** | DCDA*** | Kaolin**** |
|---|---|---|---|---|---|
| 1 | + | + | + | - | + |
| 2 | + | + | + | - | - |
| 3 | + | + | - | + | + |
| 4 | + | + | - | + | - |
| 5 | + | + | - | - | + |
| 6 | + | + | - | - | - |

* [d-AMP] =0.01 M

** [HCl] = 0.01 N

*** [NaDCA] = [DCDA] = 0.01 M

**** Kaolin = 4 mg (see section on pyrophosphate synthesis)

Aliquots of each solution were chromatographed adjacent to a d-ApAp standard (172) using t-butanol:0.02 M ammonium formate (55:45) mixture as solvent (173). These analyses appeared to indicate that no dinucleotide was synthesized under the conditions of these experiments. Adding phosphoric acid as a reagent did not help. Similarly, NaDCA was unable to promote the synthesis of isopropyl phosphate, a reaction also involving the phosphorylation of a secondary alcohol. (Compare this with the unsuccessful synthesis of AMP (2') and AMP (3') already reported.) This will be discussed further.

b) Surface catalysis

It has been suggested that polyglucosamine (chitosan) might be useful as a surface template in polynucleotide synthesis (68). This might result from an electrostatic bond being formed between the phosphate and the amino group of the respective participants under suitable conditions (see Figure 15).

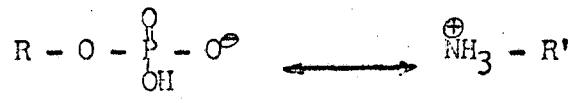


Fig. 15. Proposed bonding between a nucleotide and anmaminopolysaccharide.

Once such an arrangement would occur (sterically it is possible) with a number of nucleotides, internucleotide phosphoesterification might take place between adjacent nucleotides by suitable means (such as DCA). Since chitosan is a β-glucoside, the amino groups of adjacent units are on opposite sides of the polysaccharide chain and linking

of bonded nucleotides would result in a coil. Then the electrostatic bonds with the mucopolysaccharide skeleton would be broken by the strain of the unraveling of the growing polynucleotide chain and an independent polymer would result. Chitosan has been employed experimentally (174) as an ion exchange system for polynucleotides, undoubtedly making use of the type of bonding proposed here. Since glucosamine has a pK of 7.8 (175) and the phosphate group of AMP exhibits a pK of 6. 1 (160), it is likely that the type of bonding proposed in Figure 15 would be most favored around neutral conditions.

Therefore, experiment no. 1 (see Table III) was repeated except that 2 mg of powdered chitosan replaced the kaolin. A magnetic stirrer kept is suspended throughout the reaction period. Also, no HCl was added, and instead the solution was buffered to pH6.8 with 0.2 M cacodylic acid (176). When analyzed for dimer as before, none was observed.

c) Acid anhydride

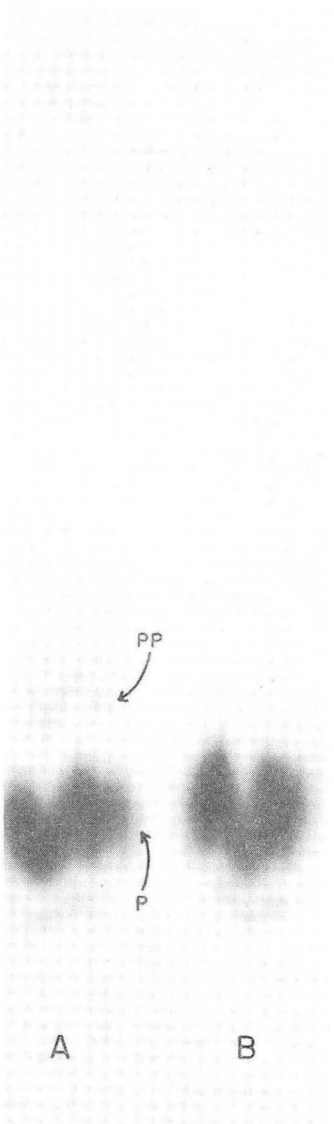
1) Pyrophosphate

The pyrophosphate bond appears to be universally employed by biological systems since this anhydride bond releases a significant amount of energy upon hydrolysis and compounds containing the bond are conveniently stored and distributed (177). The triphosphate of adenosine is the anhydride of phosphoric acid most often used by biological systems for a readily available source of energy. As an energy source, the primary function of the metabolic breakdown of glucose is the production of adenosine triphosphate. Other pyrophosphate-containing compounds, such as 5-phosphoribosyl-1-pyrophosphate, are important in biosynthetic processes (90).

Although synthetic polyphosphates have been used experimentally in the past to synthesize ATP (72), the existence or persistence of such reagents on the primitive Earth is highly questionable. The employment of simple reactants in an aqueous environment probably more closely recreates the situation existing during chemical evolution. Thepossible role of DCDA in promoting the synthesis of the pyrophosphate bond was therefore investigated.

That it was indeed possible to synthesize pyrophosphate itself under primitive Earth conditions was demonstrated by preparing an aqueous solution containing phosphoric acid (0.01 M) and DCDA (0.02 M). After it remained ten hours in the dark at room temperature, the sample was chromatographed adjacent to known standards of orthophosphate and pyrophosphate using isopropanol:water:trichloroacetic acid: conc. $NH_4OH$ (75:25:5:0.5) as solvent. Molybdate spraying of both the standards and the reaction product indicated that pyrophosphate had been produced. No pyrophosphate was observed in the reagent phosphoric acid used.

Quantitative determination of yields was possible by using labeled phosphoric acid (see Figure 16). By eluting the products and measuring them in a Packard Model 527 Tri-Carb Liquid Scintillation Counter, it was shown that when DCDA was allowed to react with phosphroic acid (labeled with $^{32}P$) for 20 hours (0.1 M concentration in both), a pyrophoshate yield of 0.2% was realized. (This figure was corroborated by Drs. Beck and Orgel of the Salk Institute (178)).




ZN-5305

Fig. 16. Autoradiogram of chromatogram demonstrating the production of pyrophosphate (PP) from orthophosphate (P). Experiment A had employed both DCDA and kaolin whereas experiment B had used only kaolin in the course of the reaction.

The suggestion of the possible use of mineral surfaces in catalyzing prebiotic syntheses has already been discussed. Kaolin, a hydrous aluminum silicate mineral, is a good starting point for an investigation of possible catalytic surfaces since the two oxides of which it is composed are in relatively large abundance on the Earth's surface (116). Also, since itis known that the hydroxyl groups on the surface of kaolin, in particular, can be replaced by the $H_2PO_4^-$ ions (179), the possible enhancement of DCDA-mediated phosphorylations by kaolin was considered. It was postulated that a local concentration of phosphate on the surface of the mineral might catalyze the phosphorylation reaction. A solution was prepared containing $^{32}P$-labeled phosphoric acid (0.1 M) and DCDA (0.1 M). Then one ml aliquots of each solution were added to 8 mg of powdered kaolin (the X-ray powder pattern of this kaolin compared well with the periodicities reported in the literature (180)) and both were continually stirred for 40 hours. The kaolin was removed by centrifugation, and a 100 λ aliquot of each sample was analyzed for pyrophosphate by the combination of paper chromatography and scintillation counting (see Figure 16). Kaolin alone did not appear to promote pyrophosphate synthesis, but the combination of kaolin and DCDA gave a net yield of 1.8%. This figure is to be compared with the value of 0.2% yield observed with DCDA alone. This increase in pyrophosphate was also documented with the molybdate colorimetric method (161).

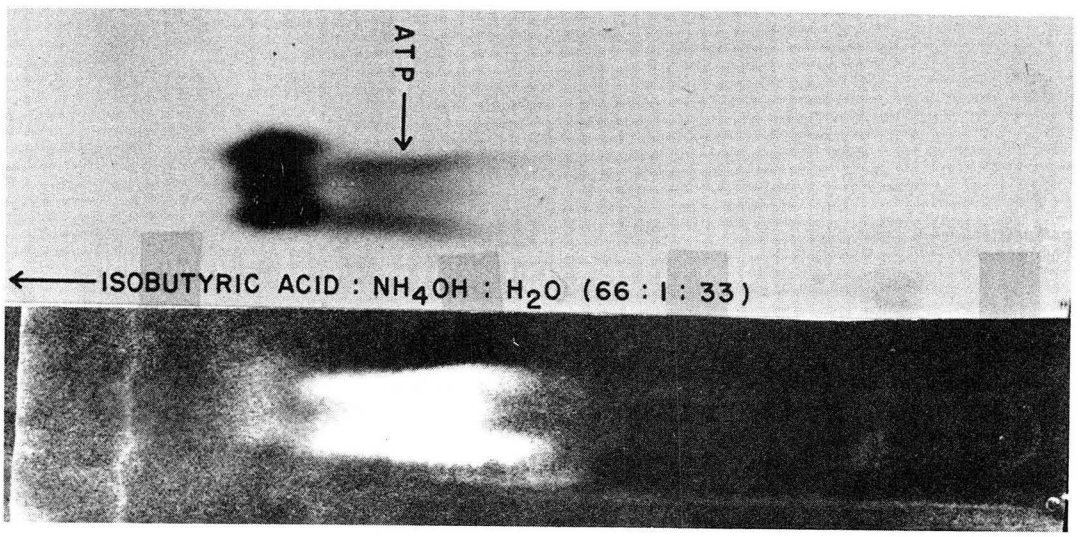
2) Adenosine diphosphate (ADP)

To explore the possibility of synthesizing the pyrophosphate of adenosine, ADP, the following experiment was carried out: A one ml solution of $^{14}C$-labeled AMP ($10^{-3}$ M), phosphoric acid ($10^{-2}$ M), and DCDA ($10^{-2}$ M) was kept at room temperature in the dark for 17.5 hours. Adjacent to the product was chromatographed an ADP standard using isobutyric acid: conc. $NH_4OH$:water (66:1:33) as solvent (160) on Whatman No. 4 paper. A comparison of the shadowgram and autoradiogram of the chromatogram indicated thatone product had very similar chromatographic properties to ADP. This compound was cut out and eluted. To it was added ADP carrier and together they were rechromatographed using the same solvent system. The resultant autoradiogram and shadowgram exhibited a common spot.

3) Adenosine triphosphate (ATP)

The previous experiment was repeated and a radioactive product which possessed a similar $R_f$ to and ATP standard run in parallel was eluted and rechromatographed with added ATP carrier. The results showing the ATP product are found in Figure 18. In this case as well as the previously described ADP case it appears that the elution and rechromatography processing contributed to an appreciable amount of hydrolysis of the desired compounds.

The use of the combination of kaolin and DCDA in the production of ATP was next examined. A one ml solution containing ADP (0.01 M), phosphoric acid (0.01 M), and DCDA (0101 M) was prepared. A similar solution containing no DCDA was also prepared. Each solution was stirred in the presence of 8 mg of kaolin for 24 hours. The kaolin




ZN-5301

Fig. 18. Autoradiogram of chromatogram demonstrating the production of ATP from AMP and phosphoric acid using DCDA. The shadowgram of the standard ATP carrier is also shown.

was then removed by centrifugation and the solution was finally neutralized with NaOH. Samples were analyzed by the luciferase method (182) where the total product solution was added to 250 $\lambda$ of the buffered enzyme solution (isolated from firefly tails) and observed in an Aminco spectrophotofluorometer at 556 m $\mu$. Standard samples of ATP were used to establish a curve to relate enzyme response to ATP concentration. The net yield (after subtracting the small effect due to ADP) of ATP with DCDA and kaolin was of the order of 0.5%. When the reaction was repeated in absence of kaolin, the ATP yield was less than 0.03%. It was demonstrated that DCDA, orthophosphate, and pyrophosphate as well as any material that may have been leached out of the kaolin by the presence of acid did not give a positive luciferase response. Therefore the conclusion that the luciferase response was due to DCDA-mediated synthesis of ATP was apparently legitimate.

It was found that kaolin retarded ATP synthesis when DCA was employed as the condensing agent. Since kaolin binds anions (179) and DCA is largely dissociated in aqueous solution, it is likely that the DCA concentration in free solution was effectively reduced by being bound to the surface of the clay and thus withheld from participating in the desired reaction.

D. Peptide

Several early attempts to synthesize peptides using dicyandiamide gave inconclusive results. For example, ultraviolet light appeared to promote the dimerization of alanine in the presence of DCDA (159). (This observation was later definitely confirmed by another laboratory (183)).

The first definite indication that this compound could effect amino acid polymerization was observed with a solution containing chromatographically purified $^{14}C$-labeled alanine (0.01 M), DCDA (0.01 M), and HCl (0.01 N). After the mixture stood in the dark for 20 hours at room temperature, the product was spotted on Whatman No. 4 paper adjacent to a dialanine standard. Chromatography was carried out using water-saturated phenol:ammonium hydroxide (200:1) mixture (184) as solvent (185). A radioactive spot, shown by X-ray film darkening, that had the same $R_f$ value as alanylalanine (0.79) was eluted from the paper. Carrier dipeptide was added, and the resultant mixture was chromatographed on paper with the organic phase of a n-butanol:acetic acid:water (4:1:5) solvent mixture in the first dimension and isopropanol:water (4:1) in the second. Ninhydrin spray indicated that the position and shape of the carrier were in coincidence with the radioactive product (see Figure 19).

The possible role of phosphate (phosphoric acid) in enhancing prebiotic reactions has already been discussed. The possible importance of phosphorous acid has also been suggested (see Historical Review). Therefore with the possibility that either one might aid the dipeptide synthesis just described, the reaction was repeated with each of the phosphorus compounds present (separately). In the presence of phosphoric or phosphorous acid no alanylalanine appeared to be synthesized, but when the only acid present was HCl the dipeptide was obtained. The absence of a dimer product may have been due to concurrent acid anhydride synthesis.

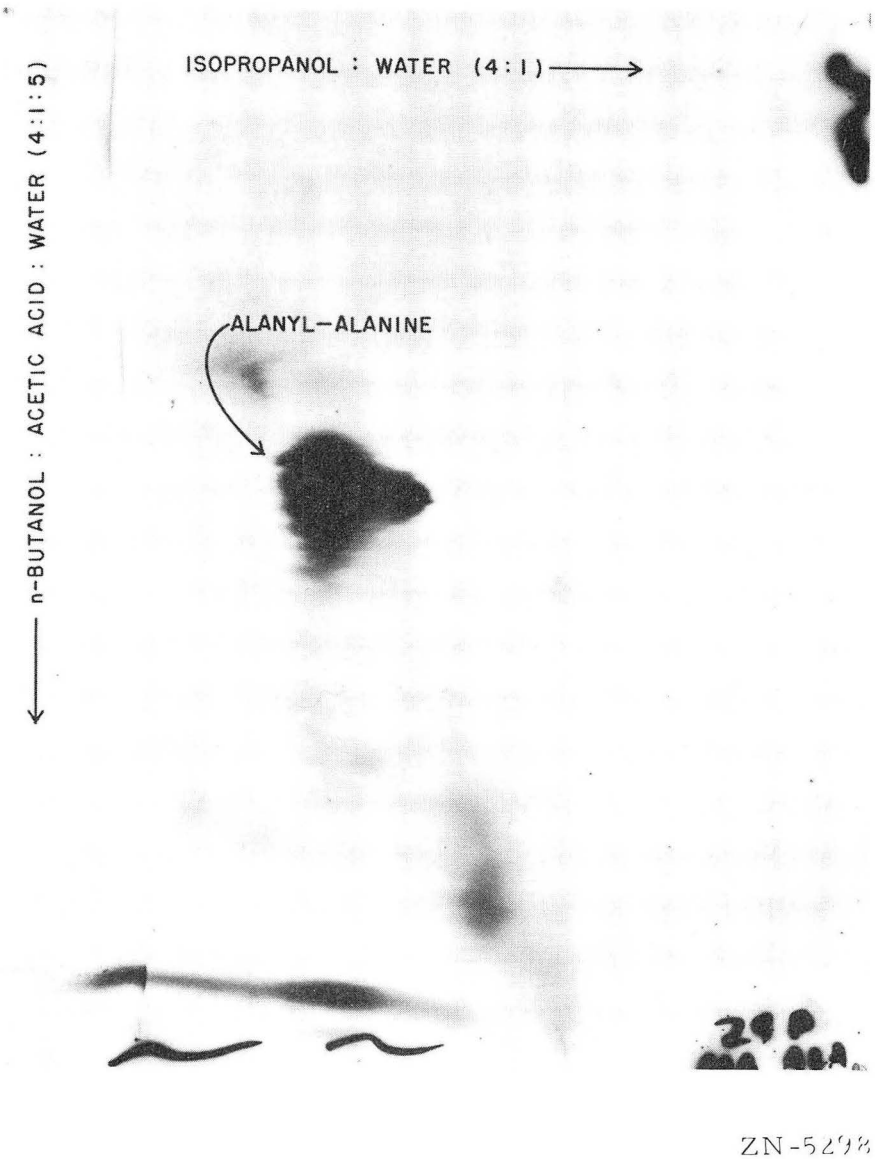



Fig. 19. Autoradiogram of chromatogram showing the alanyl-alanine product synthesized from alanine using DCDA.

It may be noted that in this, as in previous syntheses discussed, the time of reaction and the concentration of reactants were arbitrarily chosen and retained since they gave the products desired under the approximate circumstances believed to have existed on the primitive Earth. Further work — to be discussed later — determined just how correct, from a mechanistic standpoint, these conditions were. For example, it appears that a low pH is advantageous in the dicyandiamide-promoted condensations. When HCl was omitted from the amino acid reaction mixture, with all other conditions (time, temperature, concentrations) remaining the same, only a trace of dipeptide was observed.

In order to prepare product solutions for paper chromatography, their volumes had to be reduced by evaporation under partial vacuum. To determine the effect of this, if any, on the reaction (the first nonphosphorylation attempted thus far), the following experiment was performed: First, the synthesis of the dipeptide of alanine was carried out exactly as before. Second, the synthesis was repeated, but, before evaporation, the dicyandiamide was precipitated with silver acetate. Any residual silver was then removed with potassium chloride, and after filtration the volume of the resultant solution was reduced for chromatography. Third, another solution of alanine and HCl in the same proportions as before was prepared and evaporated to a small volume. However, this solution at no time contained dicyandiamide. The three solutions, after appropriate time, were chromatographed with the water-saturated phenol: conc. $NH_4OH$

(200:1) solvent. The yields were measured with the Geiger-Müller counter. The second solution exhibited the same degree of dipeptide synthesis as when DCDA had not been removed before evaporation. The third solution appeared to indicate only a tracer of dimer. The original appearance of alanylalanine may thus be largely attributed to the action of dicyandiamide.

To investigate the possibility of synthesizing tripeptides, a one ml aqueous solution was prepared containing L-alanine (0.1 M), L-alanyl-L-alanine (0.1 M), DCDA (0.1 M), and HCl (0.1 N). A similar solution was prepared without DCDA. Both solutions remained in the dark at room temperature for 24 hours. Then one ml of 0.1 N NaOH was added to each sample which was then trasferred to a 10 ml volumetric flask and diluted with water to the volume of the container. The resultant mixture was added to 5 ml of biuret reagent (500 ml of aqueous solution containing 0.75 gm of $CuSO_4$, 3.00 gm of NaK tartrate, and 15 gm of NaOH (186)) and was allowed to stand for 30 minutes. An aliquot of each solution was observed in the Beckman Model DU spectrophotometer at 570 mμ. The net results appeared to indicate that a small amount of tripeptide had been formed.

To observe this tripeptide chromatographically, a solution was prepared containing $^{14}C$-labeled alanine (0.2 M), HCl (0.01 N), and DCDA (0.2 M). After the solution remained in the dark at room temperature for 18.5 hours, an aliquot was chromatographed on Whatman No. 4 paper adjacent to a trialanine standard using the water-saturated phenol: conc. $NH_4OH$ (200:1) solvent. Appropriate locating methods permitted elution of a suspected radioactive product at the

position of the trimer. Carrier was added and the mixture was chromatographed with the same two solvent systems used earlier for this purpose. A trace of alanylalanylalanine was finally observed.

The possible catalytic role of mineral surfaces during chemical evolution has already been discussed. Attention has been given to clays such as montmorillonite and kaolin, since they are known to bind organic bases (187) and thus might provide a local concentration of reacting units. Kaolin has been successfully employed in this work as a catalytic surface in the synthesis of anhydrides of phosphoric acid (see section on acid anhydrides). Therefore, the possible use of these two clays to promote dipeptide synthesis was next considered. The montmorillonite (whose identity was also verified by X-ray analysis) was sonicated in suspension in 0.01 N HCl with a Bronwill Biosonik for 15 minutes. Since the kaolin was already powdered, it was used directly. Then, the synthesis of alanylalanine using DCDA was repeated as before except thatportions of montmorillonite, kaolin, alumina, and silicic acid were added to aliquots of the reaction mixture. The last three solutions had to be kept in suspension with continuous application of a magnetic stirrer. After the reaction period all solutions were neutralized and centrifuged to remove the clays. Paper chromatography with the same solvent system previously described indicated that the yield of dipeptide was not enhanced by the presence of the clays and in the case of montmorillonite appeared to decrease presumably due to the binding (and resultant inactivation) of the DCDA as well.

Earlier it was noted that dicyanamide (DCA) was a more effective phosphorylating agent than dicyandiamide (DCDA). (See section on glucose-6-phosphate synthesis.) Therefore, in an attempt to find compounds which might act as more effective peptide bond-synthesizing agents (since this, like phosphorylation, is a dehydration condensation reaction) than DCDA, the possible use of DCA was considered. The synthesis of alanylalanine previously described using labeled alanine was repeated using sodium dicyanamide (NaDCA) in place of dicyandiamide. In this case a yield of 1.58% of the alanine dimer was observed after 20 hours of reaction time which was three times the amount noted when DCDA was employed.

To investigate the application of dicyanamide in the synthesis of polypeptides, as well as the relative reactivity of amino acids and peptides, the following experiment was performed (165): A 100 λ aqueous solution was prepared containing glycine-2-$^{14}$C (0.12 M), triglycine (0.12 M), NaDCA (0.12 M) and hydrochloric acid(0.12 N). A similar solution was prepared containing no NaDCA. After the mixture remained 21 hours in the dark at room temperature, 20 λ aliquotes were removed from each solution and were spotted on strips of Whatman No. 1 filter paper together with standards of diglycine and tetraglycine. These samples were then subjected to low-voltage horizontal electrophoresis in borate buffer at pH 9.2 (188). X-ray film located the labeled products and ninhydrin identified the carriers. Radioactive bands coinciding with the standard diglycine and tetraglycine were observed. The determination of the activity associated

with each band indicated that the yield of tetraglycine was about 6.1% and that of diglycine was about 2.3%, both based on initial glycine.

E. Carboxylic Ester (Lipid)

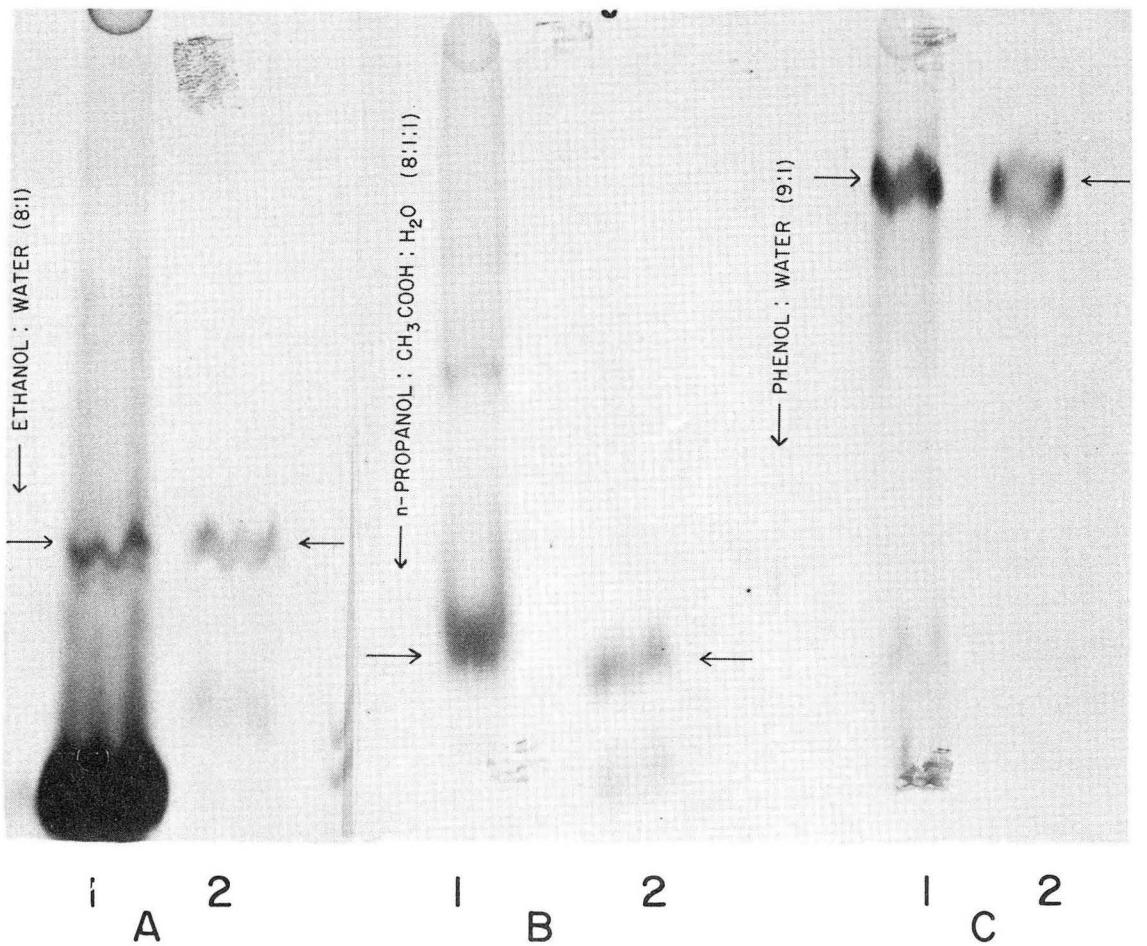
Lipids constitute an important class of compounds in biological systems (90,180). They are usually fatty acid esters of glycerol with various combinations of phosphates,amines, and alcohols. The most common characteristic linkage in acylic (and some alicylic) lipids is the ester bond between glycerol and a fatty acid. These fatty acids are all straight chain members of the acetic acid series of monocarboxylic acids. The production of a carboxylic ester is another example of a dehydration condensation reaction. So that the reaction might be carried out in an aqueous environment, glycerol-1-acetate, or monoacetin, was selected as the compound to be synthesized since it is soluble in water, the radioactive tracers of both the glycerol and acetic acid are readily available, and it is the simplest example of a condensation of a fatty acid and glycerol to form a lipid-type compound.

A solution of $^{14}C$-labeled glycerol (1.7 mM), acetic acid (0.01 M), and DCDA (0.01 M), as well as a solution of $^{14}C$-labeled acetic acid (0.01 M), glycerol (0.01 M), and DCDA (0.01 M) were both kept in the dark at room temperature for 20 hours. Aliquots were then chromatographed adjacent to one another using a mixture of ethanol:water (8:1) as solvent. A common spot was observed with X-ray film. The spot was eluted in each case, divided in half, and chromatographed in parallel with its corresponding fraction from the other experiment using n-propanol:acetic acid:water (8:1:1) as solvent (170) in one case and phenol:water (9:1) as solvent in the other. Both samples indicated

similar fractions in each solvent (see Figure 21), suggesting the production of a compound containing both acetate and glycerol (quite possibly the acetate ester of glycerol).

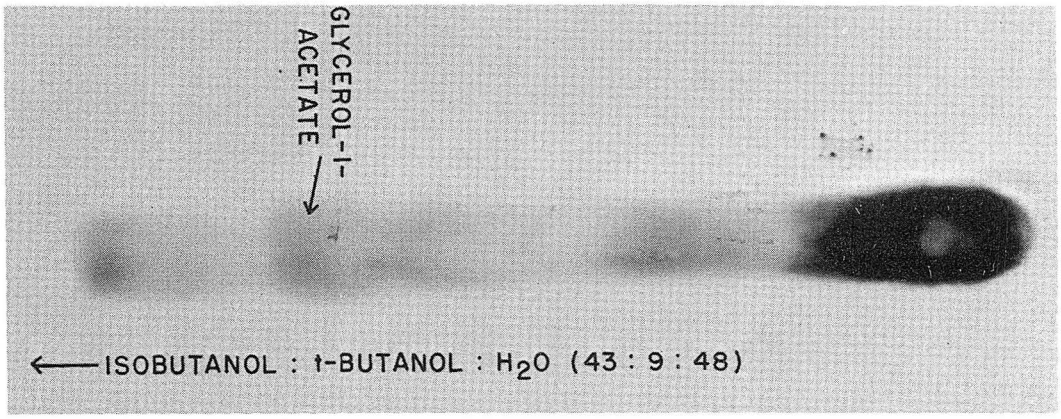
The experiment using labeled acetate was repeated with a solution compounds of the same reagents; the solution was kept in the dark at room temperature for 45.5 hours. Since the unused "hot" acetic acid is volatile, it was easily removed from the solution upon spotting on the chromatography paper and thus left only nonvolatile labeled products to be observed. Standard monoacetin was added to the product and together they were chromatographed on Whatman No. 4 paper using the upper phase of mixture containing isobutanol:t-butanol:water (43:9:48) as solvent. X-ray film located the labeled products. The standard was located by spraying the completed chromatogram with 0.1% sodium meta-periodate solution followed by a spray made up by adding 70 ml of water, 30 ml of acetone, and 1.5 ml of 1 N HCl to a solution of 2.8 gm of benzidine in 80 ml of ethanol (190). Since this reagent requires vicinal glycols, glycerol-1-acetate is the only acetate ester of glycerol thatcould be detected. The presence of glycerol-1-acetate as a product was substantiated (see Figure 22).

The previous experiment was repeated. Also, a second solution was prepared in the same manner but an equimolar amount of NaDCA replaced the DCDA. A third solution contained neither one. After 50 hours, aliquots were removed and chromatographed on Whatman No. 4 paper adjacent to glycerol and monoacetic standards. When the X-ray film was developed after three days, only the reaction which had employed NaDCA exhibited glycerol-1-acetate. (A longer exposure had




ZN-5307

Fig. 21. This illustrates an autoradiogram of three separate chromatograms suggesting the production of a compound (possibly an ester) containing glycerol and acetate (using dicyandiamide). In each chromatogram, column no. 1 exhibits the experiment in which $^{14}C$-labeled glycerol had been used and column no. 2 is the one where labeled acetic acid was employed in the same reaction scheme. The arrows indicate the products with very similar $R_f$ values. This product in chromatogram A was eluted and rechromatographed as shown in chromatograms B and C. This would suggest that the designated compound is the same in each case and contains both acetate and glycerol.




ZN-5304

Fig. 22. Autoradiogram of chromatogram showing the synthesis of glycerol-1-acetate from glycerol and $^{14}C$-labeled acetic acid with dicyandiamide.

been used in the earlier experiment with DCDA only.) This would suggest, first of all, that the synthesis could be attributed to the added condensing agent and, secondly, that NaDCA works better than DCDA in this reaction. Although the yield was small (∿ 0.01%), the acetate ester had definitely been produced. The use of acetic acid, a relatively weak acid, rather than a stronger acid to regulate pH (in addition to acting as a reactant) undoubtedly accounted for the low yield.

An attempt was made to synthesize the silyl ethers of glycerol and monoacetin, and analyze them on an Aerograph Vapor Phase Chromatography apparatus, but the few columns tried failed to give the needed separation of the two derivatives so that the method was abandoned.

F. Glycoside (Disaccharide)

1) Introduction

Polysaccharides serve a number of functions in biological systems (191). First of all, they play a structural role in plant cell walls and the shells of crustaceans. As mucopolysaccharides they act as important components of blood. Also, they serve as a means by which chemical energy can be transported and stored, such as sucrose and glycogen. The most common disaccharides are dihexoses. Linkage between the two hexoses is achieved by an oxygen bridge between the hemiacetal hydroxyl of one of the units and one of the hydroxyls of the second.

The formation of the glycoside bond is another example of a dehydration condensation, but, in contradistinction to all previous

condensations which have been successfully promoted by DCDA or NaDCA, this one involves the reaction between two hydroxyl groups rather than one acid function and one hydroxyl group. It was discussed earlier that HCN might act as an agent to promote dehydration condensation (119). Therefore, the possible application of HCN to diglucose formation was first considered.

2) Diglucose

Early results in this work suggested that the application of ultraviolet to a solution of glucose, sodium cyanide, and hydrochloric acid produced dimers of the carbohydrate. A large number of solvent systems were tried in the chromatographic analysis but did not permit positive conclusions to be drawn because of incomplete separation of products. Finally, it was found that the best solvent system for the separation of glucose from its dimers is ethanol:methanol: water (9:9:2). The application of this system permitted definite product identification. Similarly, previous results indicated that sodium dicyanamide could operate effectively as an agent to promote dehydration condensation. Both cyanide and DCA absorb (are excited by) ultraviolet light, as observed in the Cary Model 14 spectrophotometer. Therefore, a solution was prepared containing $^{14}C$-labeled glucose (0.01 M), NaCN (0.01 M), and HCl (0.01 N). The sample was divided inhalf and the first portion (A) was irradiated with ultraviolet light for 4 hours while the second (B) remained in the dark for the same period. Another solution was prepared containing $^{14}C$-labeled glucose (0.01 M), NaDCA (0.01 M), and HCl (0.01 N). This mixture was divided in half and the first portion (C) was irradiated by the UV lamp for 4 hours while the second (D)

remained in the dark. Finally, another solution was prepared containing labeled glucose (0.01 M), and HCl (0.01 N). Half of the solution (E) was irradiated for 4 hours while the other half (F) was kept in the dark. An aliquot of each product solution was applied to Whatman No. 4 paper and chromatographed in parallel with samples of maltose and gentiobiose using ethanol:methanol:water (9:9:2) as solvent. The standards were located with benzidine spray. The results are summarized in TableIV. (Other standards of diglucose were unnecessary since all have about the same $R_f$ in the solvent employed.)

Table IV. Summary of Experiments to Synthesize the Dimer of Glucose

| Expt. | Glucose | NaCN* | NaDCA* | UV** | HCl* | Diglucose Product |
|---|---|---|---|---|---|---|
| A | + | + | - | + | + | - |
| B | + | + | - | - | + | - |
| C | + | - | + | + | + | + |
| D | + | - | + | - | + | - |
| E | + | - | - | - | + | + |
| F | + | - | - | - | + | - |

* 0.01 M

** 4 hours

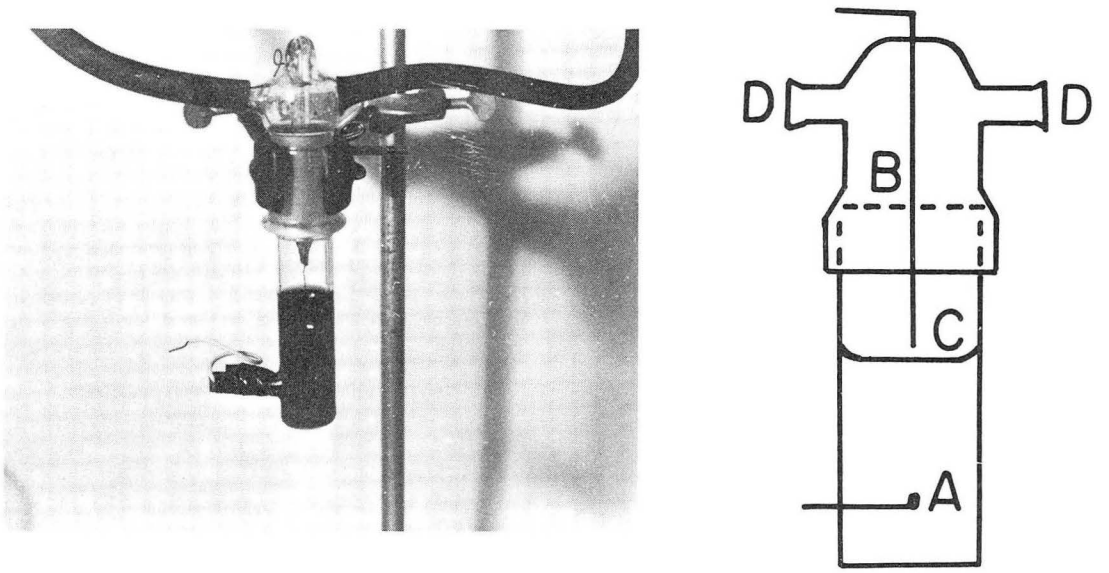
No appreciable difference in the yield was noted between the UV-irradiated sample that contained DCA and the one which did not have it. The absence of dimer when cyanide was present may have been due to the production of the cyanohydrin which prevented reaction in the desired manner. The close similarity in $R_f$ values did not permit the differentiation as to which glucose dimer had been produced. Alcohols can add across the cyano group of DCDA in the presence of copper ion (150). When the irradiation was done in the presence of DCDA or DCDA plus copper sulfate no improvement was observed. Thus, the addition of these agents (HCN, DCA, or DCDA) does not appear to aid in promoting disaccharide synthesis with or without ultraviolet irradiation. This is to be contrasted with the appearance of dimer with UV illumination alone. Thatlight in the vacuum ultraviolet region can be used to synthesize disaccharides has been observed previously (192).

3) Chitobiose

Amino sugars are important as blood factors and as structural units in crustacean shells (175). The primary interest in them here, as discussed earlier in connection with polynucleotides, is that with a periodically recurring charged group such as in polyglucosamine (also called chitosan or deacetylated chitin), with one amino group on each glucose moiety, polymers of amino sugars could conceivably have acted as a matrix for ordering and assembling other polymers of biological importance under primitive Earth conditions. It was first necessary to demonstrate that amino sugars could be synthesized on the monomer level.

Previous results (49, 68) have shown that by passing an electrical discharge into an aqueous solution containing an alcohol in the presence of nitrogen, an amino group can be added to the compound. The sparking apparatus employed (see Figure 23) had one platinum electrode submerged within the reaction solution and the other suspended about 5 mm above the liquid surface. The vessel was designed to contain a 10 ml solution within the chamber. The vessel was closed after filling and remained open only to the ventilation ports at the top which brought in an released acid- and thymol-filtered air under pressure. This arrangement removed undesired gases resulting from the sparking process. Filtration of the air before entry enhanced its microbiological purity. The spark was supplied from a high voltage transformer directly connected to the chamber's electrodes. In this manner, a visible spark passed continuously between the upper electrode and the solution surface, which served as the other electrode. A magnetic stirrer mixed the solution continuously. To minimize the possibility of contamination, the glass reaction vessel was cleansed with chromic acid solution each time before use.

A 1% aqueous solution of glucose in (0.01 N HCl) was sparked for 2 hours. Then the Moore-Stein ninhydrin test (193) and Elson-Morgan test (194) were performed on the product. (The first test was carried out by adding 1 ml of the product to 1 ml of a reagent solution prepared by adding 0.8 gm of stannous chloride in 500 ml of 4 M sodium acetate buffer to 20 gm of 1,2,3-triketohydrindene hydrate in




ZN-5309

Fig. 23. Photograph and schematic drawing illustrating the sparking vessel with submerged electrode (A), suspended electrode (B), liquid surface (C), and ventilation ports (D).

500 ml of methyl cellosolve. The mixture of the reagent and product solutions was then heated for 20 minutes in a boiling water bath and the appearance of a blue color was observed in an $\alpha$-amino sugar were present. The second test was performed by adding 0.5 ml of the product to 0.5 ml of a reagent prepared by mixing 0.75 ml of fresh acetylacetone with 25 ml of 1.25 N sodium carbonate. This combination of reagent and product was heated in a boiling water bath for 20 minutes and then added to 4 ml of 96% ethanol and 0.5 ml of a reagent prepared by dissolving 1.6 gm of p-dimethyl-aminobenzaldehyde (Erlich's reagent) in 30 ml of conc. HCl and 30 ml of 96% ethanol. After the solution stood for one hour, the appearance of a red color indicated the presence of a compound containing an amino group in the alpha position to an aldehyde carbonyl.) The sparked glucose solution exhibited positive reactions to both of these tests, as did glucosamine.

Next, a solution was prepared containing $^{14}C$-labeled glucose (0.02 mM) and HCl (0.012 N) and was sparked under filtered nitrogen for 4 hours (ammonia would probably work as well). The volume of the solution was then decreased by evaporation under reduced pressure. Standard glucosamine-HCl was added and together they were spotted on Whatman No. 4 paper. Chromatography was carried out with pyridine: ethyl acetate:acetic acid:water (5:5:3:1) as solvent (195) in the first dimension, and the organic phase of a mixture of n-butanol: acetic acid:water (4:1:5) as solvent in the second dimension. Ninhydrin spray located the carrier and X-ray film showed that one of the labeled products coincided in position and shape to the glucosamine-HCl standard.

The addition of amino groups to cellobiose, the β1-4 dimer of glucose, was next demonstrated. (Chitobiose is the β 1-4 dimer of glucosamine.) When 100 mg of cellobiose was dissolved in 10 ml of 0.012 N HCl and sparked for 2.5 hours, the product solution exhibited positive Elson-Morgan and Moore-Stein reactions.

To combine the use of ultraviolet light for dimerization of the sugars and sparking for the replacement of a hydroxyl group by an amino group, the synthesis of chitobiose, the basic unit of chitosan, was finally attempted. The sparking chamber (see Figure 23) was modified with a small quartz window placed in its side so as to permit direct UV irradiation of the solution being simultaneously sparked. A 10 ml solution was prepared containing $^{14}C$-labeled glucose (2 mM) and dicyandiamide. This mixture was irradiated (spark and UV) for 6 hours. Standard chitobiose was isolated by the usual procedure (196). When chromatographed with n-butanol:pyridine:water (6:4:3) as solvent, the isolated standard exhibited an $R_{glucosamine}$ of 0.65, whereas the published value (196) is 0.64. Thus, the identity of chitobiose was substantiated. An aliquot of this chitobiose was added to the UV- and spark-irradiated glucose sample and together they were chromatographed with the butanol-pyridine-water solvent. X-ray film located the labeled product and ninhydrin noted the position of the chitobiose carrier. Both the shape and $R_f$ of one of the labeled products matched the ninhydrin-positive spot, suggesting the production of chitobiose.

Summary and General Comments

Thus, it may be seen that dicyanamide and dicyandiamide can effect a variety of dehydration condensations in aqueous solution under presumed primitive Earth conditions. These syntheses are summarized in Table V. The designation of a particular compound with a (o) under the listing of one of the condensing agents does not indicate the inability of the reaction to occur but rather that the particular synthesis using that agent was not attempted. On the other hand, a negative sign indicates failure in the attempted synthesis.

It may be observed that dicyandiamide was unable to accomplish the dehydration condensation of adenine with ribose to produce adenosine, either by itself or with the assistance of ultraviolet light. This is to be contrasted with the successful formation of adenosine in the presence of orthophosphate and ultraviolet light. Since it is uncertain if the condensation of adenine and ribose is a simple reaction, merely involving the direct union of the two units with the concomitant removal of a molecule of water, or if it is a complex series of steps, the possible role of dicyandiamide and related compounds in the dehydration condensation of other biologically important compounds cannot be ruled out just from these first negative results. The proposed mechanism (to be discussed more fully later) of dicyandiamide action suggests that the compound would operate in those syntheses involving simple union of the compounds involved and would therefore probably be inoperative in a more complex reaction.

Table V. Compounds Synthesized with the Aid of DCA and/or DCDA in Aqueous Solution*

| Bond | Compound | DCDA | DCA |
|---|---|---|---|
| 1. Nucleoside | | | |
| | Adenosine (from adenine and ribose) | - | o |
| 2. Phosphate ester | | | |
| | Adenosine-5'-phosphate (from adenosine) | + | o |
| | Ribose-5-phosphate (from ribose) | + | o |
| | Glucose-6-phosphate (from glucose) | + | + |
| | O-phosphoserine (from serine) | + | o |
| | Glycerol-1-phosphate (from glycerol) | + | o |
| | d-ApAp (from d-adenosine-5'-phosphate) | - | - |
| | Isopropyl phosphate (from isopropanol) | o | - |
| 3. Acid anhydride | | | |
| | Pyrophosphate (from orthophosphate) | +** | o |
| | Adenosine diphosphate (from AMP) | + | o |
| | Adenosine triphosphate (from ADP) | +** | o |
| 4. Peptide | | | |
| | Dialanine (from alanine) | + | + |
| | Trialanine (from alanine) | + | o |
| | Diglycine (from glycine) | o | + |
| | Tetraglycine (from triglycine & glycine) | o | + |
| 5. Carboxylic ester | | | |
| | Glycerol-1-acetate | + | + |
| 6. Glycoside | | | |
| | Diglucose | - | - |

+ = success
- = failure
o = synthesis not attempted
* = without UV
** = synthesis enhanced by kaolin

For more data see Tables I - IV

With regard to the dinucleotide synthesis, it is significant to note that in this reaction it was necesaary to phosphorylate a secondary hydroxyl to achieve the desired linkage. This was as unsuccessful as other attempts to carry out the synthesis of compounds with secondary phosphates, in particular AMP (2'), AMP (3'), and isopropyl phosphate. It would appear that steric hindrance and reactivity (nucleophilicity) might have been the causes making the primary hydroxyl of a cyclic sugar more responsibe to phosphorylation than the secondary hydroxyls. The failure of chitosan to promote dinucleotide synthesis can be attributed to the fact that DCA apparently requires an acidic environment to operate effectively, which was not the condition in the experiment performed in this case. Condensing agents which can operate well around neutral pH might demonstrate the usefulness of the glucosamine polymer as a surface catalyst after all.

It may be recalled that the synthesis of glycerol phosphate as well as glycerol acetate was observed in the course of this work. Therefore, a reasonable explanation is arrived at for the possible primordial origin of phospholipids which may have been needed for the first biological membranes to have appeared. A major problem remaining is how longer chain fatty acids could have reacted in aqueous solution, since they are relatively insoluble, to form the more common glycerides. It is possible that eobiots utilized shorter fatty acids and that the longer ones are a comparatively recent innovation. It is also plausible that protobiological systems did not utilize lipids in their membranes but instead used polysaccharides, such as

dextrans, as found in the cell walls of plants and bacteria (191), or some other agent that could have served the purpose on a primitive level.

It is interesting to note that the successful reactions employing DCDA and DCA involved acids as reactants and generally gave yields of the order of 1%. That diglucose synthesis, involving the union of two hydroxyl-reacting compounds, was unsuccessful with just the mediation of either condensing agent is not surprising. For example, the dialkylcarbodiimides are able to react with hydroxyl compounds only in the presence of appropriate catalysts and rather vigorous conditions (197).

With regard to polysaccharide synthesis, in particular, it does not seem reasonable that by the random syntheses available during primeval chemical evolution a long chain of glucose units could be linked by 1-4 bonds exclusively as would be needed in a cellulose molecule, for example. The results already cited in fact appear to favor the 1-6 linkage in random syntheses (112). The work reported here on the phosphorylation of glucose would also seem to suggest the greater kinetic and steric availability of the primary hydroxyl of the no. 6 carbon in contradistinction to the other hydroxyl groups. The 1-6 linkage in biological systems is usually restricted to energy-storing polysaccharides, such as glycogen, but is also found in the usual dextrans of bacteria and fungi (191). Some of the dextrans serve as food reserves and others are incorporated in the capsular sheath of bacteria, while another group of these polysaccharides apparently have no function other than being side-products of meta-

bolism. Analysis of a large number of dextrans indicates that the primary linkage between adjacent glucose units is an α 1-6 bond (50-97%) while the 1-4 bond (0-50%) and 1-3 bond (0-40%) are found to a lesser extent (212). A 1-4, 1-5 linked glucose polymer has also been isolated (213).

With the possible exception of these seemingly random bacterial dextrans, it does not appear likely that sugar polymers with only one type of repeating linkage could have been synthesized without the usual biological mediators, i.e., enzymes (polypeptide catalysts). If, for example, two glucose molecules join by a 1-4 linkage, there does not appear to be anything to prevent the formation of a 1-2, 1-3, or 1-6 linkage of a third glucose unit to the dimer as easily as another 1-4 bond. A 1-1 linkage is also plausible. Also, many present day polysaccharides involve regular alternations between two different sugars (191). Such regularity is unlikely in a random synthesis. On the other hand, dextran-like polymers may have been quite useful to eobiots for employment in the construction of cell barriers. It thus appears that from the point of view of the means conceivably available during chemical evolution (acid, UV light, and the like) that the regular polysaccharides, such as cellulose, chondroitin, and amylose, probably had to wait for the appearance of biocatalysts, whereas random polymers such as the dextrans could very well have played an instrumental role in bringing about the initial formation of unique protobiological systems.

It would appear a priori that a nonspecific dimerization of glucose could produce any of the eleven possible dimers, thus lacking the bond specificity essential for the synthesis of a particularly required disaccharide. In the case of diglucose, eleven pyranosyl dimers are possible since there are four reactive hydroxyls and also the hemiacetal hydroxyl can assume an α or a β configuration. In natural polysaccharides, the 4-linkage and the 6-linkage appear most often with a predominance of the former (191). It may be noted that by acid-reversion (0.08 N HCl at 98° C for 10 hours) of the D-glucose, eight of the eleven possible dimers have been produced and identified (112,214-218). The other three were thought to be present as well. Although the yields were similar, there appeared to be a preference for the 6-linked disaccharides (not the 4). Thus, one would expect a nearly random distribution of glycoside linkages formed during chemical evolution.

In DCA-mediated peptide synthesis it was reported here that when both glycine and triglycine are available in the reaction solution the tetramer is preferentially produced over the dimer of glycine. In support of this it is known that the (positive) free energy of formation of the tetramer from the dimer, for example, is about half as great as for the synthesis of the dimer of glycine from the monomer (218a). This is probably due to the fact that in the monomer a high charge field is set up by the proximity of the terminal charges of the zwitterion, tending to repel an incoming ammonium or carboxylate group. In the trimer, these charges are much further removed, thus offering less resistance.

It is evident that members of the cyanamide group of compounds are indeed effective in the promotion of a variety of condensations in aqueous solution under presumed primitive Earth conditions. The experiments described have held to the restrictions set up by the attempt to simulate and duplicate chemical evolution, including:

1) simple, relatively stable reactants,
2) aqueous environment,
3) moderate temperature, and
4) dilute concentrations.

These restrictions would eliminate the employment of the more complex dialkylcarbodiimides, the less stable polymetaphosphates, the hotter, kinetically desirable circumstance of an anhydrous volcanic environment, and the more favorable condition of a nonaqueous solvent for carrying out a dehydration condensation reaction.

By the use of cyanamide, dicyandiamide, and dicyanamide various esters and anhydrides have been synthesized. Although the yields were small from a preparative standpoint, the prospect of greater yields over the long time period in which chemical evolution is believed to have occurred (198) would compensate for this.

The possible role of cyanamide chemistry during chemical evolution discussed earlier is now emphasized by the successful application of compounds derived from cyanide to further problems. It is quite possible that additional compounds or conditions will be found to effect the syntheses described here. It must be emphasized that these experiments were designed to demonstrate one plausible means by which such compounds could have been formed on the prebiotic Earth, thus providing materials needed for the origin of living systems. It was

necessary materials needed for the origin of living systems. It was intended that these demonstrations might give a clue to the general means by which condensations were effected by these condensing agents or related compounds. It is also possible that the types of bonds synthesized here arose by two or three different means operating at the same time, including the method described here.

That both dicyandiamide and dicyanamide could have existed on the primitive Earth has been demonstrated in the laboratory. First of all, DCDA was synthesized by UV-irradiation of an ammonium cyanide atmosphere (199). It is significant to recall that cyanide is a key intermediate when an electrical discharge is passed through this type of gaseous mixture (26) and probably played an important role in the synthesis of DCDA just mentioned. Also, melamine, the trimer of cyanide, has been reported in the Orgueil meteorite (200), and it is known that dicyandiamide is readily converted to melamine at about 200° C (130). Recently DCA was synthesized by γ-irradiation of an ammonia-carbon tetrachloride solution (201). Preliminary results from this laboratory suggest that DCA can be produced by UV-irradiation of ammonium cyanide solutions.

Since DCDA and DCA appear to operate as condensing agents best at low pH, the question remains as to the likelihood of such a situation on the primitive Earth. The presence of carbonates in mineral deposits (116) would favor an alkaline environment. However, it has been pointed out, for example, that the redox reaction between hydrogen gas and cupric ion to give hydronium and cuprous ions becames

autocatalytic once initiated and can lead to an accumulation of product ($H^+$) if given sufficient reagnet (202). The presence of $H_2$ in the primitive atmosphere is now commonly accepted (8), giving credence to the suggestion. As will be reported in the next section, DCA-mediated reactions, although most active at low pH, give detectable yields under neutral conditions as well. Thus, the plausibility of these experiments duplicating primordial events is realized.

# Part II:

## STUDIES ON THE MECHANISM OF OPERATION OF DICYANAMIDE AND DICYANDIAMIDE AS CONDENSING AGENTS

### Introduction

Now that the observations were made of the capacity of dicyanamide and dicyandiamide to act as agents in promoting dehydration condensation in aqueous solution, it was necessary to establish the mechanism by which these compounds operate. This information would permit further insight into the nature of the reactions so as to allow additional applications and improvements of the procedure.

The first step in the analysis was to study the physical properties of dicyandiamide to learn more of the nature of the compound and thus perhaps be able to better understand the mode of operation of it. These properties were studied by nuclear magnetic resonance spectroscopy as well as ultraviolet and infrared spectrophotometry.

The next step was to pick out one of the condensation reactions known to be promoted by DCA and DCDA (in this case, dipeptide synthesis) and to examine it in detail to elucidate the nature of the reaction, its response to various conditions, and the possible role of catalysts in it.

The resultant data permitted certain conclusiosn to be drawn about the reaction, as will be discussed.

Experimental Methods — General

1) Chromatography

The chromatography (paper) used in this group of experiments was carried out in the same general manner as described in Part I.

2) Spectroscopy

UV spectra were observed by the usual method in fused silica cuvettes using a Cary Model 14 apparatus. IR spectra were observed in a Perkin-Elmer Model 137 or Beckman IR7 spectrophotometer with the compound under study dissolved in an appropriate solvent or pressed into a KBr pellet. NMR spectra were recorded with a Varian Model A-60 spectrometer. Besides giving characteristic hydrogen patterns, the NMR procedure was used to determine the Arrhenius activation energy of protonation. The following scheme, using standard equations (203), was employed:

$$\frac{S}{S_\tau} = \left[\frac{1}{2\pi^2\tau^2(\nu_A - \nu_B)^2}\right]^{1/2}$$

$$\tau = \left[\frac{1}{2\pi^2(\nu_A - \nu_B)^2\left(1 - \frac{S}{S_\tau}\right)^2}\right]^{1/2}$$

Data collected: $\nu_A$, $\nu_B$, T

T = absolute temperature

$\nu_A - \nu_B$ = separation of peaks in cps (cycles per second)

S = separation of peaks at given T

$S_\tau$ = separation of peaks at large $\tau$ (exchange time)

Also, $\tau = 1/K$, where K is the first-order reaction rate constant. In addition, $\ln \tau = E_a/kT$, where $E_a$ is the energy of activation and k is the Boltzmann constant. A graphic plot of the data gives $E_a/k$, which in turn gives $E_a$.

3. Paper Electrophoresis

The primary analytical method used in the analysis of the mechanism based on the yield of products under various conditions was low voltage, horizontal paper electrophoresis with borate buffer at pH 9.2 (188). Strips of Whatman No. 1 paper, measuring 1.5 inches by 21 inches, were used. Electrophoresis was carried on for 8.5 hours at 300 volts and 5 ma using a Hewlett-Packard Model 712B power supply. X-ray film located the labeled products and appropriate sprays located the carriers.

4. Spectrophotometric Determination of pK

To determine spectrophotometrically the pK of a compound it is first necessary to find an absorption maximum which has different extinction coefficients for the protonated and ionized forms. Then the compound is dissolved in solutions of various acid concentrations. The absorbancy of each resulting solution is read in a Beckman Model DU spectrophotometer at the selected wavelength where each zero point adjustment is made with a comparable acid solution. The resultant data are used in the formula:

$$pK = H_o - \log \frac{(A_\lambda - A_{AH})}{(A_{A^-} - A_\lambda)}$$

where $A_{AH}$ is the absorbancy for the protonated form of the molecule, $A_{A^-}$ is the absorbancy for the ionized form, and $A_\lambda$ is the absorbancy

at the given wavelength of a value about midway between the two plateaus (see Figure 29) at a given acidity which then also defines the $H_o$ of the equation (204). The pK is thus determined.

NOTE:

The experiments described in this section are arranged in terms of the sequence in which each provided information leading to the next. For example, the study of the time-course of the reaction laid the foundation for work on varying the concentrations of the reactants. Similarly, the infrared studies led to an investigation of the effect of pH on the reaction.

As before, each experiment is introduced with a comment on the particular relevance of the procedure being studied to the overall problem, followed by a description of methods used and the data collected. Finally, a short discussion is presented of the results, their ramifications, and significance to the elucidation of the overall mechanism.

## Experimental Data and Discussion

### Physical Studies on Dicyandiamide

#### 1) Ultraviolet spectrum and effect of UV irradiation

It was first necessary to establish the UV spectrum of dicyandiamide. An aqueous solution of DCDA ($10^{-4}$ M) was placed in a fused silica cuvette and was measured in a Cary Model 14 spectrophotometer against a water reference. Also, another solution of DCDA ($10^{-2}$ M) which had remained at room temperature for six months at neutral pH was diluted to $10^{-4}$ M and observed in the same manner. A major peak was noted in both cases at 214 mμ; it has a molar extinction coefficient of $9.74 \times 10^3$ (see Figure 25). A moderate change was observed in the spectrum of the DCDA that had remained in aqueous solution at neutrality for half a year, attesting to its stability. When the pH was lowered the peak remained, but was shifted to about 204 mμ at pH 2. On the other hand, as the pH was raised, the 214 mμ peak disappeared. A reasonable explanation would be that the 214 mμ peak is due to conjugation of the diamine form of DCDA mentioned earlier. Removing a proton by raising the pH would destroy the conjugation of the system.

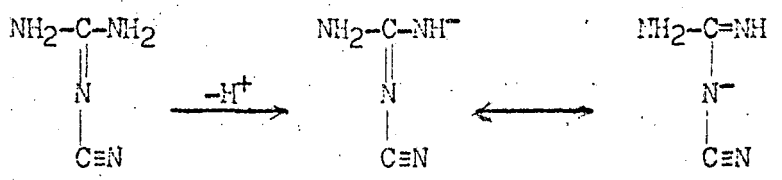



Fig. 24. Suggested effect of raising the pH of an aqueous solution of DCDA.

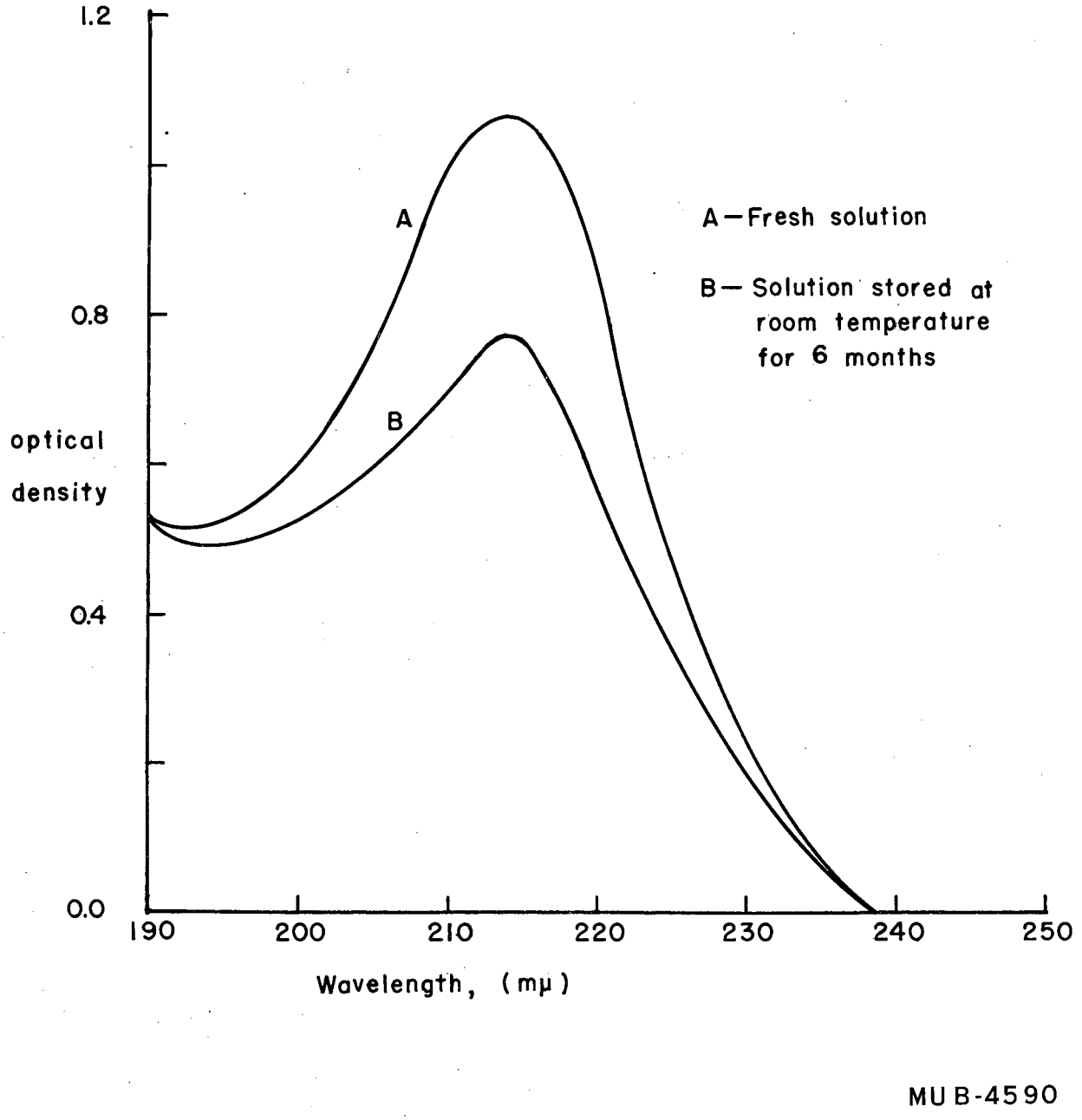



Fig. 25. Ultraviolet spectrum of aqueous solutions of diayandiamide.

To study the effect of ultraviolet irradiation on dicyandiamide, a $10^{-2}$ M aqueous solution of $^{14}C$-labeled DCDA was prepared and irradiated in a sealed fused silica cuvette for 4 hours with a high pressure General Electric Type A-H6 mercury lamp at a distance of 7.5 centimeters. Throughout the illumination period the cuvette was cooled by a steady air stream blown across the surface of the vessel. The product solution was spotted on Whatman No. 4 paper next to a similar sample which had not been illuminated. Resolution of the products was achieved by employing a mixture of n-butanol:ethanol:water (4:1:1) as solvent. The suspected products, for which chromatographic standards were procured, included melamine, cyanamide, guanylurea, dicyandiamide, guanidine, and urea. Since only the sulfates of guanidine and guanylurea were readily available, the free compounds were prepared by ion exchange chromatography. The first three compounds (melamine, cyanamide, and guanylurea) were located on chromatography paper with nitroprusside spray. (This consists of one part 10% NaOH, one part 10% sodium nitroprusside, one part potassium hexacyanoferrate (III) and three parts water by volume (190)). Guanidine was first sprayed with 1% $FeCl_3$ solution and then nitroprusside, giving a deep red-orange color. Urea and dicyandiamide were identified with the Erlich spray which was prepared by dissolving 0.25 gm of p-dimetylaminobenzaldehyde and 0.5 ml of conc. HCl in 50 ml of ethanol (205).

The production of melamine, the trimer of cyanamide, by the heating of dicyandiamide, the dimer of cyanamide, is believed due to the formation of the monomer which subsequently trimerizes (206). Therefore, melamine was one of the first compounds looked for as a product of the

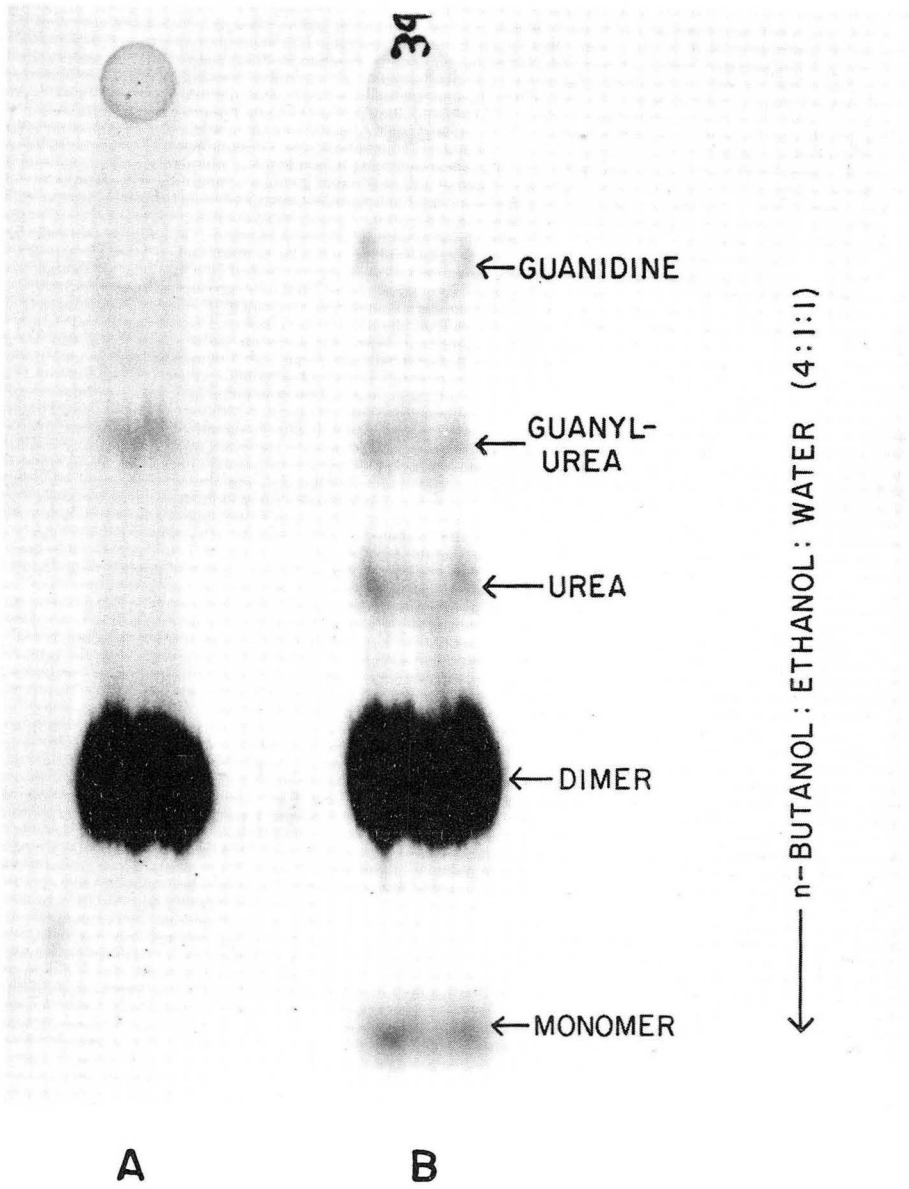
UV irradiation of DCDA. However, it was not found. The new products identified were cyanamide monomer and the urea of cyanamide. The starting material was slightly contaminated with guanylurea and guanidine. Increased amounts of these compounds were found following the irradiation. The results are summarized in Table VI. It is interesting to note that a large amount of the product is hydrolyzed to ureas. This effect might suggest that UV excitation of DCDA could conceivably promote dehydration condensation reactions, as originally suggested, but as yet this has not been observed.

Table VI: Summary of Products Formed by Ultraviolet Irradiation of Dicyandiamide

| Compound | % in Reagent* | % Product* | $R_f$** |
|---|---|---|---|
| guanidine | 0.1 | 0.3 | 0.25 |
| guanylurea | 0.4 | 0.8 | 0.40 |
| urea | 0 | 1.4 | 0.52 |
| DCDA | 99.5 | 97.1 | 0.69 |
| cyanamide | 0 | 0.4 | 0.87 |

** Solvent = n-butanol:ethanol:water (4:1:10); see Figure 26.

* Yields were recorded with a Baird-Atomic Geiger-Müller counter Model 123.




ZN-5308

Fig. 26. Autoradiogram of chromatogram exhibiting the products resulting from the ultraviolet irradiation of $^{14}C$-labeled DCDA. Column A shows the original reagent and column B the products.

2) Infrared Spectrum

An infrared spectral study of dicyandiamide was carried out to determine if evidence could be found for the cyclic form of the compound. The spectra were observed with solutions of DCDA in $H_2O$ and also $D_2O$ in a Perkin-Elmer Model 137 spectrophotometer using matched $BaF_2$ cells. The total spectrum compared well with that appearing in the literature (140). Varying the pH did not give an indication of the cyclic form in the regions observed.

A more exacting study of DCDA was carried out in a Beckman IR7 infrared spectrophotometer. The results indicated that, in particular, DCDA, cyanamide, and DCA (of subsequent interest) exhibit shoulders at 2115 $cm^{-1}$, the place at which dicyclohexylcarbodiimide has its carbodiimide absorption (197).

3) Nuclear Magnetic Resonance

Further evidence was sought for the structure of dicyandiamide in solution. Since NMR requires somewhat concentrated solutions, water absorbs near the region of interest, and DCDA is only 2% soluble in water at room temperature (140), additional solvents were considered for these studies. First it was noted that $D_2O$ wasn't useful since the hydrogens of DCDA exchange too rapidly. Acetone was useless since it, too, absorbs heavily in the region of concern. DCDA was found not to be soluble in chloroform, dioxane, and tetrahydrofuran, and only somewhat soluble to 2-methoxyethanol. Finally, DCDA was shown to be very soluble in dimethylsulfoxide and dimethylformamide, neither of which obstruct the region which was to be observed.

It was decided to find an acidity at which DCDA exchanged a proton in sufficient amount to allow low temperature studies. Protonation also helped to classify the hydrogens of the molecule more easily. By adding various amounts of conc. HCl and measuring the resultant apparent pH, it was shown in dimethylsulfoxide that around pH 2 such an exchange took place. Since dimethylsulfoxide (DMSO) has a higher freezing point than dimethylformamide (DMFA), work was now switched to the latter solvent. All NMR spectra were made on a Varian Model A-60 analytical spectrometer. An examination of the structure of DCDA indicates thatprotonation of the molecule should result in only two types of hydrogens in a 4:1 ratio for the carbodiimide form to be possible. A 50% solution of DCDA in DMFA was prepared and adjusted to pH 2. NMR spectra were taken at various temperatures down to -50° C. (Below this, the solution froze.) Figure 28 shows the two peaks observed at the various temperatures. The peak around 275 cps was attributed to the water in the added conc. HCl (35% in $H_2O$). As the temperature was lowered the proton exchange rate slowed and permitted observation of the two species involved in the exchange, $H_2O$ and DCDA. It may be noted that at -50° C the DCDA peak at 411 cps is beginning to split, probably due to a slowing of the hydrogen exchange between the sites of the molecule itself. It was hoped that this resolution would occur at a higher temperature to permit observation of the species within DCDA itself. However, since the nature of the solution prevented further lowering of the temperature, it was impossible to achieve this.

In the course of this study, data were collected at a number of temperatuares whereby it was possible to calculate the Arrhenius

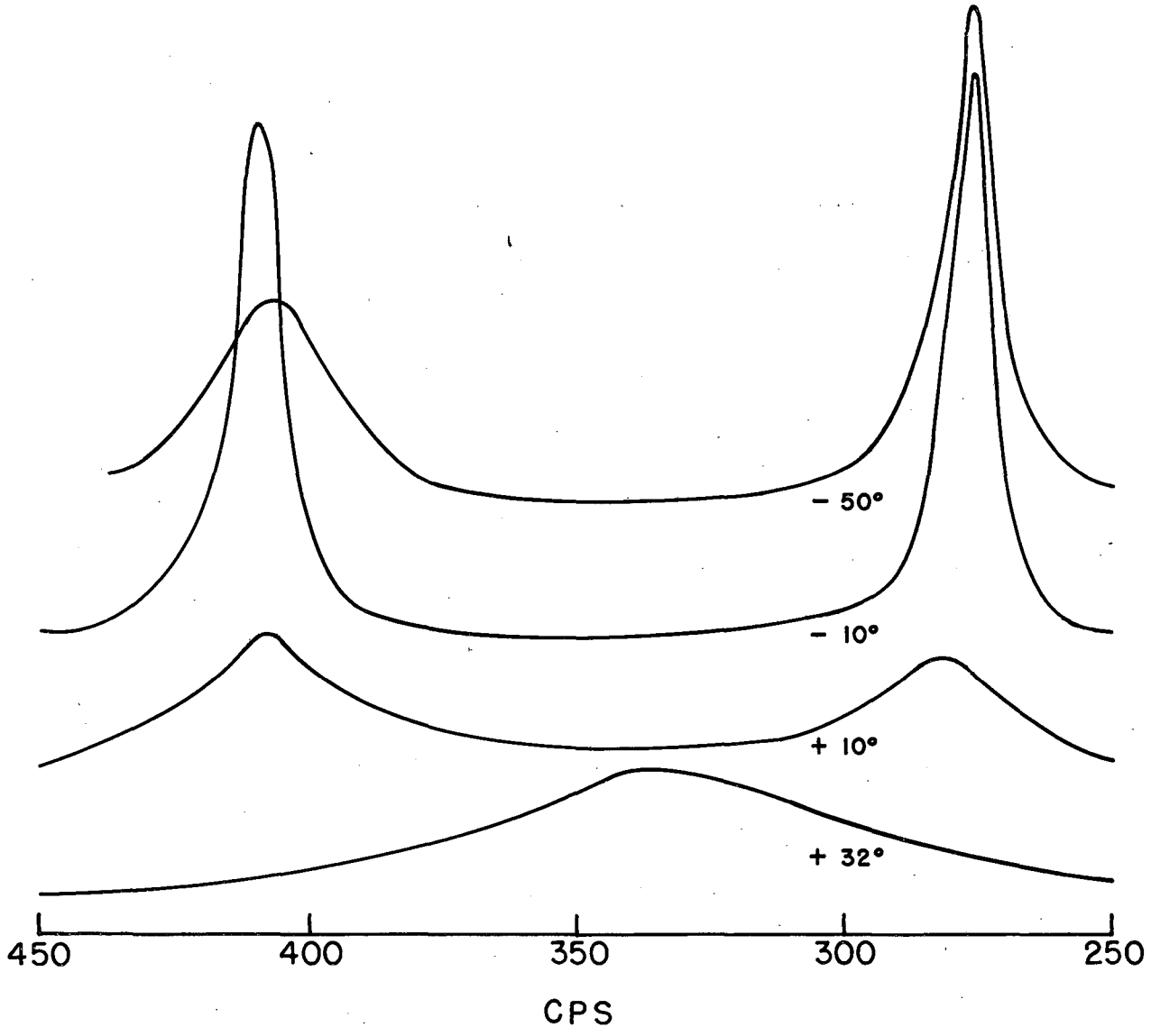



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Fig. 28. NMR spectra of dicyandiamide in dimethylformamide adjusted to pH 2 at various temperatures.

activation energy of the protonation. The data and results are as follows:

Table VII: Data from NMR Studies on DCDA at Various Temperatures

| T | $\gamma_A$ | $\gamma_B$ | $\tau \times 10^2$ |
|---|---|---|---|
| 293 | 388 | 285 | 0.349 |
| 283 | 408 | 282 | 0.472 |
| 273 | 415 | 287 | 0.520 |
| 263 | 411 | 275 | |

At 263° K, the $\gamma_A - \gamma_B$ value (411 - 275 = 136) equals $S_\gamma$ since it is the maximum spread encountered; the separation was no greater at 223° K, for example. By graphic plot of the data, $E_a/k = 1.84 \times 10^3$ and $E_a$ = 3.66 kcal/mole. A similar value was found when the width of the individual curves was considered.

Although the experiment did not give any further insight into the structure of DCDA, the data on the activation energy of protonation proved interesting and worthy of noting.

B. The Mechanism of Dipeptide Synthesis

1) Quantitative aspects of DCDA-mediated alanylalanine synthesis

As was already noted, dicyandiamide can successfully promote the dehydration condensation of alanine to give alanylalanine. This reaction was carried out in dilute aqueous solution in the dark. Guanylurea was demonstrated as a concomitant product of dipeptide synthesis using dicyandiamide in the following experiment: A stock aqueous solution was prepared containing hydrochloric acid (0.02 N), DCDA

(0.02 M), and alanine (0.02 M). A 50 λ aliquot of this solution was added to 50 λ of an aqueous solution containing $^{14}C$-labeled alanine. Also, 50 λ of the stock solution was added to 50 λ of an aqueous solution which contained $^{14}C$-labeled dicyandiamide. The solutions remained in the dark at room temperature for 20 hr. Each was directly applied to a separate sheet of Whatman No. 4 paper. Standard samples of guanylurea and alanylalanine were placed in parallel to the test samples on the appropriate sheets. The chromatogram containing labeled alanine was run with water-saturated phenol: conc. $NH_4OH$ (200:1) as solvent, whereas the other, containing labeled DCDA, was run with n-butanol: ethanol:water (4:1:1) as solvent. The standard sample of dipeptide was located with ninhydrin, the guanylurea was found with a nitroprusside spray, and the labeled products were located by means of X-ray film. The results indicated that 0.51% of the original alanine was converted to the dimer. After subtracting the amount of excess guanylurea formed by direct acid hydrolysis of DCDA, it was found that the amount of excess guanylurea formed was equal to the number of peptide bonds formed.

2) Introduction to DCA studies

Similarly, dicyanamide (DCA) proved effective in promoting dipeptide synthesis. In fact, dicyanamide appeared to be better than dicyandiamide in terms of the amount of dipeptide produced. The experiment described here using labeled alanine was repeated using sodium dicyanamide in place of dicyandiamide. A yield of 1.6% of the alanine dimer was observed, as compared with 0.5% in the case of DCDA.

In order to estimate roughly the rate at which polypeptides synthesized with the aid of DCA might have accumulated on the primitive Earth, it is necessary to have some insight into the mechanism of the reaction and its possible catalysis by inorganic ions likely to have been present in abundance in the primitive oceans. Peptide synthesis from glycine using dicyanamide was chosen as the reaction to study in greater detail, since the yields are good and the separation of reaction products is easily carried out. The facts observed earlier from the studies with dicyandiamide proved useful in evaluating the DCA-mediated reaction.

It was postulated a priori that cyanamide and related compounds, such as dicyanamide and dicyandiamide, operate as condensing agents by a mechanism similar to that encountered with the dialkylcarbodiimides. This would mean that the key intermediate to the reaction would be an acylisourea (see Figure 38) formed by the carboxyl group (in the case of dipeptide synthesis) adding across one of the carbodiimide double bonds. This would then be subject to nucleophilic attack by the amino group of another amino acid, resulting in the production of the condensation bond (peptide bond, in the case of amino acids) and the corresponding urea.

3) pK of dicyanamide

An essential piece of information needed for further mechanistic studies was the pK of dicyanamide. Related evidence, to be discussed, indicates that the rates of hydrolysis and polymerization at room temperature of DCA in acid are quite slow and that the compound is apparently unaffected by brief (3 minutes) exposure to acid with regard to its capacity to enter into the synthesis of peptide bonds.

A spectrum of NaDCA ($10^{-4}$ M) measured in a Cary Model 14 spectrophotometer indicated that the compound has a maximum at 190 mμ which tails off to about 230 mμ. To determine the pK of DCA by spectrophotometry, solutions were separately prepared of various concentrations of HCl in which the NaDCA ($10^{-4}$ M) was dissolved. Within less than half a minute following preparation of each solution, its absorbancy was measured at 211 mμ.

The resultant data are found in Figure 29 and Table VIII. For the present experiment, $A_{AH} = 0.381$, $A_{A^-} = 0.087$, $A_{\lambda} = 0.211$, and

Table VIII: Determination of pK of DCA

| HCl normality | $H_o$* | O.D.$_{211\ m\mu}$ |
|---|---|---|
| 0 | — | 0.087 |
| 1.0 | -0.20 | 0.072 |
| 2.0 | -0.69 | 0.211 |
| 3.0 | -1.05 | 0.362 |
| 4.0 | -1.40 | 0.382 |
| 6.0 | -2.12 | 0.381 |

*$H_o$ values from Ref. 204a.

$H_o = 0.69$. This gives a value for the pK of -0.8. When the determination was made at 220 mμ, the same pK value was found. This figure proved useful in experiments where the DCA concentration was varied and it was desired to maintain the same initial pH in each procedure.

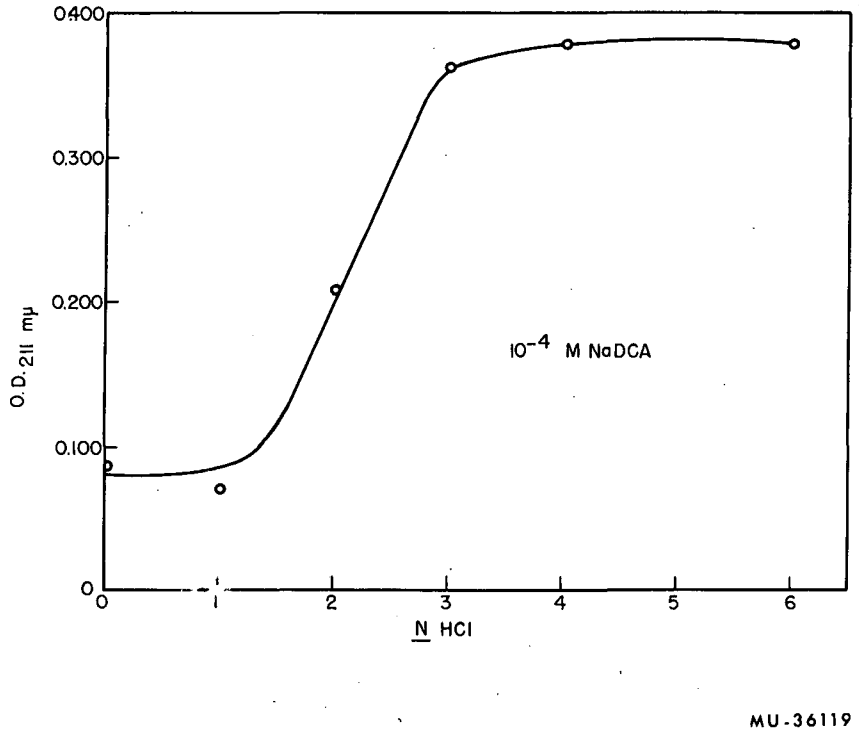


Fig. 29. "Titration curve" of sodium dicyanamide measured at 211 mμ. The NaDCA was dissolved in solutions of various acid normalities and the resultant absorbancies were measured.

4) Effect of acid on peptide synthesis

It may be recalled that with the DCDA-promoted phosphorylation of glucose, significant yields were observed only when the pH was adjusted to 2 or lower. Similarly, with the other reactions attempted using DCDA as well as DCA, the most successful synthese were achieved when the conditions were acidic. To determine what effect, if any, pH has on DCA-mediated dipeptide synthesis, the production of diglycine was carried out in a variety of acid concentrations. In each case, the concentrations of NaDCA and labeled glycine were kept the same initially at 0.01 M. Aliquots were taken after 10 minutes of reaction time and analyzed by low-voltage horizontal paper electrophoresis in borate buffer at pH 9.2. The results, summarized on the graph in Figure 30, suggest that the reaction is promoted by acidic conditions.

When the reaction was carried out in the absence of HCl, the yield of diglycine was about 1%. (This observation is significant in that it also demonstrates that while DCA-mediated dipeptide synthesis goes best at low pH, it gives detectable yields under neutral conditions.)

5) Effect of DCA concentration on dipeptide synthesis

That dicyanamide is indeed the agent promoting dipeptide synthesis was demonstrated very easily with corresponding control experiments in which the DCA was absent (all other conditions being held the same) whereby no diglycine was observed. Then, the effect which DCA concentration has on the yield of glycine dimerization was

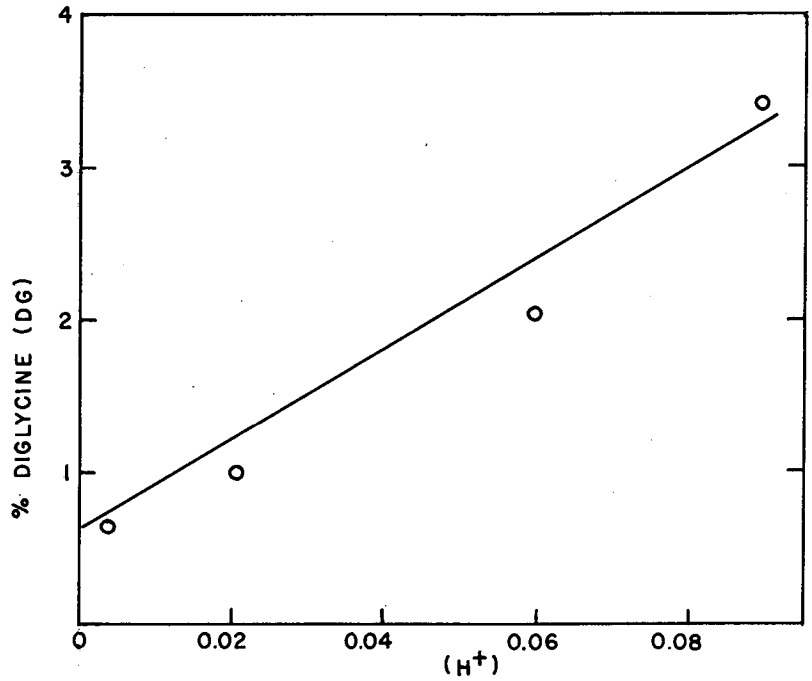


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Fig. 30. Effect of increasing acid concentration on dicyanamide-mediated diglycine synthesis. In each experiment the glycine and sodium dicyanamide concentrations were both 0.01 M.

charted. A number of solutions were prepared in which the labeled glycine concentration was 0.1 M in each and the pH was adjusted with HCl to 1.3 The concentration of sodium dicyanamide was varied between 0.01 M and 0.1 M in the array of solutions. After 10 minutes, aliquots were removed, applied to strips of Whatman No. 1 paper, and analyzed by electrophoresis. The results are noted in Figure 31. Yields are based on total glycine. These data indicate that the dipeptide synthesis is directly dependent on the concentration of dicyanamide and the yield of diglycine is enhanced by an increased initial concentration of dicyanamide.

6) Effect of glycine concentration on dipeptide synthesis

The next variable to be studied in the synthesis of diglycine from glycine using dicyanamide was the initial amino acid concentration. A number of solutions were prepared containing NaDCA (0.1 M) and were adjusted to pH 1 with HCl. Each solution also contained an amount of labeled glycine whose concentration was between 0.01 and 0.07 M. After 10 minutes, aliquots were withdrawn and resolved by paper electrophoresis. The results are exhibited in Figure 32. Thus, the direct dependence of dipeptide synthesis on initial glycine concentration is evident.

Hydrolysis of the diglycine product (6 N HCl for 8 hours at 100° C) gave back the original glycine monomer, further substantiating the identity of the dipeptide (see dipeptide synthesis in Part I).

It was of interest to know if the carboxyl group of the glycine reacts with the dicyanamide to form the acylisourea intermediate

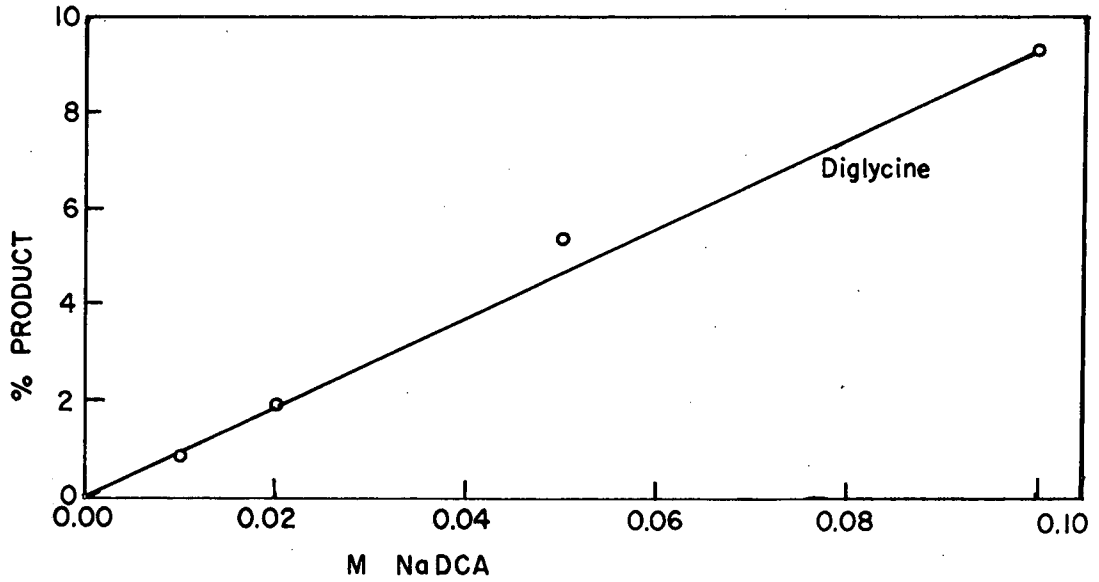


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Fig. 31. Effect of varying the concentration of sodium dicyanamide in the synthesis of diglycine from glycine. In each experiment the glycine concentration was 0.1 M, and the acid normality was adjusted to attain pH 1.3.

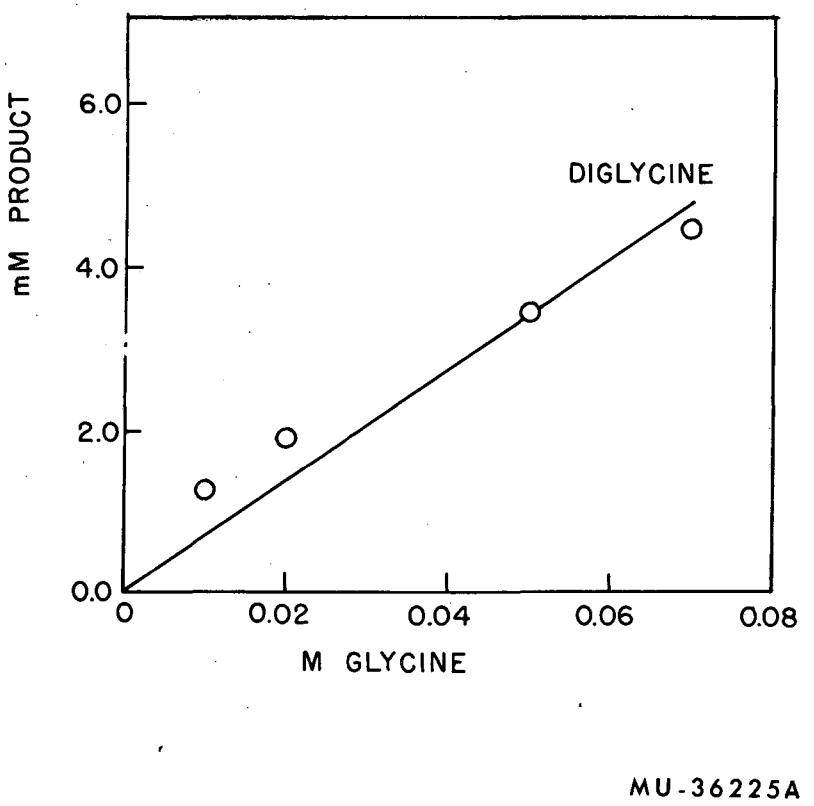



Fig. 32. Effect of varying the initial concentration of glycine in the DCA-mediated synthesis of diglycine. In each experiment the concentration of NaDCA was 0.1 M and the acid normality was adjusted to attain a pH of 1.

as a carboxylate anion or in the undissociated form. It is known that the pK of glycine (carboxyl) is 2.34 whereas that of diglycine is 3.06 (207). By choosing appropriate pH's it would be possible to prepare solutions in which the glycine carboxyl would be mainly dissociated and the diglycine carboxyl would be mostly protonated. By calculation it was determined that at pH 1.40 glycine carboxyl would be 90% undissociated and diglycine 98%. On the other hand, at pH 2.60 glycine would be 35% and diglycine would be 74% undissociated in its carboxyl group. At both pH's the amino groups of both compounds would be considered similar for all practical purposes. In comparing glycine and its dimer, steric factors would also need to be considered sources of difference in the reactivities of the two compounds. Therefore, in order to subtract this and leave only the degree of dissociation of the carboxyl as the differentiating factor, it was decided to carry out peptide synthesis in solutions containing glycine and diglycine at both pH 1.40, where there is essentially no difference in the degree of carboxylate protonation, and pH 2.60, where the difference is quite evident. For this experiment $^{14}C$-labeled diglycine was prepared from labeled glycine with sodium dicyanamide and isolated by paper chromatography. By adding appropriate amounts of HCl solutions were prepared containing glycine (0.01 M), diglycine (0.01 M), and NaDCA (0.1 M) at pH 1.40 and 2.60. One set of solutions at the two pH's containing $^{14}C$-labeled glycine while another set containing $^{14}C$-labeled diglycine. After 5 minutes of reaction time, 100 λ aliquots of the product solutions were applied to strips of Whatman No. 1 paper and analyzed by electrophoresis in borate buffer at pH 9.2.

Where labeled glycine had been used, the yield of diglycine was noted; in the experiments where labeled diglycine had been a reactant, the yield of tetraglycine was noted, since with the triglycine produced it could not be ascertained if it had been the monomer or dimer which had formed the acylisourea intermediate with dicyanamide or which had been the nucleophile attacking this intermediate.

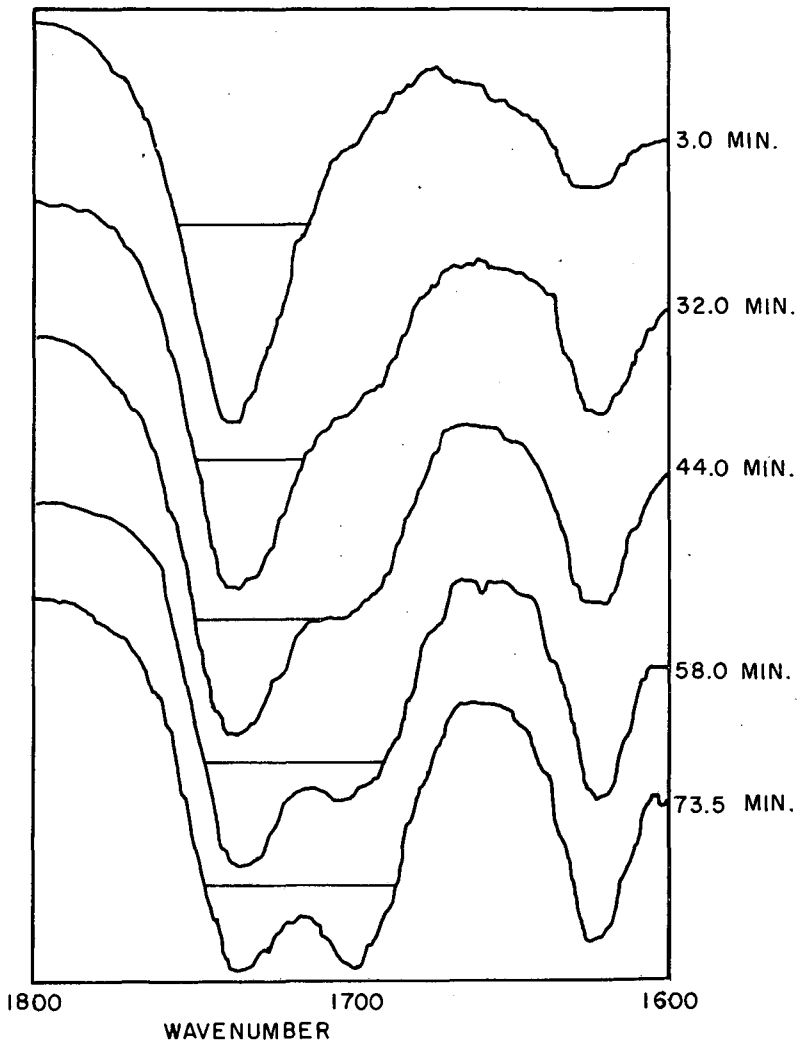
The amount of glycine going to tetramer was probably negligible under the conditions of the reaction. In the figures for the degree of protonation of the monomer and dimer at the two pH's noted above it can be calculated that for glycine the degree of dissociation going from pH 1.40 to 2.60 changes by 61.1% whereas for diglycine it changes by 24.5%. The results of the present experiment showed that going from pH 1.40 to 2.60 the yield of diglycine from glycine changes by 57.0% whereas the yield of tetraglycine from diglycine changes by 29.6%. These results suggest that it is the undissociated form of the reacting carboxyl that participates in the formation of acylisourea intermediate which is thought to be necessary for the subsequent synthesis of the peptide bond.

7) <u>IR studies</u>

Infrared spectrophotometry was employed in an attempt to observe the hypothetical acylisourea intermediate. A solution was prepared in $D_2O$ containing glycine (0.05 <u>M</u>), HCl (0.1 <u>N</u>) and sodium dicyanamide (0.1 <u>M</u>). The sample was placed in a $BaF_2$ cell and observed (50 seconds after mixing) in a Beckman Model IR7 spectrophotometer between 1600 and 1800 $cm^{-1}$. Although no absorption suggesting

such a compound appeared in sufficient magnitude to be observed in this region, even when the temperature of the system was lowered to 3° C, other interesting data were recorded. At room temperature one peak at 1735 $cm^{-1}$ gradually disappeared with time of reaction while two other absorption maxima appeared at 1698 $cm^{-1}$ and 1620 $cm^{-1}$ (see Fig. 33). Studies with standard compounds indicated that none of these peaks was due to dicyanamide (which is transparent in this region), biuret, diketopiperazine, or diglycine. It was also shown that if the reaction had given a 3% yield of the glycine dimer (which it does under these conditions, as shown by an independent experiment), the dimer could not have been detected in the presence of the other bands with the sensitivity employed. By studies of glycine monomer at appropriate pH values, two of the peaks were identified. The 1735 $cm^{-1}$ maximum was attributed to undissociated carboxyl groups, whereas the 1620 $cm^{-1}$ peak was attributed to carboxylate anions. The decrease of the first peak and the increase of the second during the course of the observation period appeared to suggest that the pH of the medium was slowly changing. A similar shifting of peaks was observed when N,N-dimethylglycine replaced glycine under the same conditions.

With the possibility that the remaining 1698 $cm^{-1}$ maximum might indeed be due to an adduct of the dicyanamide and glycine, three 0.1 M solutions were prepared in $D_2O$. The first contained sodium dicyanamide, HCl, and $CH_3NH_2 \cdot HCl$. The second contained sodium dicyanamide, HCl, and acetic acid. These two considered the possibility of a carboxyl and an amine adduct with the dicyanamide. The third solution contained only sodium dicyanamide and HCl. All three samples exhibited the 1698 $cm^{-1}$ maximum when observed at 30 minutes after mixing, and it was thus suggested




MU-35596

Fig. 33. Infrared spectra of a mixture of glycine (0.05 M), sodium dicyanamide (0.1 M), and HCl (0.1 N) in $D_2O$ observed at various periods during the course of the reaction. The 70% transmission line has been included for reference.

that this peak might be involved in the hydrolysis of dicyanamide. The third solution showed a similar slowly rising value for the time-rate of appearance of the 1698 $cm^{-1}$ peak as when glycine had also been present, but with the absence of the other two maxima observed previously. It has already been demonstrated that the peak was not due to biuret. Therefore, cyanurea was synthesized by the reaction between cyanamide and potassium cyanate (133). The identity of the product was verified by elemental analysis as well as its hydrolysis to biuret (see Fig. 10 for hydrolysis scheme of dicyanamide). The IR spectrum of this independently synthesized cyanurea exhibited the 1698 $cm^{-1}$ peak. Therefore, the appearance of the 1698 $cm^{-1}$ peak in the system appeared to reflect the hydrolysis of DCA to cyanurea. (This will be discussed further.) Sodium dicyanamide was observed to be quite resistant to hydrolysis in neutral solution. The urea, a more basic compound than DCA (pK 4.6 as compared to pK -0.8), could affect the pH as it is synthesized and might thus explain the slow apparent pH change.

It should also be remembered that sodium dicyanamide itself is subject to hydrolysis even in the absence of concomitant peptide synthesis. Apparently once peptide synthesis has stopped (which was shown to be a few minutes after mixing reactants, as discussed in the next section), urea production continues. The sensitivity of the infrared spectrophotometer used as well as the time involved between mixing reagents and observing them in the apparatus prevented the recording of the initial small (1.5%) increase of cyanurea which (if the proposed mechanism is correct) took place due to peptide synthesis. This reaction step is reasonable since it was previously demonstrated in the case of dicyandiamide that the production of its urea matched the synthesis of peptides

during the period in which the reaction was taking place.

It was hoped that it would be possible to run the diglycine synthesis using glycine labeled with $O^{18}$ in its carboxyl group and then look by infrared spectrophotometry for the label in the cyanurea formed. The labeled glycine was prepared by hydrolyzing glycylglycine in the presence of $H_2O^{18}$. However, it was found that under the acidic conditions required for the dipeptide synthesis, the oxygen exchanges too rapidly with the water solvent to permit definite conclusions.

8) Time-course of dipeptide synthesis

The kinetics of the synthesis of dipeptide using dicyanamide was next studied. A solution was prepared containing glycine-2-$C^{14}$ (0.01 M), sodium dicyanamide (0.01 M), and HCl (0.02 N). Samples were withdrawn at 10 and 30 minutes, and analyzed by paper electrophoresis. No significant difference was observed in the yield of diglycine between the two. This would suggest that the reaction was rapid after mixing and quickly reached a maximum, much the same as is observed with the carbodiimides.

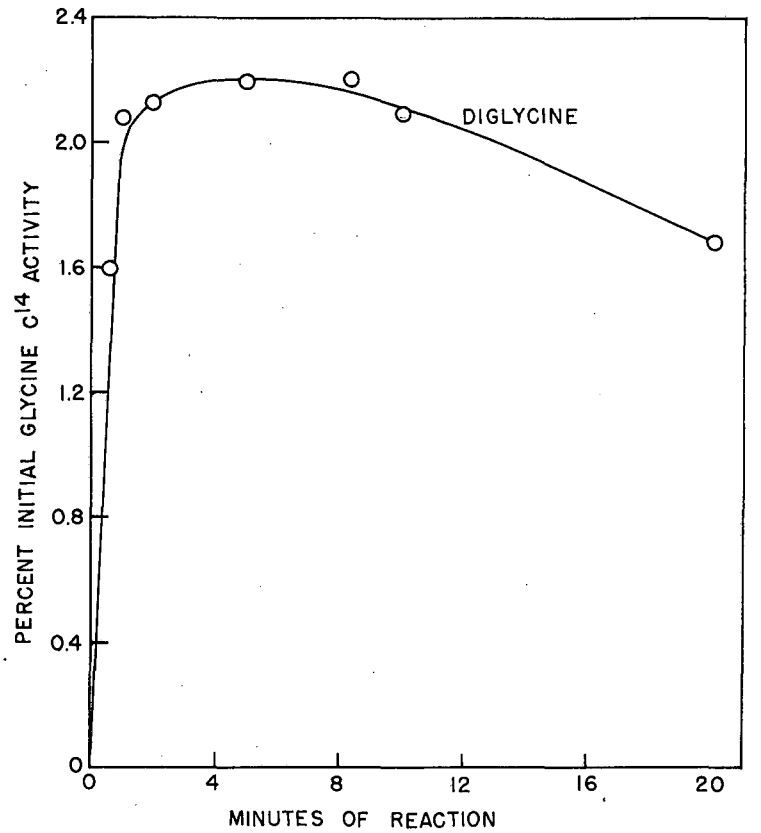
It was therefore of interest to know the actual course of diglycine appearance with time of reaction. A solution was prepared containing labeled glycine, NaDCA, and HCl (all at 0.1 M). Aliquots were withdrawn at various times during the reaction period, immediately applied to the electrophoresis paper, and very rapidly dried by a nitrogen stream. This drying procedure took about 20 seconds in each case and undoubtedly stopped the reaction in each applied sample. The results of this experiment where the concentration of the three reagents was 0.1 M in each appeared to indicate that the reaction was complete within the first minute after mixing of the reactants. Next, the DCA concentration was changed to 0.03 M and the HCl and glycine were kept the same as before. Again the

reaction terminated quickly. Two other experiments were carried out, one where the DCA was kept at 0.03 M and the glycine concentration was lowered to 0.01 M, and the other where the glycine was 0.01 M and the DCA was 0.1 M (both had HCl at 0.1 N). These cases also demonstrated a rapid appearance of diblycine which abruptly stopped after a minute or two following mixing, even though not all the glycine was used up (see Fig. 34). It was also observed that the appearance of diglycine followed the same time course as the disappearance of glycine monomer.

It was thus necessary to determine why the reaction ended after such a short period of time. One possibility was a change of pH. Since the synthesis is so pH-dependent, a small change in acidity could alter the reaction considerably (see section on acid-dependence). The IR data suggested such a pH change but it was quite slow and continued long after the diglycine synthesis was known to have ceased. To resolve this point, a solution was prepared containing glycine (0.1 M), NaDCA (0.1 M), and HCl (0.1 N). The pH of the solution was measured with a Beckman pH meter (calomel reference electrode) during and after mixing of the components. No change was observed in the pH within the first few minutes after mixing. Thus, a changing pH was ruled out as the process which was halting the dipeptide synthesis.

9) Catalysis of dipeptide synthesis by divalent cations

The cause of the termination of the dipeptide synthesis remained to be elucidated. Another possible explanation of this phenomenon was investigated. It is known that free (undissociated) dicyanamide tends to polymerize and precipitate out of solution (208). Therefore, a solution was prepared containing sodium dicyanamide (0.3 M) and HCl (0.3 N). After several hours, the solution was observed to become cloudy and finally form a definite precipitate. Measurement of the final pH of the solution indicated




MU-36183

Fig. 34. Kinetics of diglycine synthesis from glycine using dicyanamide. The initial solution contained glycine (0.01 M), NaDCA (0.03 M), and HCl (0.1 N).

that it was now approximately neutral. It has already been noted that DCA has a pK value of -0.8. Therefore, the polymerization of the dicyanamide upon protonation of DCA anion (addition of acid to the medium) might explain the short reaction period for the synthesis of diglycine.

If the synthesis of dipeptide is terminated mainly by the polymerization of undissociated DCA, the amino acid dimerization might be aided by an agent that could prevent or retard the polymerization of the condensing compound. Metal cations, by their likely presence on the primitive Earth, known importance in biological systems, and ability to stabilize polyelectrolytes, would a priori seem to be good candidates. Therefore, solutions were prepared containing sodium dicyanamide (0.3 M), HCl (0.3 N), and metal chlorides (0.3 M) including magnesium, calcium, copper, and manganese. The copper solution immediately displayed a precipitate probably due to the formation of an insoluble copper salt of dicyanamide. After it remained overnight at room temperature, the standard solution (containing no divalent metal) appeared cloudy, as was the tube containing magnesium. However, the solutions containing calcium and manganese remained clear.

These results suggested that $Ca^{++}$ and $Mn^{++}$ effectively retarded the acid-induced polymerization of dicyanamide and that the addition of these cations to the glycine-dicyanamide-HCl system might significantly increase the yield of dipeptide by retarding the loss of DCA due to polymerization. In order to test this hypothesis, the following experiment was performed: A stock solution was prepared containing labeled glycine (0.02 M) and NaDCA (0.2 M). A 100 λ aliquot of this solution was added to 100 λ of 0.2 N HCl and 0.2 M $CaCl_2$. A similar solution contained 0.2 M $MnCl_2$ in place of the calcium. The results taken from samples analyzed after

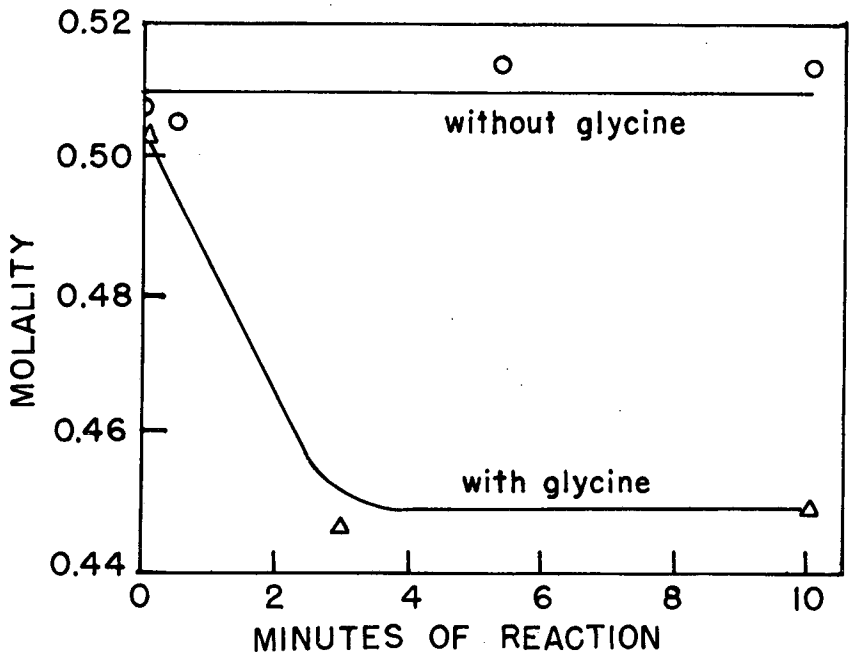
30 minutes indicated that the added salts did not enhance dipeptide synthesis. (This apparent discrepancy will be discussed further.)

10) Fate of dicyanamide

Since at the end of the reaction period much glycine remained unreacted and the pH was not observed to change, the limiting factor to the reaction was therefore attributed to the DCA. Since undissociated dicyanamide is very prone to polymerize (208) and since its known trimer, as a model compound, was not observed to promote peptide synthesis in the same manner as dicyanamide itself (to be discussed), a reasonable hypothesis would be that a consequence of polymerization of the DCA was to reduce the concentration of the condensing agent (DCA monomer) and thus reduce the dipeptide formation.

To test this suggestion, a solution was prepared containing NaDCA (0.4 M) and HCl (0.1 N). At various times after mixing of the reagents, 1 ml aliquots were withdrawn and added to 0.1 ml portions of 1 N NaOH. A "0" time standard was prepared with a solution containing an amount of NaDCA and NaCl equivalent to the other samples. The samples were analyzed on a Mekro Labs Vapor Pressure Osmometer and the results were presented as solute molality. No significant change was observed in the course of the reaction (see Figure 35).

The reaction was repeated except that the solution now contained glycine (0.04 M) as well. Aliquots were withdrawn at various times and neutralized as before. An amount of glycylglycine was added to the "0" time standard equivalent to the known yield of the reaction (with a corresponding decrease of glycine). The results indicated a sharp decrease in the molality within the first three minutes of the reaction which decreased no further with additional time. This change




MU-36466

Fig. 35. The change of total solute molality with time after mixing, as measured by vapor pressure osometry, of an aqueous solution containing glycine (0.04 M), NaDCA (0.04 M), and HCl (0.1 N). The "O" time standards are corrected for the amount of diglycine known to be produced under these conditions and the "without glycine" values are corrected for the absent amino acid.

could not be attributed to the glycine since there was not enough present to account for the magnitude of the drop and since the yield of diglycine had already been accounted for anyway. To compare the results of this experiment with the previous one where glycine was absent, the data ofthe procedure with no glycine were corrected for the absent glycine (see Figure 35). More initial glycine made no change in the magnitude of the overall molality drop (corrected for dipeptide). These observations were reproducible.

It was next necessary to determine if the change (presumed due to the polymerization of DCA with a glycine catalyst) was due to glycine alone or if acid was also necessary. A 90 λ solution was prepared containing NaDCA (0.1 M) and HCl (0.11 N). After three minutes, 10 λ of $^{14}C$-labeled glycine was added (bringing the glycine concentration to 0.1 M). Three minutes later the solution was applied to a strip of Whatman No. 1 paper and analyzed by electrophoresis. A similar solution which initially contained the DCA and glycine, and to which the acid was added after three minutes, was analyzed in the same fashion. Both samples displayed a diglycine product, suggesting that both glycine and acid are necessary to bring about the change noted with vapor pressure osmometry which paralleled the termination of dipeptide synthesis.

It was then necessary to determine if the effect was due to catalysis by the amino or carboxyl group of the glycine. First the carboxyl group was tested. Solutions were prepared for analysis by vapor pressure osmometry as before except that the glycine was re-

placed by an equivalent amount of formic acid in one case (as a model compound containing a carboxyl group) and acetic acid in another case. No molality drop was observed in either case.

Therefore, since it was established that it was not the carboxyl group which promoted the drop in solute molality, the amino group remained as the possible cause of this effect. A solution was prepared containing NaDCA (0.4 M), HCl (0.1 N), and, as a model compound for a substance containing an amino group, methylamine hydrochloride (0.04 M). Samples were taken and neutralized at periods following mixing, as before. An appropriate "0" time standard was prepared. Contrary to the earlier negative results from those systems containing only carboxyl groups, the present combination exhibited the same molality drop in a very short period of time (three minutes) as had been observed with the solutions containing glycine. Thus, it was concluded that it was the amino group which was responsible for promoting the observed effect on the solute molality (believed to be due to the polymerization of the dicyanamide).

One way in which an amino group-containing compound might effect this type of response would be to form an amino adduct with the dicyanamide. (N,N-dimethylglycine did not promote the type of molality decrease of a DCA solution noted when glycine was present.) This amino addition compound could then be subject to nucleophilic displacement by another dicyanamide molecule. Such amino addition compounds are known in cyanamide (130) and carbodiimide (197) chemistry. For example, sarcosine can react with cyanamide to form creatine (130). Evidence has been collected for such a compound existing in the NaDCA-

glycine system. (NC-NH-C(=NH)-NH-$CH_2$-COOH) The data supporting the suggested structure of the substance are as follows:

a) The compound (to be designated here as "A") was first defined in the reaction system containing $^{14}C$-labeled glycine, NaDCA, and HCl. Compound A appeared as a radioactive band with a greater mobility than diglycine when analyzed by paper electrohporesis with borate buffer at pH 9.2. (Glycylglycine has the greatest mobility of all the glycine peptides, including the monomer, in this buffer system.) This indicated that compound A contains carbon atoms derived from glycine. Similarly, when labeled NaDCA was used, the band with the same mobility as compound A was radioactive. When both $^{14}C$-labeled NaDCA and $^{3}H$-labeled glycine were employed together, compound A was isolated by paper electrohporesis and its activity was measured by scintillation counting. The $C^{14}/H^{3}$ ratio of compound A equalled 0.10 whereas the ratio of the initial specific activity of $C^{14}$ to $H^{3}$ of the reactants was 0.12. This supported the proposal of a 1:1 proportion of glycine to DCA in the structure of compound A.

b) The electrophoretic mobility of compound A in borate buffer at pH 9.2 is toward the anode, indicating that it has a net negative charge at this pH.

c) Compound A was isolated by curtain electrophoresis in borate buffer. In the ultraviolet spectrophotometer, it exhibited a peak at 217.5 mμ. (It is important to recall that dicyandiamide, also a cyanoguanidine, has a UV absorption at 214 mμ.)

d) Compound A is ninhydrin-negative.

e) Compound A was isolated by paper chromatography. It was recrystallized from 95% ethanol and then dissolved in $D_2O$. Analysis was carried out by infrared spectrophotometry with matched $BaF_2$ cells in a Beckman IR-7 apparatus using cyanoguanidine ($NC-NH-C(=NH)-NH_2$), guanidoacetic acid ($NH_2-C(=NH)-NH-CH_2-COOH$), and glycine as reference compounds. Assignments made to distinctive absorption maxima for the isolated compound included 1610 $cm^{-1}$ (carboxylate), 1700 $cm^{-1}$ (carboxyl), and 2180 $cm^{-1}$ (cyano). As the pH was lowered, the magnitude of the 1700 $cm^{-1}$ peak increased whereas that of the 1610 $cm^{-1}$ peak decreased. In KBr, it was also possible to discern the imine bond (1650 $cm^{-1}$) as well as several amines (3160, 3280, 3345, and 3440 $cm^{-1}$).

The quantitative values observed in this infrared spectrum were compared with those for cyanoguanidine (both in KBr). The maxima compared were those of the imine bond (1650 $cm^{-1}$) and the cyano group (2230 $cm^{-1}$). (Both cyanoguanidine and the proposed structure for compound A exhibit one cyano group for each imine group per molecule). By assuming that the extinction coefficients for the corresponding bonds in the two compounds are similar, the ratio of the absorbancies of the two groups in each molecule ($A_{C\equiv N}/A_{C=N}$) was calculated. This would, of course, not depend on the total concentration of the compound itself but rather the abundance of each group in the molecule. The ratio for compound A (0.102/0.155) was divided by the ratio for DCDA (0.061/0.076). A value of 0.82 was calculated, further supporting the proposed structure of compound A.

It is known that hydrogenation of cyanoguanidine produces guanidine and methylamine (140). Therefore, compound A was synthesized from $^{14}C$-labeled glycine, NaDCA, and HCl, then isolated by paper electrophoresis, and finally reduced by sodium in alcohol. Paper chromatography of the product, using guanidoacetic acid as a carrier standard, supported the proposal that compound A is indeed a cyanoguanidine. (The identity of the standard was substantiated by the correspondence of its melting point to published values.)

Radioactive sodium dicyanamide was prepared in the following manner: A total of 17.7 mg of $^{14}C$-labeled barium cyanamide was dissolved in 1 ml of water and to this was added 45 mg of sodium sulfate in 5 ml of water. The precipitate of barium sulfate was removed by centrifugation. The supernatant was added to 10.6 mg of cyanogen bromide while in an ice bath. The mixture remained overnight at room temperature. The product was then chromatographed on Whatman No. 1 paper using n-butanol:ethanol:water (4:1:1) as solvent. X-ray film located the labeled compounds and an adjacent standard sample of NaDCA was located with Erlich's spray. The suspected product exhibiting a similar $R_f$ to the standard was eluted with water. An aliquot was hydrolyzed in 6 N HCl for 10 minutes at 100°C. Paper chromatography revealed that the predominant product of the hydrolysis was biuret (>90%). Another aliquot of the eluate was added to carrier NaDCA and together they were chromatographed on Whatman No. 4 paper using n-butanol:ethanol:water (4:1:1) solvent in the first direction and isopropanol:water (4:1) in the second. The results indicated perfect coincidence

between the carrier and the labeled product.

One of the applications of the labeled NaDCA was to determine the actual fate of the dicyanamide during dipeptide synthesis. Dipeptide synthesis was carried out as before except that labeled NaDCA was included as a reagent. The glycine in the reaction solution was nonlabeled. The products were resolved by electrophoresis (paper). The major band, which ran much faster than diglycine, was eluted. Paper chromatography revealed that the substance was not sodium dicyanamide nor dicyandiamide, cyanurea, or biuret. Electrophoresis also showed that it was not the amino addition compound (A) nor the acylurea side product to be discussed. The fraction accounted for about 80% of the original DCA when labeled dicyanamide was employed. (Cyanurea was identified as another product of the reaction.) When labeled glycine was used, the fraction was not radioactive. Addition of $CaCl_2$ did not retard the production of the compound (refer to section on divalent cation catalysis).

Therefore, a solution was prepared containing labeled NaDCA (1 M), glycine (0.1 M), and HCl (0.16 N). Five minutes after mixing, the product solution was resolved by chromatography on Whatman No. 3MM paper using n-butanol:ethanol:water (4:1:1) as solvent. The major fraction (which, in this solvent system, has a very different $R_f$ from glycine or diglycine) was located by X-ray film, eluted, and dried by lyophilization. It was firmly established that this compound was not dicyanamide itself since the substance failed to give an insoluble copper salt, as DCA does. However, it did give an insoluble silver salt, as do most cyanoguanidines (130). It was

also certain that the compound was not tricyanomelamine, the cyclic trimer of DCA, since the UV spectra of the isolated compound and the trimer were quite different. The isolated product was unable to promote detectable dipeptide synthesis.

A portion of the isolated compound was mixed with KBr and pressed into a pellet for analysis by infrared spectrophotometry. By comparison with a number of known reference compounds, the following assignments appeared evident for these observed absorption peaks: 1345 $cm^{-1}$ (secondary amine), 1385 $cm^{-1}$ (tertiary amine), 1645 $cm^{-1}$ (imine), 2180 $cm^{-1}$, 2230 $cm^{-1}$, and 2300 $cm^{-1}$ (cyano), and a number of maxima between 3060 and 3540 $cm^{-1}$ (amines). These spectra are in accord with the structure suggested for the product (N,N,N'-tricyanoguanidine). Such a compound is not at all unrealistic since both cyanoguanidine and dicyanoguanidine are already known (140). Elemental analysis also suggested that the compound was the dimer of dicyanamide.

The quantitative values observed in the infrared spectrum noted above were compared with those for cyanoguanidine (in KBr). The maxima compared were those of the imine bond (1640 $cm^{-1}$ in DCDA) and the cyano group (2230 $cm^{-1}$ in both). (Cyanoguanidine has one of each group whereas it is proposed that the DCA dimer has three cyano groups to one imine group per molecule.) The calculations were carried out in the same manner as had been done in the case of compound A, already discussed. The ratio of the cyano absorbancy to the imine absorbancy was calculated for each compound. The ratio for the DCA product (0.301/0.114) was divided by the ratio for DCDA (0.061/0.076). A value of 3.29 was calculated, further supporting the proposed structure. The production of such a compound is con-

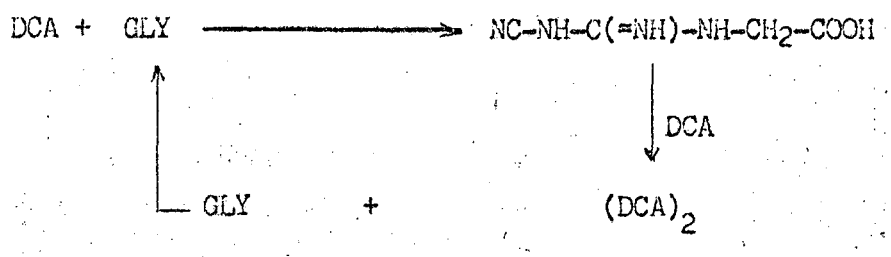
sistent with the molality drop observed in a NaDCA-glycine solution noted earlier.

The compound is retained by both Dowex-1 (anion exchanger) and Dowex-50 (cation exchanger)columns. This would suggest that it is a zwitterion in aqueous solution.

As pointed out earlier, hydrogenation is an effective means for removing cyano groups in cyanoguanidines. Therefore, using $^{14}C$-labeled NaDCA, the compound believed to be tricyanoguanidine, the dimer of dicyanamide produced during dipeptide synthesis, was synthesized as before and isolated by paper chromatography. Mild hydrogenation (sodium in alcohol) was employed so as to permit the isolation of the intermediate products of cyanolysis. Thus, one would expect the production of the mono and dicyano derivatives of guanidine, among others. This would permit confirmatory identification with known standards. Paper chromatographic analysis revealed that, in particular, cyanoguanidine (dicyandiamide) was one of the products of hydrogenation, supporting the proposed structure of the DCA product.

Therefore, the limiting factor in the DCA-mediated synthesis of dipeptide is evidently the dimerization of dicyanamide to tricyanoguanidine. The latter reaction is apparently amine catalyzed. The data already presented indicate that one concurrent product of dipeptide synthesis is compound A, the amino adduct formed between glycine and dicyanamide. Such a substance could be seen as the intermediate in the polymerization of dicyanamide whereby the glycine would be available at the end of this reaction for reuse and would

thus be needed in only catalytic amounts, which is consistent with the observations made thus far.




By varying the concentrations of the reactants it was determined that the percent of glycine found in the form of this amino addition compound (A) in the final product solution (5 minutes reaction time) is minimized by increasing the initial amount of NaDCA in excess of the glycine. This would be expected by the mechanism just proposed since compound A could act as a reactive intermediate if more DCA is still available or as a dead-end sideproduct if all the DCA is accounted for.

Compound A was prepared from $^{14}C$-labeled NaDCA, $^{3}H$-labeled glycine, and HCl, and was isolated by paper electrophoresis. To this isolated fraction was added fresh unlabeled NaDCA and HCl. Paper chromatography revealed that label was now found in dicyanamide dimer as well as glycine. This corroborates the suggested mechanism for compound A to act as an intermediate in DCA dimerization whereby the glycine is released at the end of the reaction for reuse. Also, when both NaDCA and glycine, with HCl, are added to the isolated compound A (whose glycine moiety is labeled), it is possible to detect by paper electrophoresis the production of diglycine as well.

It was observed that methylamine could promote the same decrease of molality of a NaDCA solution as could glycine. To be certain that the chemical effect was the same in the two cases, the following experiment was carried out: Two solutions were prepared. The first contained glycine, HCl, and labeled NaDCA, while the second contained methylamine, HCl, and labeled NaDCA. When analyzed by paper chromatography, both systems exhibited the same major product.

If the key to the cessation of dipeptide synthesis is the polymerization of the dicyanamide and if the condensing agent is added in portions over a period of time rather than all at once, one would expect an enhancement of diglycine yield. Such an event has actually been observed. In one case, a solution was prepared containing glycine (0.01 M), HCl (0.1 N), and NaDCA (0.1 M), whereas the reactants were mixed together at the same time. In another case, a similar solution was prepared except that the concentration of dicyanamide was 0.05 M. Five minutes after mixing, the solution was transferred to another vessel containing enough fresh NaDCA to bring the concentration (total initial) of the reactant to 0.1 M. The samples were analyzed after five more minutes of reaction time by paper electrophoresis. The results indicated that the latter experiment, where the dicyanamide was added in two equal parts, gave twice as much dipeptide as the former experiment, where the total condensing agent was added at one time. It was then shown that by adding small amounts of dicyanamide continuously during the reaction period it was

possible to use up nearly all of the original glycine in the form of peptides and side products.

Another approach to the slow addition of the dicyanamide was to use the clay mineral, kaolin. As pointed out earlier, kaolin has the capacity to exchange anions with its surface hydroxyls. Since dicyanamide is a strong anion (in the dissociated form), it would seem reasonable to expect the condensing agent to be bound to the surface of the kaolin and in dynamic equilibrium with the solution phase. Such an experiment was carried out whereby a solution was prepared (1 ml) containing glycine-2-$^{14}C$ (0.01 M), NaDCA (0.1 M), and HCl (0.1 N). To this was added 8 mg of powdered kaolin and the total mixture was kept in suspension by a magnetic stirrer. Aliquots were taken at various periods following initial mixing of the reactants and were analyzed by paper electrophoresis. The results indicated that, in fact, the reaction did not terminate within the first minute or two after mixing, but rather that the yield of diglycine continued to increase long after it would have stopped with kaolin absent. Although the yield was initially lower than without kaolin present after 3 minutes of reaction time), it is quite possible that the build up of diglycine with time (which was probably the case during chemical evolution) would give a larger overall yield than if kaolin had been present.

11) Role of reaction side products

A pertinent experimental observation involved the possible synthesis of glycylglycine using cyanurea. An aqueous solution was prepared with all reactants (glycine-2-$^{14}C$, cyanurea, and HCl) in 0.1 M concentration. After 30 minutes the products were resolved by paper electrophoresis. No dipeptide was observed.

It is necessary to understand why, in the IR studies previously noted, the production of a cyanurea-like absorption increased well after the time at which dipeptide synthesis had stopped. Using trisodium tricyanomelamine (NaTCM) as a model compound, this point was considered. A sample of NaTCM was hydrolyzed in 1 N HCl at 100°C for four hours. The resultant product exhibited a new 1698 $cm^{-1}$ peak in the spectrophotometer. This might suggest that this same maximum observed during the course of dipeptide synthesis could be due to the slow hydrolysis of the available cyano group of DCA or its polymers (dimer,trimer, etc.). It is significant to recall that even though a NaDCA solution containing N,N-dimethylglycine exhibited the same 1698 $cm^{-1}$ peak after 30 minutes as one containing glycine, polymerization was apparently the primary fate of the dicyanamide in the latter solution but occurred only to a very small extent in the former solution. The apparent slow pH change in a NaDCA-glycine-HCl solution could also be attributed to the formation of these ureas. (For example, the pK of DCA is -0.8 whereas the pK of cyanurea is 4.6.)

A portion of the dimer produced from dicyanamide, described in the previous section, was dissolved in $D_2O$. Enough HCl was added

to bring the concentration of the acid to 0.1 N. The solution was placed in a $BaF_2$ cell and observed in the infrared spectrophotometer against a $D_2O$ reference. The same carbonyl peak at 1698 $cm^{-1}$ gradually appeared just as it had with the system containing NaDCA, glycine, and HCl. This would support the suggestion in the previous paragraph.

A reaction mixture was prepared containing NaDCA, glycine-2-$C^{14}$, and HCl, and after 5 minutes, standard diketopiperazine (glycine anhydride) was added. The resulting mixture was applied to a sheet of Whatman No. 1 paper and chromatographed with a mixture of n-butanol:acetic acid:water (4:1:5-organic phase) as solvent. After it was dried, the paper was cut to the size of the electrophoresis apparatus and the products were further resolved in the second dimension with the borate buffer (pH 9.2) at 300 volts. The resultant separation was charted with X-ray film and the carrier was located with iodine vapors. None of the glycine anhydride was observed in the product.

Assuming that the reaction of DCA-mediated dipeptide synthesis follows a mechanism similar to that of the carbodiimides, one of the side products of the reaction might be expected to be a rearranged urea (197). This would result from the proposed acylisourea rearranging to a stable form other than cyanurea and dipeptide (see Figure 38). The standard acylurea was prepared in the following manner: To 5.0 ml of dimethylformamide was added 85 mg of cyanurea, 330 mg of N-carbobenzoxyglycine-p-nitrophenyl ester, and 0.3 ml of triethylamine. After it remained 40 hours at room temperature, the solution was added to 1 ml of 1 N $NH_4OH$. After one hour, to this solution was added 1 ml of a 20% (w/v) solution of silver nitrate. The mixture was centrifuged and the supernatant was decanted. The silver

salt precipitate was washed five times with water and was then dispersed in hot water. To this was added ten drops of concentrated HCl. The resultant mixture was centrifuged and the supernatant was decanted, filtered, and evaporated to dryness under reduced pressure. The residue was taken up in 5 ml of glacial acetic acid and enough concentrated HBr was added to bring the concentration of the latter compound to 2 normal. (Cyanurea is soluble in this solvent mixture.) After 35 minutes, the product was isolated by centrifuging the mixture and decanting the supernatant. The precipitated product was recrystallized from glacial acetic acid and was dried in the oven at 75°C. The identity of the product was substantiated by elemental analysis (calc'd: C-26.89, H-3.79, N-31.37; found: C-26.68, H-3.46, N-31.05). The prepared compound was also analyzed by infrared spectrophotometry. Using cyanurea, glycine, diglycine, and triglycine as reference compounds, the following assignments were made for distinctive maxima observed in the spectrum of the synthesized compound in $D_2O$: 1450 $cm^{-1}$ (amide), 1475 $cm^{-1}$ (amide), 1677 $cm^{-1}$ (amide carbonyl), 1700 $cm^{-1}$ (urea carbonyl), and 2180 $cm^{-1}$ (cyano). Neither the 1677 $cm^{-1}$ nor the 1700 $cm^{-1}$ peak varied in magnitude with changes in solution pH. In acetonitrile, the two carbonyl peaks were observed at 1735 and 1755 $cm^{-1}$. Also, the compound is ninhydrin-positive, whereas cyanurea is ninhydrin-negative.

A mixture of $^{14}C$-labeled glycine, NaDCA, and HCl was prepared as in the diketopiperazine experiment just described. A portion of the standard acylurea compound just synthesized was added to the product solution after 5 minutes of reaction time. Analysis was carried out in the same manner as in the diketopiperazine experiment

using a combination of electrophoresis and paper chromatography. The acylurea carrier was located with nitroprusside spray. The results indicated that the rearranged urea was indeed synthesized and the implications of this on the elucidation of the mechanism are apparent (see Figure 38). The acylurea's mobility by electrophoresis at pH 9.2 is less than that of glycine. Using labeled glycine in one case and labeled NaDCA in another, it was ascertained that the product contained both reactants. A portion of this fraction was isolated from the NaDCA-glycine-HCl system by electrophoresis. Analysis by infrared spectrophotometry showed that the compound had the cyanurea-like carbonyl absorption at 1700 $cm^{-1}$ in particular. It was certain that the fraction was not cyanurea itself since the latter compound has a much greater mobility in the borate buffer used than the isolated material.

By referring to the diagram of the proposed structure of the acylisourea intermediate (see Figure 38), it would seem a priori that rearrangement of the acyl group to form the acylurea sideproduct could proceed either to the terminal imino nitrogen ($NC-NH-CO-NH$ $CO-CH_2-NH_2$) or to the central secondary amino nitrogen ($NC-N(CO-CH_2-NH_2)-CO-NH_2$). It is not unambiguously certain which form had been produced in the synthesis of the standards as well. Therefore, the acylurea was prepared from NaDCA, $^{14}C$-labeled glycine, and HCl, and was isolated by paper electrophoresis. From the two proposed structures of the acylurea, it is apparent that the labeled products of partial hydrolysis would probably be only glycine in the second case and both glycine and glycinamide in the first case. Hydrolysis of the isolated labeled acylurea was

carried out in 2 N HCl at 95°C for three hours. Paper chromatography with known standards revealed thathydrolysis of the acylurea did yield glycinamide, tending to support the first structure proposed above. Also, it may be recalled that the synthesis of the standard acylurea included a step involving the production of the silver salt of the carbobenzoxy derivative. This would require an acidic hydrogen. Such would more likely be the case with the first structure proposed for the acylurea than the second.

The form in which the dicyanamide and dicyandiamide are reactive, carbodiimide or cyanamide, although not critical to the understanding of the mechanism, is also of interest to ascertain. Two model compounds were chosen for this study: cyanamide and aminoacetonitrile. The former compound ($NH_2CN$) differs from the latter ($NH_2CH_2CN$) by one methylene group, whereby cyanamide has the potential for existing as carbodiimide, its tautomer, but aminoacetonitrile, with its less labile methylene hydrogen, has less chance of containing a paired double bond system and is more likely in the cyano form. Solutions were prepared containing $^{14}C$-labeled glycine (0.01 M), HCl (0.1 N), and cyanamide or aminoacetonitrile (0.1 M). Analysis was carried out by means of paper electrophoresis. Diglycine was observed only in the experiment in which cyanamide had been used, corroborating the suggestion that it is the carbodiimide form of the cyanamide group of compounds that makes them reactive as condensing agents.

It was definitely established that the major compound produced from the dicyanamide is not tricyanomelamine, the cyclic trimer of DCA. The trisodium salt of tricyanomelamine (NaTCM) was prepared by

heating NaDCA to a dull redness (208). As a side point of interest, diglycine synthesis was repeated except that TCM replaced dicyanamide. No dipeptide product was observed.

D. Discussion

The data recorded in this section were collected with the intention of shedding light on the mechanism of peptide synthesis promoted with dicyanamide. As with any mechanistic study, absolute certainty of the actual course of the reaction is never unequivocally achieved but rather evidence is compiled to lend support for a suggested scheme and rule out others. For example, the main synthesis under study was, of course, DCA-mediated peptide synthesis. However, it is reasonable to assume that compounds closely related to dicyanamide, such as cyanamide and dicyandiamide, operate by a similar mechanism as the primary agent under study. Since the reaction using DCDA is apparently slower than with DCA, certain types of evidence collected from the former system provide useful information with regard to the latter.

In the case of the experiments noted in the first part of this section involving dicyandiamide, it was observed that the magnitude of the production of the urea of DCDA equals the concurrent synthesis of dipeptide. In terms of the energetics of the reaction, these data suggest that the energy needed to form a peptide bond comes from the energy released by hydrolysis of the DCDA.

The experimental observation of the advantage of low pH's for the DCDA-promoted condensations gives some indications of the mechanism of the reaction. Also, at least in the solid state, the presence of the carbodiimide group has been established in DCDA (141). These two facts, taken together, might suggest that it is the carbodiimide form of DCDA which is the effective dehydrating agent and that it is effective by virtue of its ability to add a carboxylic acid group across one of the imide double bonds. Thus, one might expect with cyanamide dimer an amidinium (acylisourea) intermediate which, presumably, is involved in the dehydration reaction.

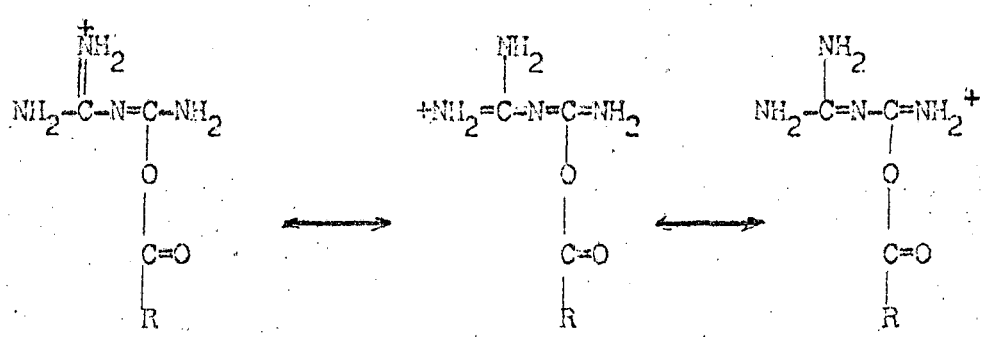


Fig. 36. Three of the principal resonance forms of the proposed amidinium (acylisourea) intermediate.

Similar intermediates have been suggested for the operation of the dialkylcarbodiimides (197).

As previously mentioned, it has been suggested that the primary form of DCDA in neutral solution involves the diamine structure in contradistinction to the amidinium form (139), which is consistent with the proposed intermediate of Figure 36. In the neutral diamine

form it is apparent that the carbodiimide structure, believed to be the key to the dehydration reaction, cannot easily form. However, with protonation, a new situation is realized in which the carbodiimide might very well be a dominant species.

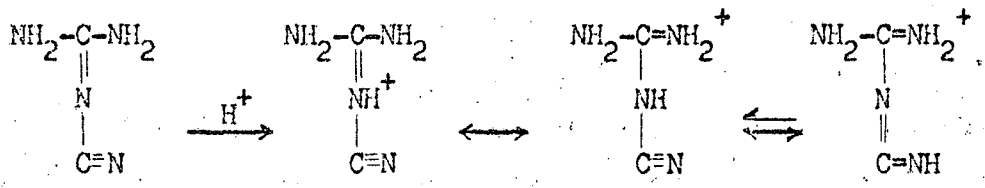
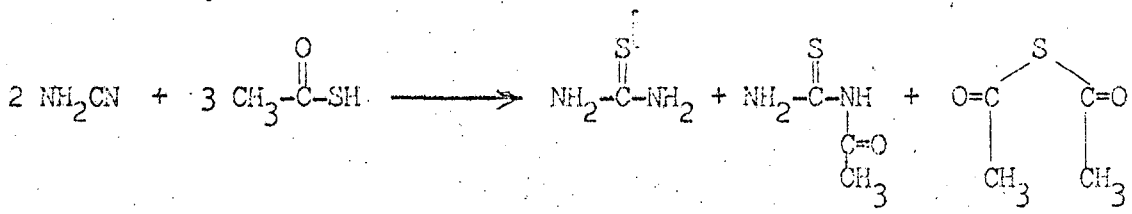


Fig. 37. Suggested protonation scheme of DCDA.

By varying the concentrations of the reactants involved in the sythesis of diglycine using dicyanamide, a number of useful observations were made. For example, the variation of solution acidity promoted a rise in the yield of product as the pH was lowered. Since the pK of DCA is -0.8, these data appear to indicate that the protonation of dicyanamide anion is necessary for its participation in dipeptide synthesis. A plot of the data found in Figure 30 with regard to the solution pH indicates a continuous rise in yield below pH 1.5. The carboxyl pK of glycine is 2.4 (207). Therefore, these data suggest that the key to yield enhancement is critically involved with the protonation of the dicyanamide anion. (Also, it was demonstrated that the carboxyl group of the glycine reacts in the undissociated form.) Upon addition of a hydrogen ion to a DCA anion, it is evident from the structure of the molecule that the neutral carbodiimide form would now be possible. Apparently the additional hydrogen

is essential for the carbodiimide structure to appear (assuming it is the active species).

The data collected in varying the availability of all three reactants suggest that the rate of appearance of diglycine, disregarding possible side reactions, is proportional to the concentrations of acid, glycine, and dicyanamide (NaDCA). Thus, the acid, glycine, and DCA are directly implicated with the steps of the reaction. It was hypothesized that the key intermediate of the reaction was formed by the carboxyl group of the amino acid adding across the CN group of the condensing agent giving an acylisourea. It is significant that thioacetic acid reacts vigorously with cyanamide to give thiourea and acetylthiourea (211).



Also, as noted earlier in the case of DCDA-mediated dipeptide synthesis, the production of the corresponding urea equals the amount of dipeptide formed, which argues for a C-O bond in the intermediate of the reaction. Combining this with the information about thioacetic acid, since it is assumed that the various members of the cyanamide group of compounds react by a similar mechanism, the suggestion of an acylisourea intermediate is apparently well-taken. In addition, the observation of the rearranged acylurea sideproduct in the course of DCA-mediated peptide synthesis, like the acetylthiourea noted above, corroborates the point. It is difficult to imagine another mechanism by which such a

compound could have been produced under the conditions of the reaction.

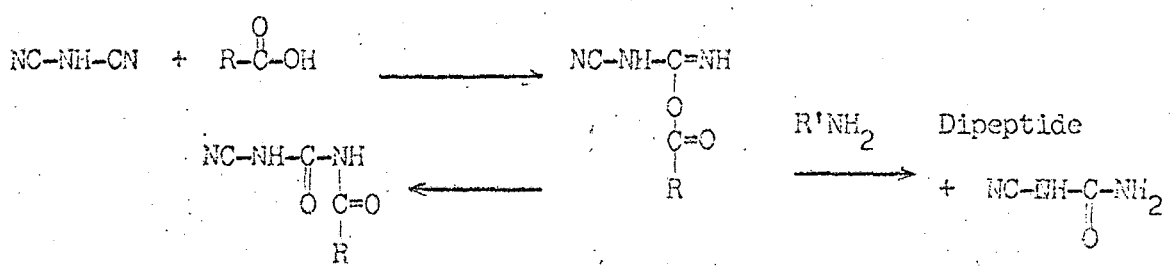



Fig. 38. Formation and fate of acylisourea intermediate

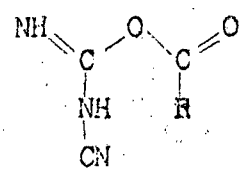
Another piece of data supporting this isourea-type of formation involves the reaction of NaDCA in absolute alcohol with an equivalent amount of dry HCl at room temperature (208). By this process, ethylcyanoisourea is synthesized. ($NC-NH-C(=NH)-OC_2H_5$)

It can also be argued that the predominant form of the intermediate is, in fact, a tautomer of the structure in Figure 38, whereby the double bond is shifted and would result in a seemingly more stable conjugated system. ($N\equiv C-N=C(-NH_2)-O-\overset{O}{\overset{\|}{C}}-R$). Similarly, the most favorable form of dicyanamide would be, by this argument, the carbodiimide since it too would then be conjugated. ($N\equiv C-N=C=NH$). The experiments with aminoacetonitrile and the IR data tend to support this suggestion.

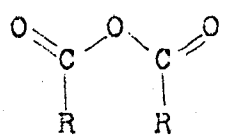
Dicyanamide is reactive in aqueous systems containing reactants with acid functions (carboxyl, for example) but is unreactive in systems containing only alcoholic hydroxyls, as shown quite clearly in the first section of this work. Similarly, it can be recalled that in the experiments where the reactivity of glycine peptides was compared at pH 1.4 and 2.6, a difference inyields of appropriate products could

only be accounted for by the variation in the degree of protonation of carboxylate groups. Since the nature of the amino groups of the participating amino acids was essentially the same at both pH's, the difference in reactivity could therefore be explained by the variation in the capacity of the carboxyl group (rather than the amino group) to react with the dicyanamide to form a species reactive in peptide synthesis. Both sets of experiments tend to support the suggestion of an acylisourea-type of intermediate.

The structure of the proposed acylisourea is much like that of an acid



anhydride. This would undoubtedly contribute to high reactivity and



relative instability in acidic solution. This is probably why the isourea intermediate could not be isolated.

The proposed mechanism for the amine-promoted dimerization of dicyanamide is much like that noted in the case of cyanamide (209). The production of dicyandiamide from its monomer is greatly facilitated by the presence of ammonia. It is also important to note the experimental observation that upon warming in aqueous solution, ammonium dicyanamide rearranges to dicyandiamide and dicyanamide polymer (210).

$NC\text{-}N(NH_4)\text{-}CN \longrightarrow NC\text{-}NH\text{-}C(=NH)\text{-}NH_2 + (NC\text{-}NH\text{-}CN)_n$. These products indicate that much of the ammonia which was part of the original reactant is released at the end of the reaction. Similarly, many amines form dicyanamide salts with DCA which spontaneously rearrange to substituted dicyandiamides (cyanoguanidines) at room temperature (210).

$$HN(CN)_2 + RNH_2 \longrightarrow RNH_3^{+}\,{}^{-}N(CN)_2 \longrightarrow NC\text{-}NH\text{-}C(=NH)\text{-}NH\text{-}R$$

It can be noted that the structure of this substituted cyanoguanidine is the type of product (A) proposed here inthe NaDCA-glycine-HCl system. The similarity of the substituted cyanoguanidine in the second reaction diagrammed above and the cyanoguanidine product accompanying DCA polymer synthesis during the ammonium dicyanamide rearrangement is also evident. Therefore, it is consistent with observations made in related reactions that the course of inactivation of the dicyanamide (as a condensing agent) involves an amine catalyst which promotes the polymerization of the DCA. With the molality studies already discussed here it was shown that whereas dicyanamide in dilute HCl polymerizes slowly, probably due to the small degree of protonation of DCA anion under the conditions of the experiment described, the rate of dimerization of the DCA is greatly enhanced by the presence of an amine. The reactions just cited above would suggest that DCA anion forms a salt with the added ammonium ion (glycine). This then rearranges to a cyanoguanidine (compound A) which participates in the subsequent polymerization of the dicyanamide. This would also suggest that the kinetics of dimer formation using a molecule of compound A and a molecule of dicyanamide are faster than with just two molecules of DCA.

It was established that the major compound produced from dicyanamide during dipeptide synthesis, later identified as tricyanoguanidine, was not tricyanomelamine (TCM). Of significance is the observation that the polymerization of DCA probably does not utilize the cyclic trimer as an intermediate (209). This was concluded from the fact that the properties of TCM do not change very quickly when it is isolated, whereas the appearance of an insoluble polymer from free dicyanamide is realized after only a very short period of time.

It was shown that calcium and manganese were able to retard the slow appearance of cloudiness in a solution of NaDCA in 0.3 N HCl, suggesting a decrease in the rate of polymerization of the dicyanamide. However, the yield of dipeptide using DCA as a condensing agent was not increased by addition of either cation. On the other hand, the presence of calcium failed to diminish the rate of synthesis of tricyanoguanidine, the dimer of DCA, which is unable to promote peptide synthesis under the conditions described. It must be recalled that cloudiness does not appear in a solution of NaDCA in 0.1 N HCl. These results would suggest that whereas dicyanamide goes to a high polymer in 0.3 N HCl, probably due to the protonation of DCA anion, the amine-catalyzed production of dicyanamide dimer even in 0.1 N HCl is sufficient to terminate peptide synthesis. Going from 0.1 to 0.3 N HCl increases the degree of protonation of the tricyanoguanidine (TCG), a strong acid, which then can go to insoluble high polymers. It is known that when free dicyanoguanidine (DCG) is prepared by placing the potassium salt of the compound, a stable substance, in strong acid, an insoluble polymer results (210a). It would therefore seem that certain divalent cations are able to slow down the formation of high

polymers from dicyanamide but have little, if any, effect on the amine-catalyzed synthesis of DCA dimer. It is this synthesis of TCG which is apparently critical to the termination of DCA-mediated peptide synthesis.

It was previously noted that dicyanamide gives greater yields of dipeptide than dicyandiamide. By examination of the structures of these two compounds, it is evident that since a cyano group has greater electron-withdrawing properties (as found in dicyanamide) than an amidinium group (as found in dicyandiamide), one would expect a greater share of positive charge on the carbon of the reactive carbodiimide group. Thus, since a major difference between these two compounds appears to involve the potential for formation of positive sites, the corresponding difference in reactivity could be explained by a mechanism involving nucleophilic attack. Similarly, this property would aid in electron withdrawal in the acylisourea intermediate, thus favoring subsequent amino group attack.

It has been suggested that one possible intermediate step in the synthesis of peptides with dialkylcarbodiimides in nonaqueous solvents involves the formation of an anhydride between the carboxyl groups of the participating amino acids (120). This would then be subject to attack by the amino group of another amino acid and would result in the synthesis of the peptide bond. As yet there is no evidence for such an anhydride in the present aqueous system. It is unlikely that the compound would be synthesized in any appreciable quantity since such anhydrides, in general, are subject to hydrolysis in aqueous solution. Also, because of the low pH, the amino groups of both members would be charged by protonation and would discourage maintenance of the anhydride by their mutual repulsion.

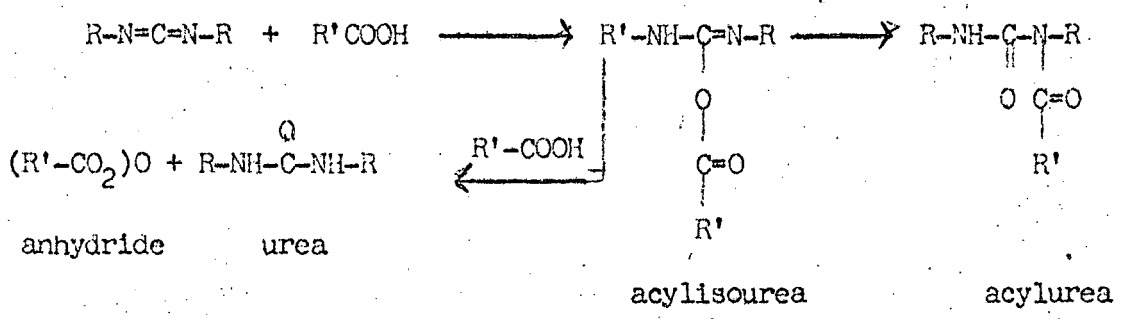
It was observed that during DCA-promoted dipeptide synthesis the primary fate ofthe condensing agent was the production of tricyanoguanidine. Since cyanoguanidine was shown here to be an effective condensing agent, it at first appears to be a discrepancy that the tricyano species is ineffective. However, it is known that whereas guanidine is a strong base and cyanoguanidine is a neutral compound in aqueous solution, dicyanoguanidine is a strong acid (140). The consequence of this makes tricyanoguanidine (TCG) a very strong acid and therefore highly ionized in aqueous solution. Since the formation of the acylisourea intermediate results from a nucleophilic reaction, the participation of an anionic reagent (TCG) in such a reaction is unlikely. This would also tend to explain why polymerization of DCA stops mainly at the dimer level under the conditions of the reaction.

Since it was initially postulated that dicyanamide could effect dehydration condensation by a mechanism similar to that found with the dialkylcarbodiimides (that is, dicyanamide is believed to tautomerize to a carbodiimide: N≡C-NH-C≡N ⟷ N≡C-N=C=NH), a consideration of the following experimental evidence reported for the latter group of compounds (197) offers helpful guidelines for comparison to the present study:

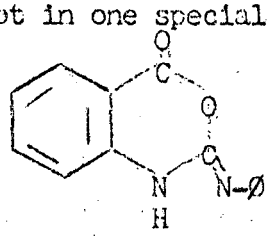
1) Dicyclohexylcarbodiimide (DCCD) is really hydrolyzed by dilute aqueous mineral acids. However, DCCD is relatively unreactive to water in neutral or basic solutions.

2) The reaction of DCCD with acylated amino acids (in acetonitrile) is 90% complete in the first minute at initial concentrations of 0.01 - 0.1 M.

3) The commonly accepted rea ction of DCCD to pro mote anhydride synthesis is indicated in the following scheme:




O-methyl isoureas can be isolated (120). The rate ofthe reaction varies with the nucleophilicity of the attacking group. This mechanism is backed up by kinetic data (197). Although all the products of the reaction have been identified, as yet the proposed acylisourea intermediate has not been isolated except in one special case.



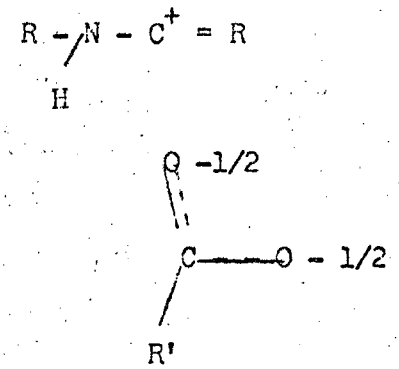
Kinetic data suggest that the disappearance of the intermediate, once formed, has, an an approximate lower limit, a reaction rate constant of 10 $mole^{-1}l\ sec^{-1}$. The presence of the acylisourea is inferred as a plausible chemical structure with regard to the known reactants and products of the reaction. It is possible to increase the amount of acylurea formed by having less organic acid available initially. Since the reaction of DCCD and acetic acid to form acetic anhydride is carried out in nonaqueous solvents such as benzene, $CCl_4$, ethylacetate and acetonitrile, free ionic species, such as those shown be-

low, probably do not occur in the formation of the acylisourea intermediate.

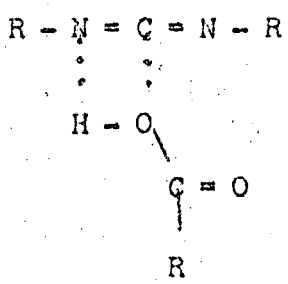
$$R\text{-}N{=}C{=}N\text{-}R \;+\; RCOOH \longrightarrow (R\text{-}NH\text{-}C{=}NR)^{+} + RCOO^{-}$$

4) Organic bases decrease the production of anhydride by apparently removing free acid from the system (as ion pairs).

5) Carboxylic acids work much better than phenols in the reaction. It is evident that the former are generally stronger proton donors than the latter. Therefore, it is possible to postulate that the formation of the acylisourea involves the following complex intermediate,

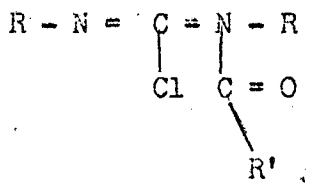



as an ion pair, rather than the four-centered system:




6) The synthesis of the acylurea does not seem to involve a reaction between the urea and anhydride products since a system initially containing urea and anhydride does not produce the acyl-

urea. Similarly, DCCD and acid chlorides give the following product:

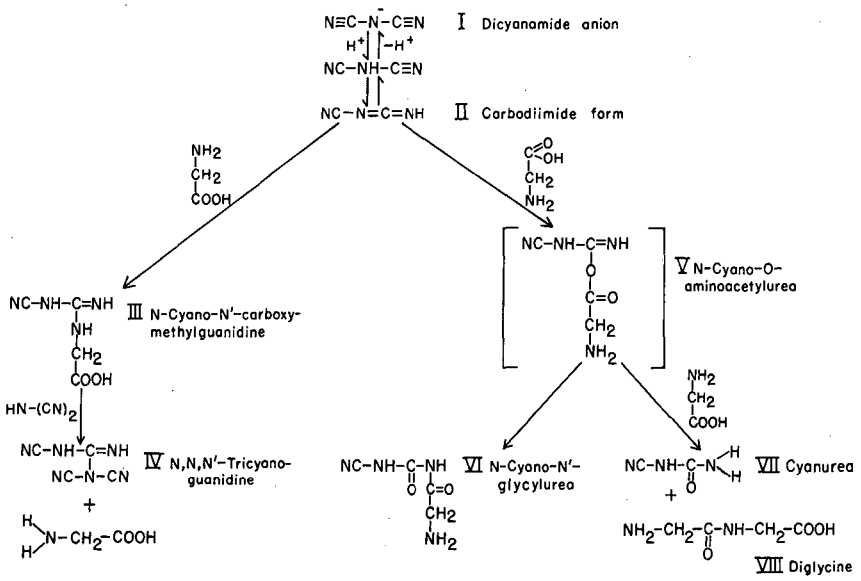


7) Ammonia and amines react with carbodiimides to give guanidines. (It may be recalled that cyanamide and sarcosine can react to give creatine (130)).

8) Alcohols can add to carbodiimides but this requires catalysts and vigorous conditions. (As yet, only those systems which have reagents with carboxylic and phosphoric acid groups appear to react with dicyanamide and dicyandiamide in aqueous solution).

D. Summary

Both the cited and experimental data noted in this report, when taken together, present a picture of the mechanism of DCA-mediated dipeptide synthesis. Each experiment was designed to provide information about one or more steps of the reaction scheme. This overall process is presented in Figure 39 and is the result of the considerations discussed here.




ZN-5306

Fig. 39. Proposed mechanism of operation of dicyanamide.

## ACKNOWLEDGMENTS

The author is greatly indebted to Dr. Melvin Calvin for his inspiring direction of the research work reported here. His scientific acumen provided stimulating guidance for carrying out this investigation.

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## APPENDIX I - pH TABLE

In the studies made on the mechanism of DCA-mediated dipeptide synthesis, a number of experiments were carried in which it was desirable to maintain a certain pH while varying the concentrations of the reactants, in particular DCA and glycine. Therefore, it was necessary to know how much acid to add in order to attain the desired pH under the designated conditions. To facilitate this, a Fortran computer program was designed to supply a table listing the various combinations of glycine and DCA, and the calculated acid normality necessary to achieve particular pH values for the solution.

Such a program is exhibited in Fig. 40. In the table resulting from this program, the concentrations of glycine and DCA are varied between 0.12 M and 0.01 M. The pH is varied between 1.0 and 2.1, in increments of 0.1 pH units. The established pK values for glycine and DCA are employed. The calculation performed by the program gives the normality of HCl that must be established in the solution in order to attain the particular pH under consideration. Thus, the resultant table lists the pH in the first column, the glycine concentration in the second column, the DCA concentration in the third, and the required HCl normality in the fourth column.

The actual computation was performed on an IBM model 7044 computer. The resultant data provided information for 1728 possible combinations of reactants and pH.

```
STEINMAN          PH TABLE                          FORTRAN SOURCE LIST MAIN
      ISN          SOURCE STATEMENT

        1          DIMENSION PH(13),A(13),B(13),C(13,13,13),X(13),T(13),W(13),Y(13),
                  $U(13)
          C        A IS GLY       B IS DCA
          C        PH-2.35 = -LOG(AH)/A
          C        2.35-PH = LOG(AH)/A      10**(2.35-PH(I))=AH/A=X      Y=X/(X+1)
          C        0.83+PH = LOG(BH)/B      10**(-0.83-PH(I))=BH/B=T     U=T/(T+1)
          C        W = H+ NEEDED TO PROTONATE WATER TO DESIRED PH
          C        C = H= TOTAL HCL NEEDED TO ACHIEVE PH  SCAN PH 1.0 TO 2.0
        2          DO 50 I=1,12
        3          DO 50 J=1,12
        4          DO 50 K=1,12
        5          PH(1) = 0.9
        6          A(1) = 0.00
        7          B(1) = 0.00
       10          B(K+1) = B(K) + 0.01
       11          A(J+1) = A(J) + 0.01
       12          PH(I+1) = PH(I) + 0.1
       13          X(I+1) = 10.0**(2.35 - PH(I+1))
       14          T(I+1) = 10.0**(-0.83 - PH(I+1))
       15          W(I+1) = 10.0**(-PH(I+1))
       16          Y(I+1) = X(I+1)/(X(I+1) + 1.0)
       17          U(I+1) = T(I+1)/(T(I+1) + 1.0)
       20          C(I+1,J+1,K+1) = A(J+1)*Y(I+1) + B(K+1)*U(I+1) + W(I+1)
       21    50    CONTINUE
       25          PRINT 60, (((PH(I+1),A(J+1),B(K+1),C(I+1,J+1,K+1),K=1,12),J=1,12),
                  1I=1,12)
       42    60    FORMAT (11H1 PH TABLE //4X,3H PH,20X,4H GLY,20X,4H DCA,20X,4H HCL,
                  1//(4X,F3.1,20X,F4.2,20X,F4.2,20X,F5.3))
       43          STOP
       44          END
```

Figure 40. Fortran program for calculating a pH table of solutions utilizing various concentrations of glycine, NaDCA, and HCl

## APPENDIX II - SPECULATION ON THE FORMATION OF MASS AND ENERGY

The real world, as is evident to any astute observer, is a conglomerate of various physical and thermodynamic interrelationships. These would include material transfer, interconversion of the various forms of energy, constructive and destructive co-activities, and the like.

A reflection upon these functions entices one to analyze the causes behind the observed effects. For example, the conception of the forces in the physical world rests entirely upon sense perception. Therefore, no matter what may be the actual conditions of a given event, absolutes, even into the realm of abstractions, must be proposed within the framework of sensual limitations, whether this be by first-hand observation or with the assistance of analytical instruments and aids.

Mental codification is based on interpretations of external impressions by which perception determines resultant values. Thus, for example, the degree to which an observer realizes the organization or disorganization of the units of a given system is subject to the manner in which the stimuli are received, the relative magnitudes of each class of data reaching the observer, and the value system he employs in drawing conclusions, whether this be arbitrary or prejudiced. Since man must draw conclusions from perceptions received, the expanse of these conceptions is subject to the limitations and the inaccuracies of the receptor or the means of transmission so that what is concluded to occur in a certain physical event may either not represent the actual phenomenon or may not give a large enough overview of all the factors significant to defining the meaning and relevance of the given event.

The law of conservation of matter and energy, for example, is a law only as long as no observation is made of some phenomenon which

disobeys it. Thus, even if such phenomena do occur but have not been observed either because of limitations of observations or lack of sufficient sampling experience, the law would still be quoted even though it was not actually true. Also, it is possible that the law is true today but at some previous time it was not, yielding the plausibility of spontaneous generation of mass and/or energy. In this case, a previously undefined concept of absolute nothingness would have to be postulated. This principle is more easily understood if instead of customarily accepting time as being infinitely divisible, it should rather be considered quantized. In other words, a point of division of time increments could be reached by which no further segmentation would be possible. The unit at that point would be the fundamental quantum of time. Combinations of these quanta constitute various segments of time passage such as the second. Undoubtedly, the fundamental quantum of time is much smaller than the second. It is here assumed that primordially the fundamental quantum was not the same as it is now and was in fact infinitely large.

Different velocities may be compared in two ways:

1) Constant distance with variable time;

2) variable distance with constant time.

The first case can be applied to the point at hand. Since $c = \Delta x/\Delta t$ and $\Delta x$, the distance traversed by a light photon during a quantum of time, is held constant, the only way c, the speed of the light photon, can vary would be if and when the quantum of time ($\Delta t$) is not constant because c is a function of $\Delta t$. Since it has been postulated already that primordially this $\Delta t$ was infinite, by the Einstein expression, energy was then nonexistent:

$$E = mc^2 = m(\Delta x/\Delta t)^2 = mx^2/\infty = 0 .$$

For time to pass, this quantum would have had to become finite, which would likewise cause energy, and from this, mass, to appear.

The primordial state of absolute nothingness, where the time quantum was infinitely large, was not a realm of an absence of something since time patterns were lacking and the realm itself did not even exist in which something could function in the first place. By this deficiency, physical laws were also nonexistent. This motionless primordial state gradually took on the dimension of moving time ($\Delta t < \infty$), which led to the appearance of energy. From the concept of absolute nothingness, it may be noted that energy is as interrelated with time as it is with mass. The apparent stationary character of the physical world today with regard to the constancy of total mass and energy would indicate that the quantum of time is, for all practical purposes, now constant on the Earth.

The concept of quantized time could also be used to explain other physical phenomena. For example, it has been observed that light emitted from distant galaxies has a tendency to appear shifted toward the red end of the spectrum when suitably analyzed (1). One explanation of this may be found in the following derivation where a hypothetical finite system is considered.

$h$ = Planck's constant

$\nu$ = light frequency

$\lambda$ = light wavelength

$\Delta x$ = distance traversed by a light photon during a quantum of time

$\Delta t$ = fundamental quantum of time

1) $E = mc^2$ (given)

2) $E = h\nu$ (given)

3) $\nu = c/\lambda$ (given)

4) $mc^2 = h\nu = hc/\lambda$

5) $\lambda = h/mc = h/m\Delta x/\Delta t$

(h, m, $\Delta x$ held constant)

6) $h/m\Delta x = k$

7) $\lambda = k\Delta t$

Thus, as $\Delta t$ goes from large to small,

$\lambda$ goes from I.R. to visible.

It has been observed that the red shift increases the greater the distance of the light source is from our Earth (1). Therefore, it may be hypothesized that somewhere in the vicinity of our solar system was the location of the first point in evolution where the quantum of time went from infinity to some finite value. By drawing concentric spheres about that point, the spread of this phenomenon may be visualized as outflowing waves. This would account for the observation of the increasing magnitude of the red shift with distance away from our Earth. Consequently, the universe is expanding in relation to the rate at which the realm of absolute nothingness is overcome by the spherically spreading deviation of the quanta of time from infinity.

If it is tentatively accepted that initially the real physical world was a giant blob of energy lacking mass, an additional factor must be included at this juncture. Such a possibility has empirical and theoretical foundations. The materialization of energy (2) has been observed in the case of high energy photons bombarded upon a heavy nucleus from which results a positron-negatron pair. In 1900, Planck proposed the concept of quantized energy which Einstein took up in 1905 to explain the photoelectric effect (3). This is quantitatively expressed by $E = h\nu$ where $\nu$ is the frequency, h is Planck's constant, and E is the energy of the photon. Thus, the photon is considered a quantum of energy which, when bombarded upon a suitable tar-

get, induces an expulsion of electrons (2). If the energy of the photon is above 1.02 Mev, the photon disappears within the field of the nucleus and a positron-negatron pair results, conforming to the equation $h\nu = 2mc^2 + E_1 + E_2$ where E is the kinetic energy of the particle at the time of production, $mc^2$ equals 0.51 Mev or the rest-mass energy of each of the resultant particles, and $h\nu$ is the energy of the photon.

Annihilation of matter is observed when a positron collides with a negatron, yielding two gamma rays each of energy 0.51 Mev. Similarly, it is possible for a positron-negatron collision to result in the production of a neutrino-antineutrino pair which also has no mass (4).

The positron may be viewed as an electron of higher positive energy, greater than $2mc^2$, while bearing positive charge and the same rest mass as the electron (5). On the other hand, a photon has no charge, no rest mass, and possesses energy by virtue of its motion at the speed of light. The photon is a quantum of energy at any of a number of frequencies, so that to define a gamma ray or an X-ray is merely to designate the frequency of the given group of photons.

The production of a positron-negatron pair by photon-photon collision has been proposed (6). Such a process requires sufficient energy. It is also significant that when the energy of the photons is of the order of billions of electron volts (Bev), the products of their collision can be a proton and an antiproton, each possessing identifiable mass and charge. As a result of the collision process, both photons disappear.

Pair production by photon-photon collision is very difficult to observe and perform experimentally because of the high energy ($h\nu_1 + h\nu_2 > 2mc^2$) and the very sensitive equipment required (7). However, a

positron-negatron pair production system becomes appreciable in the interior of stars where radiation density and temperature are both very high. The value kT is approximately equal to $mc^2$ at $T = 5 \times 10^9$ C° and above this the density of beta particle pairs becomes equal to the density of the light quanta themselves.

In photon-photon collision, the production of identifiable mass from energy without the need for an original mass has fundamental theoretical significance, especially in the hypothesis now under consideration. Not only does such a process give a plausible explanation for the materialization of non-matter from primordial energy sources, but it also suggests strongly the real significance of the electrical charge dichotomy observable in nature. One of the most outstanding examples is the electron and proton.

## APPENDIX III — PROSPECTUS

The following section has been included in this work to represent some general thoughts on the future of the investigation of chemical evolution. The experiments recorded to this point have been mentioned in order to indicate thatmeans are at hand for synthesizing biological monomers and polymers under presumed primitive Earth conditions. Therefore, the next step is to design means by which these compounds could have become organized into systems exhibiting the characteristics and properties of living organisms.

This section of discussion includes cited work from other laboratories as well as the suggestions of the present author. It is designed overall as a medium for speculation rather than an exact presentation of verified experimental results exclusively.

The reactions, syntheses, and experiments discussed here tend to indicate that possible means have been demonstrated by which most of the groups of compounds (and especially, in the light of the present study, phosphates, pyrophosphates, carboxylic esters, and peptides) needed forliving systems may have evolved on the primitive Earth. It is quite likely that the exact methods employed can, at best, only approximate prebiotic events but, taken as a whole, these syntheses indicate that essential biochemicals can be formed by the energy sources and simple reactants most likely present on the young Earth. The recreation of these syntheses is therefore restricted by

the probable lack of both initial complexity and specificty of the primordial environment, chemicals available for reaction, and the means for catalysis. Such restrictions are far from ideal from a synthetic organic chemistry point of view. However, a system (i.e., chemical evolution) able to make the transition from elementary gaseous units to complex macromolecules exhibits a large number of interesting and instructive chemical and physical phenomena.

Aside from the methane, ammonia, nitrogen, hydrogen, and water believed to have been the primary synthetic units, the central role of such compounds as cyanide and formaldehyde as key intermediates has been elucidated and emphasized by the experiments already discussed. That the simple methods employed produce just the chemicals presently utilized by biological systems tends to fortify the suggested validity of the approach to the problem. This is not to say that all successful systems truly reflected the course of evolution. However, that the general types of synthetic procedures, with all their restrictions, lead to chemical compounds of current biological significance gives credence to the suggested course of chemical evolution.

Perhaps the most significant synthesis reported here, with regard to its ramifications and applications to the problems of biogenesis, is the production of the peptide bond. The linking of amino acids gives rise to many possible new functions and operations.

Macromolecular polypeptides, or proteins, are important constituents of living systems (219). Their functions range from structural, such as keratin and collagen, to catalytic, as in the case

of the enzymes and cytochromes. Since the primary linkage (α-peptide) of nearly all amino acids in a polypeptide chain is of the same chemical nature, the factors which differentiate the physicochemical properties of one polymer from another involve the side chains of the constituent amino acids. In addition to providing a large number of possible linear combinations, it has been demonstrated (in the case of ribonuclease) that the primary sequence of amino acids appears to have great importance in determining the secondary and tertiary structure of the protein (220).

The diverse functions of polypeptides in biological systems make the elucidation of the means by which they may have been synthesized during chemical evolution very important. A number of definitions (to be discussed later) have been given to define at which stage evolving systems could be considered as living. Most seem to require autocatalytic systems that can reproduce, mutate, and reproduce mutated characteristics. Present knowledge indicates that such functions are determined by nucleic acids (221). However, the replication of nucleic acids requires enzymes (222). On the other hand, the biosynthesis of enzymatic proteins is controlled by nucleic acids. With regard to chemical evolution, this situation presents a dilemma as to which came first, proteins or nucleic acids.

The nontemplate-controlled random chemical synthesis of ribonucleic acid would not, a priori, appear to yield the specificity required in the 3'-5' phosphodiester linkage (although some as yet unconfirmed claims have been made of syntheses refuting this point (118)). On the other hand, nearly all peptide bonds observed to

date are of the same bond type (α-peptide). Also, metabolic functions are dependent on the presence of enzymes, and if such enzymes are available in sufficient (and autocatalytically increasing) quantity, nucleic acids are not as essential. If a protobiochemical system could be found by which peptide synthesis would be autocatalytic and somehow would favor certain, perhaps useful, amino acids sequences over others, the emphasis on the protobiotic necessity of nucleic acids would be diminished. The ultimate appearance of nucleic acids would merely serve to increase the survival potential of evolving systems by helping to preserve desirable traits. One could therefore envision first the development of useful polypeptide systems which could also serve as templates for ribonucleic acids whose information, in turn, could be translated and stored in the more permanent deoxyribonucleic acids. This is the reverse of the present day operation of these compounds. It is potentially dangerous to be prejudiced by present biosynthetic pathways in an attempt to elucidate the coure of protobiochemical evolution since such thinking may constrict one's field of vision.

It is likely that the earliest enzymic peptides were hydrolytic in nature. Since the operation of catalysts is reversible, it is evident that when a much higher concentration of monomer, such as amino acids, than dimer is present, the normal hydrolytic operation of the enzyme would be reversed and polymerization favored (223). Assuming preferential synthesis of the enzyme, this would be one type of autocatalytic activity. Also, it is known that the hydrolysis

of polypeptides goes rapidly down to the dimer and then relatively slower to the monomer level (194). This is apparently due to the fact that since amino acids are zwitterions in solution, the close proximity of the terminal charges in the dimer hinders the attack of the hydronium ion. In a synthetic procedure going in the other direction, it would appear that a similar situation would be encountered. Thus, as the chain grew longer, terminal charges would be separated further and the joining of new units would apparently become easier. Such a situation has actually been demonstrated (224). Whereas the enzymic (hydrolytic) equilibrium constant between glycine and diglycine is 0.001, the constant between diglycine and tetraglycine is 0.05. This is believed due to the free energy values of the larger peptides being more favorable for condensation. This would be another contribution to autocatalysis.

It is not necessary for high molecular weight peptides to have appeared for significant catalytic processes to have been going on very early in the course of chemical evolution. For example, a five amino acid segment of melanocyte-stimulating hormone has been prepared and demonstrated a detectable amount of biological activity (225). Histidine alone has chymotrypsin-like esterase activity (226). Also, it is possible to remove about two-thirds of the amino acids from the N-terminus of papain without loss of enzymatic activity (227). Similarly, most enzymes appear to operate at a specific site of activity within the protein's structure, usually involving a very small number of amino acid residues (228). This would suggest that the residues making up the large majority of the macromolecule merely serve to maintain stereospecificity in the positioning of the few amino acids constituting the active site. These examples

point out that small polypeptides, of the type that would be found in the early stages of biopoietic development, could have readily served as useful participants in protometabolic systems. Evolving biological units made more efficient by these organic catalysts, which probably replaced earlier forms of simpler catalysts such as divalent cations, were not only improved by the presence of them but also protected them from the perils of the external environment. Thus, the importance of the demonstration of a possible means by which the peptide bond appeared on the primitive Earth is recognized.

A number of methods have been employed in the laboratory to chemically synthesize polypeptides. These usually require the blocking of the appropriate nonreacting amino and carboxyl groups followed by activation of the portions to be joined (228). These syntheses are usually carried out in nonaqueous solvents. Some of these schemes include the carbobenzoxy method, where the carboxyl group is converted to the reactive azide (228), the N-carboxy anhydride method which gives long chains of homopolymers (229), and the method employing dialkylcarbodiimides (120). It is quite unlikely, however, that such complex systems were present and operative to any great extent on the primitive Earth. The use of unprotected amino acids with simple activating systems was the most likely course of events. As discussed earlier, a few laboratories have successfully demonstrated peptide synthesis under these conditions. However, most of these have either required unrealistic activating agents (118) or unlikely experimental conditions (92).

In line with earlier discussion, it would appear that the most likely duplication of prebiotic chemistry would involve unprotected amino acids, simple reagents, dilute aqueous solutions, and moderate temperatures, such as that demonstrated here with dicyanamide.

One important question in abiotic polypeptide synthesis involves those amino acids which have reactive groups in their side chains, such as the $\varepsilon$-amino group of lysine, the $\gamma$-carboxyl group of glutamic acid, and the $\beta$-hydroxyl group of serine. It would appear that any synthesis which could involve the $\alpha$-amino or carboxyl group of the amino acids could just as easily utilize the reactive sides of the type noted here. Therefore, since these side chains were available as well, a mechanism for polypeptide synthesis must also consider the way in which reactive groups could be incorporated in the chain without disrupting the primary unit-linking process. As noted earlier, it has been suggested that reaction of appropriate aldehydes and ketones with a polyglycine chain could result in the formation of a polymer bearing the characteristic side chains of polypeptides (107). This has been demonstrated experimentally with formaldehyde and acetaldehyde, yielding seryl and theonyl residues, respectively. Mechanisms were proposed for the synthesis of other reactive side chains. Thus, if such a mechanism were operative, the initial polypeptide chain formation would involve the simple amino acids (such as glycine, alanine, and the like) and reaction of the resultant polymer with appropriate aldehydes would lead to the variety of residues known in polypeptides today. The likely presence of aldehydes on the primitive Earth has also been demonstrated (26). It

is interesting to note that, as expected, random syntheses of amino acids under proposed primitive Earth conditions usually lead to a predominance of the simple amino acids with less reactive side chains (see Ref. 25,37, and 49, for example).

On the basis of information theory, a good argument can be made for the early effective use of polypeptides. Nucleic acids are recognized as the primary means by which biological systems now store information. However, they are effective in this capacity only by virtue of appropriate enzymes which can interpret and translate the nucleic acids for useful purposes (221). Information, I, is defined as (230);

$$I = - kN \sum_{i=1}^{m} P_i \log P_i$$

where m is the number of kinds of units available for association (such as letters of the alphabet), N is the number of units in the message (such as letters in a word), and $P_i$ is the probability of occurrence of each unit in any given message. If the probabilities of all units are equal, then the definition of maximum information becomes:

$$I = kN \log m$$

where k, as before, equals 3.31 ( $\frac{1}{\log 2}$).

Assuming for the moment that during chemical evolution associations of amino acids to form polypeptides, on the one hand, and nucleotides to form nucleic acids, on the other hand, are random, the probabilities of occurrence of each unit in the appropriate polymer chain would be equal. Therefore, in the case of a three-unit polynucleotide chain where four different bases are available, the maxi-

mum information* would be 6.02 bits whereas with a tripeptide the maximum information, assuming twenty different amino acids equally available, would be 12.9 bits. Thus it may be seen that polypeptides by comparison to polynucleotides can serve as effective information storing systems and that if an eobiotic system is able to collect and utilize similar polypeptides, the information inherent within these polymers could more than make up for the initial absence of nucleic acids. This would also be aided by the preferential sequencing and accumulation of potentially useful polypeptides in contrast to a hodge-podge of every association of amino acids imaginable.

The problem of the origin of optical activity in natural amino acids as well as other compounds of biological significance has never been clearly resolved (245a). One school of thought holds that stereospecific mineral surfaces such as quartz could catalyze the production of one enantiomer over another, but this would, of course, be restricted to a small region of space. Another suggestion has been that the Earth's magnetic field leads to a small preference of one direction of circularly polarized solar light over the other. This would apparently affect the outcome of light-promoted syntheses on the Earth's surface and in the water, but such a phenomenon has not yet been clearly demonstrated.

It is assumed, and such an assumption is certainly not without experimental backing, that a number of means have been put forth, some or all of which could represent historical phenomena, by which

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* Information may be defined as "that which sets or reflects a pattern of organization" (68).

essential biochemicals could have appeared on the primitive Earth. Them, the next problem that remains is how this molecule became organized into a metabolizing system. This is perhaps the most difficult problem to overcome in the ultimate elucidation of the origin of life. The appearance of systems that could be termed "living" was undoubtedly a very gradual chain of events leading to increased complexity rather than being an abrupt occurrence. In other words, evolving systems went through increasing degrees of becoming "alive". It is evident that to determine at which stage an evolving body could finally be considered definitely living, in contradistinction to inanimate, requires a consideration of the attributes of systems now designated as "living" and thus establish a definition to serve as a criterion for the test. Such an unequivocal definition is very difficult to establish. Living cells carry on metabolism which involves ingestion, digestion, secretion, energy conversion, excretion, and assimilation (231). Cells respond to stimuli and are able to reproduce. However, response to stimuli is not unique to living cells since, for example, a cube of sugar in the presence of carbon, heat, and oxygen responds by going to an oxidized product. Similarly, sulfur vacillates between its various allotropes with changes in temperature (232). Individually each biological function can be re-enacted in the test tube with the appropriate selection of reagents and catalysts, and is apparently subject to the usual standards of chemical and physical phenomena. However, as such the reaction is often less efficient than it had

been *in vivo*. For example, it has been calculated that the known rates of respiration could not occur if the enzyme participants were dissolved in free solution rather than arranged in their solid state array on the mitochondrial membrane (233). Also, the colloidal properties found in the cell are known to enhance catalytic action by the resultant heterogeneous character of the system which can promote adsorption and orientation of the reacting molecules (234). Changes in the colloidal properties of cells can directly affect enzyme activity (15). Similarly, DNA synthesized *in vitro* with DNA polymerase lacks the biological activity possessed by its natural template (221). A mixture of all the cellular compounds in a test tube obviously lacks the characteristics exhibited by the cell itself (177). There is nothing mystical about the way a cell is able to organize isolated reactions into a coordinated whole. This association is undoubtedly affected by the usual laws of chemistry and physics. The ignorance of how this operates is merely a function of the limited experimental elucidation of the nature of the phenomenon. Therefore, a major consideration in deciding whether a system is living or not is the observation of whether it bears the inherent properties giving it the potential for organizing the necessary component reactions into a coordinated system of events.

Another attribute of living systems is autocatalysis (235). As discussed earlier, it is conceivable that primordial polypeptides indeed possessed this property and effectively employed it in the promotion of eobiotic evolution.

The requirement that living bodies are able to grow is not unique to these systems, since crystals exhibit this function as well (235). Similarly, reproduction is not an essential attribute for a whole organism to be classified as living, since a mule is certainly alive but is unable to reproduce itself. An amoeba can be denucleated and is thus reproductively dead. However, it certainly goes on with its normal metabolic functions for an extended period and bears the usual properties of a living system (except reproduction )(177). However, the capacity to change and also reproduce such a change is apparently unique to living systems. The current excitement over nucleic acids has led many to suggest that no system could be classified as living unless it possesses these compounds to direct its course of existence (236). However, just as the essence of biological evolution is the survival of those organisms which have by chance acquired traits which enhance their survival potential (e.g., by genetic mutation) (231), so too the evolution of protobiological systems could depend on the chance acquisition of some valuable trait, such as a useful catalyst. Growth would result from continuous accumulation of similar material through selective membranes and catalytically enhanced alteration of these materials into useful constituents. Reproduction could occur once the unit passed a critical size of expansion resulting in separation into small units carrying portions of the acquired characteristics (e.g., catalysts, membranes, and the like). Thus, primitive evolution need not be restricted by the (possible) initial absence of

nucleic acids. The main problem now seems to be the solving of the question of how isolated events became coordinated into an evolving organism. However, the validity of the suggestions put forth must still be subjected to experimental verification.

The first step involves the identification of biological activity in compounds produced under primitive Earth conditions. Heating glucose together with asparagine has been reported to give nicotinic acid (237). The product exhibited activity in bioassay. The tripeptide, glutathione, has limited redox activity by itself (237a). Ferredoxin is a small polypeptide which contains twelve of the usual twenty or so amino acids (238). Seven moles of iron are bound by sulfur in it. It has been suggested that this compound may be the ancestor to the more complex hematoporphyrins.

A primitive form of enzymatic activity has been demonstrated with styrene (239). First, the styrene was polymerized in the presence of CO-heme diethyl ester. Then the CO was removed. It was found that the polymer could bind $O_2$ reversibly like hemoglobin. Also, the heterogeneous nature of a water-oil interphase causes linoleic acid to be rapidly oxidized by $O_2$ whereas no oxidation takes place when added ethanol makes the system homogeneous (240).

The proteinoid, discussed earlier, synthesized by anhydrous heating of a mixture of amino acids, appears to possess some enzymatic activity. First of all, when glucose is added to the polymer, $CO_2$ is detected (241). Also, the proteinoid seems to catalyze the hydrolysis of p-nitrophenyl acetate (242), although this can be promoted by a number of simpler substances (226).

The nature of protometabolic development presents another problem to be considered. It seems likely that heterotrophic nourishment was the most primitive form of metabolism (15). Chemical evolution undoubtedly provided great stores of ready-made food initially for the evolving systems. One good piece of supporting evidence that heterotrophic metabolism was the most primitive is that many autotrophic organisms now are also able to function as heterotrophs, if necessary, but the opposite arrangement is not observed Once the most easily utilized nutrients (A) were used up, those eobiots able to make A from other accessible compounds (B) probably had the greatest survival potential (243). Once the supply of B diminished, it was necessary to make A and B from C, and so forth. These early reactions were most likely fermentations. The direct (autotrophic) utilization of sulfate, nitrogen, carbon dioxide, hydrogen, and oxygen required the appearance of appropriate porphyrins, probably at a later date. One possibility for nutrient B is glycerate-2-phosphate (A would be like ATP or something of that sort) (244). It is argued that this acid phosphate could lead to the evolution of the major phases of metabolism such as high energy phosphorylation, oxidation-reduction (triose phosphate would act as the H carrier), amino acid and sugar synthesis, and the production of metabolic intermediates. That such is possible nonenzymatically has not been demonstrated experimentally as yet. However, if this type of scheme did indeed occur, it would represent the evolution of metabolic pathways in reverse of, as well as in, the direction in which they take place now whereby one catalyst (or enzyme) was added at a time. Such a suggestion is quite plausible.

Those organisms which then developed pigment-sensitizers had a supplementary means for more efficient utilization of organic substances (15). An example of this would be direct photosynthetic phosphorylation. It is noted that some obligate anaerobes can consume $O_2$ but this eventually leads to their death (245). These organisms are found to contain flavin pigments which could have once utilized oxygen in H transfer but don't have this function now.

Early protometabolic reactions, such as oxidation-reduction, probably involved metal cations in various states. Later, with the addition of porphyrin-like moieties, the catalytic efficiency of the process went up as much as one thousand times (202). Iron porphyrins, for example, might even oxidatively promote their own duplication by autocatalysis. Other metals could then replace the iron.

Thus it is likely, because of the suspected initial lack of oxygen and excess of hydrogen, that the first metabolizing systems were anaerobic (13). The energy sources available to these primitive systems included ultraviolet and visible light, ionizing radiation, heat, and chemical bond energy. The reactions of the systems probably involved simple compounds. Next, as greater amounts of oxygen appeared and hydrogen decreased, metabolism remained anaerobic but since compounds being used by the evolving systems, which by this time reflected the characteristics of living entities, were now more complex visible light could be utilized effectively. Organic reservoirs from prebiotic chemical evolution became depleted and living cells had to resort to photoreduction to satisfy energy

requirements. Ozone now helped to protect these units from the hazards of ultraviolet radiation. With increasing complexity, the fate of these eobiots became subject to Darwinian-type evolution. Finally photosynthesis appeared to greatly increase the availability of oxygen, making aerobic respiratory systems possible. In terms of the types of metabolic systems involved, this hypothetical (since, to say the least, it has not been repeated experimentally) course of evolution can be outlined in the following manner (246):

1) fermentation: glucose $\rightarrow C_2H_5OH + CO_2 + \sim P$ (energy source).
2) hexose monophosphate cycle: 6 glucose $+ 6H_2O + 12 \sim P \rightarrow 6CO_2 +$ 5 glucose + 24 H (available for metabolic reductions).
3) photophosphorylations: direct use of sunlight to get ATP (requires appearance of metalloporphyrins).
4) photosynthesis: capture of sun's energy for the synthesis of needed organic compounds with $O_2$ as a byproduct.
5) aerobic respiration: most efficient use of chemical energy.

Since the oceans are too immense to assume that chemical evolution provided a sufficient concentration of compounds to make these bodies of water one big unit going through the steps of metabolic evolution just outlined, a major problem that remains is how local agglomerations of compounds occurred. In other words, it is necessary to elucidate the means by which barriers appeared to provide local concentraions of chemicals which were shielded from the external environment. The semi-permeable membrane seems to have been adopted by all organisms as such a barrier (247). This membrane, as discussed earlier, is usually a double layer of lipid covered on each external side by protein. It has been suggested, as shown in Figure 41, that such a membrane could arise from the agitation of a protein solution covered by a lipid scum (248).

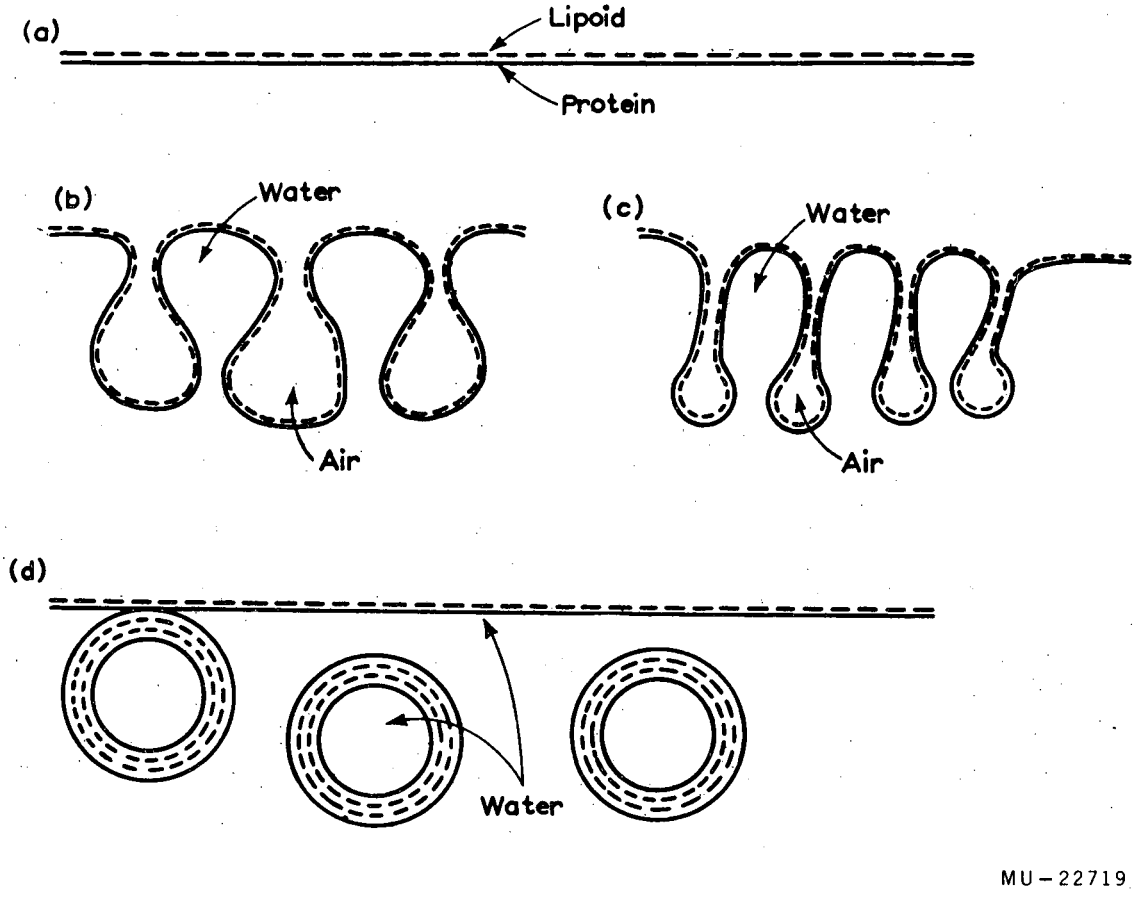


Fig. 41. Droplet formation by film collapse (from Ref. 248).

This phenomenon has been observed in the laboratory. Inorganic models for the semi-permeable membrane have been produced as well (249). Mixing $CuSO_4$ and $K_3Fe(CN)_6$ produces a membrane of $CuFe(CN)_5$. This structure is found to be selectively permeable and is also self-repairing to damage that may occur.

Another approach to the evolution of the isolated cell-type unit has been with the proteinoid, discussed earlier, which is synthesized by the anhydrous heating of a mixture of amino acids. When this protein-like product is boiled in water, membraneous microparticles appear (250, 251). They are usually 1 to 3 μ in diameter although some are as much as 80 μ. In the electron microscope, these structures appear to be double layered. They are stable enough to be sectioned and stained. When zinc is incorporated into the microspheres, they exhibit ATPase activity (252). However, as noted before, the likelihood of the idealized conditions under which the proteinoid is formed is highly questionable from a primitive Earth point of view.

By bubbling air through filtered sea water, aggregation of organic material into particles takes place (253,254). These units are capable of sustaining growth since they serve as a point of concentration of nutrient. Phosphate has been specifically shown to accumulate on the surface of the bubbles. Similarly, the surfaces of clays, such as kaolin, have been shown experimentally in the present work to also concentrate phosphate-containing compounds. Montmorillonite adsorbs amino acids (179).

The significance of colloid chemistry to biological phenomena has been pointed out (254a). It is from this field of study, therefore, that clues to the origin of living systems can be extracted. The association of the molecules and polymers formed during the preceding period

of chemical evolution may have depended to a great extent on the colloidal nature of the general environment and the ability of colloidal units to provide local milieux for accelerated development.

Oparin noted five physical phenomena which made coacervates possible candidates for the origin of primordial cells (255). First of all, they are able to selectively adsorb large amounts of substances onto their surfaces from the surrounding medium. Secondly, since it is postulated that the primordial bodies of water contained various amounts of polymers (as proposed in this dissertation as well as elsewhere), the material for the formation of coacervates was probably available.

Thirdly, coacervates can form from very dilute solutions of polymers. Also, these colloidal structures can freely interact with the environment.

Finally, protoplasm of present day cells has coacervate-like colloidal properties.

In suggesting that the coacervates provided the means by which a division was made between certain associations of macromolecular material and the general environment, Oparin also proposed the means by which these units developed into a stable, surviving system. His outline, although intended to apply specifically to coacervates, could equally describe the evolution of practically any of the proposed primordial biogenic units. By its physico-chemical nature, a coacervate droplet is only moderately stable and is prone to dissolution with changes in the environment. However, since the coacervate is able to exchange materials with its environment, it may possibly take in substances which are beneficial to its existence and thus sustain its existence. For example, it may acquire metal ions which might act as catalysts for simple internal reactions that could fortify its properties such as

surface tension. Those coacervates which gain such added stability have a greater likelihood for survival than those that do not.

Once having achieved self-preservation, the next step essential for perpetuation would require a means for growth. By taking in and utilizing materials from the environment, already rich in organic material, the mass of the coacervate population would increase. Those units exhibiting greater rates of mass accumulation would evidently displace less progressive systems. This increase of internal activity with the acceleration and assemblage of beneficial chemical reactions would rapidly alter the properties of the coacervate, promoting disturbances and ultimately fragmentation of the units, or division. The coordination of growth with this division would amount to reproduction. The available supply of organic compounds for direct use was, of course, limited and only those coacervates whose development had led to the acquisition of traits superior to those of other units would continue to exist. The ability to repeat successful acts would enhance this survival. Once repeated acts became coordinated into the system of the unit, self-duplication became possible and "life" in its most basic sense appeared.

The "purposiveness" of the system became the development of superior traits with which to cope with and utilize the environment so as by "natural selection" to survive less successful competitors. Catalytic functions were improved by sophistication and modification of the original inorganic materials. Further specialization led to regions of the biological unit taking on individualized functions and structure. Association of units led to interdependent colonies.

Oparin's outline was by no means the only plausible explanation for the sequence of steps leading to the appearance of the living cell. Oparin saw the original biogenic unit subdividing within itself in order to develop intracellular structures. However, it is also possible that the nucleus, mitochondria, and the like may have developed as independent organisms and later came together in a symbiotic manner. The finding of extranuclear DNA has helped to support this contention (256). Viruses may therefore be no more than "homeless" nuclei.

Coacervates have the property by which their internal environment strives to come into equilibrium with materials dissolved in the surrounding medium so that the internal concentration of a given substance is about the same as in the external medium. Living cells, on the other hand, often maintain a given concentration of a substance against a gradient which would ordinarily mean an escape of some of the given substance. Thus, the appearance of living cells required the development of the capacity for active (energy-consuming) transport against a concentration gradient. Likewise, whereas coacervates depended on unregulated chance to effect multiplication by division, the transition to orderly, systematic division, at least on a primitive level, was necessary in order for primordial biological units to bear the characteristics of living cells.

The Earth is believed to be 4.7 billion years old (257). A study of fossils and ancient organic deposits permits the determination of the length of time available in which prebiotic chemical evolution could have taken place. It is evident that the older the evidence for living organisms, the shorter was the time for the occurence of pre-

biotic events. Fossils have been studied by chromatographic analysis (258,259,260). Amino acids were detected in specimens as old as 450 million years. Plant-like cells have been observed in Pre-Cambrian rocks possibly dating to 2 billion years ago (261). Analysis of the Pre-Cambrian Nonesuch deposit, dated at 1 billion years, gave evidence of the isoprenoids, pristane and phytane (262,263). Material taken from this sedimentary rock also suggested the presence of sterane-type and optically active alkanes (264). The Soudan shale, believed to be 2.5 billion years old, similarly exhibited pristane and phytane (265). Electron microscopic studies have found evidence of organisms in rocks as old as 3 billion years (264a). The seemingly biological nature of these compounds and structures suggests that they reflect the presence of living organisms at a period in which the rocks were laid down. This could be taken to mean that prebiotic chemical evolution took place in something less than 2 billion years.

By definition, enzymes accelerate reactions which would occur even if the catalysts were not there. However, in the absence of these accelerators, most biochemical reactions would turn over so slowly that their progress would be exceedingly small. Therefore, given the necessary starting materials and an extensive amount of time, it is conceivable that by equilibrium considerations small quantities of required compounds would result (265a). As the period allowable for the occurence of chemical evolution is found to be shorter by new discoveries, more reliance is placed upon substances and conditions that were quite possibly present on the primitive Earth (such as dicyanamide) which could have accelerated the formation of biochemicals

essential for the ultimate appearance of living organisms.

The next phase of research into the problem of the origin of life is perhaps the most critical and most difficult of all: the assembling of the components into a coordinated, metabolizing unit. The following discussion of objectives listed by the Space Science Board of the National Academy of Sciences (266) describes quite well the future direction of this type of investigation:

> "The modern, naturalistic view of life's origin and evolution dates from the foundations of modern biology a century ago. Implicit in the evolutionary treatment of life is the proposition that the first appearance of organisms was only a chapter in the natural history of the planet as a whole. Oparin later made this notion explicit in his view that the origin of life was a fully natural, perhaps inevitable, step in the ontogeny of the Earth. Systems capable of self-replication and controlled energy transfer - living organisms - had their origin in the sequence of chemical changes that were part of the planet's early history. . .
>
> "To a significant extent the discussion of life's origin must concern the origin of those molecular types that are crucial in cellular organization: the origin of nucleic acids, of proteins, of carbohydrates, and so on.
>
> "In the 1950's a series of experiments was initiated in which the synthesis of biologically important compounds was accomplished by application of energy to presumptive primitive environments. The list includes: amino acids and their polymers; carbohydrates and fatty acids; purines and pyrimidines; nucleotides, including adenosine triphosphate (ATP), and oligonucleotides - every major category of molecular sub-unit of which the cell is built.
>
> "The credibility of the naturalistic, evolutionary view of life's <u>origin</u> as an exploitation of previous chemical evolution on a sterile Earth is greatly heightened by these results: the great chemical complexity of its molecular constituents does not, in the last analysis, require the intervention of the cell itself.
>
> "The general tenet that life involves no qualitative novelty - no <u>élan vital</u> - goes hand in hand with the more explicit proposition that it is the molecular <u>organization</u>,

as such, of living things that alone distinguishes them from the non-living. The central issue in discussing origins now concerns not so much the prior evolution of complexity in molecular constituents as the attainment of their organization into a system that is alive. It is here we lack any sure guides. . .

"It remains unclear, of course, what precise sequence of events exploited the opportunities afforded by the purely chemical evolution of the Earth's surface and atmosphere. But at some point in the unknown sequence a community of molecules would have been fully recognizable to us as a living as against a non-living thing: it would have been bounded from its environment by a membrane, capable of controlled energy expenditure in fabricating more of itself. . . We cannot fully know the precise course of the Earth's early chemical evolution, and the degree of contingency involved in the subsequent transition to a living organization of molecules. . . What is at stake in this uncertainty is nothing less than knowledge of our place in nature."

## REFERENCES


1. G.C. McVittie, Fact and Theory in Cosmology, Eyre and Spottiswoode, London, 1961.

2. P. Dirac, Quantum Mechanics, Oxford, 1935.

3. S. Glasstone and D. Lewis, Elements of Physical Chemistry, D. Van Nostrand Company, Inc., Princeton, New Jersey, 1960.

4. H. Semat, Introduction to Atomic and Nuclear Physics, Rinehardt and Winston, New York, 1962.

5. G. Friedlander and J. Kennedy, Introduction to Radiochemistry, John Wiley and Sons, Inc., New York, 1949.

6. J. Jauch and F. Rohrlich, Theory of Photons and Electrons, Addison-Wesley Publishing Company, Inc., Reading, Mass., 1959.

7. W. Heitler, Quantum Theory of Radiation, Oxford University Press, Great Britain, 1944.

8. H.C. Urey, The Planets, Yale University Press, New Haven, Conn., 1952.

8a. O. Struve, The Astronomical Universe, Condon Lectures, Eugene, Oregon, 1958.

9. A.P. Vinogradov, in Origin of Life on the Earth, A.I. Oparin, ed., Pergamon Press, Inc., New York, 1959.

10. R.S. Young and C. Ponnamperuma, Early Evolution of Life, D.C. Heath and Company, Boston, 1964.

11. S.L. Miller and H.C. Urey, Science, 130, 245 (1959).

12. H.C. Urey, in Origin of Life on the Earth, A.I. Oparin, ed., Pergamon Press, Inc., New York, 1959.

13. H. Gaffron, The Graduate Journal, 4, 82 (1961).

14. H.D. Holland, in Petrologic Studies: A Volume to Honor A.F. Buddington, Geological Society of America, New York, 1962.

15. A.I. Oparin, Origin of Life, Dover Publications, New York, 1938.

16. J.B.S. Haldane, Rationalist Ann., 148 (1928).

17. D.E. Hull, Nature, 186, 693 (1960).

18. J. Keosian, Origin of Life, Reinhold Publishing Corp., London, 1964.

19. W. Loeb, Ber., 46, 690 (1913).

20. O. Baudisch, Z. angew. Chem., 26, 612 (1913).

21. E.C.C. Baly, I.M. Heibron, and D.P. Hudson, J. Chem. Soc., 121, 1078 (1922).

22. N.R. Dhar and S.K. Mukherjee, Nature, 134, 499 (1934).

23. K. Bahadur , Nature, 173, 1141 (1954).

24. K. Bahadur et al, Nature, 182, 1668 (1958).

25. S.L. Miller, J. Am. Chem. Soc., 77, 2351 (1955).

26. S.L. Miller, Biochim. Biophys. Acta, 23, 480 (1957).

27. J.D. Bernal, Proc. Phys. Soc. (London), 62A, 537 (1949).

28. H. Urey, Proc. Natl. Acad. Sci., 38, 351 (1952).

29. P.H. Abelson, Science, 124, 935 (1956).

30. K. Heyns, W. Walter, and E. Meyer, Naturwiss., 44, 385 (1957).

31. T.E. Pavlovskaya and A.G. Pasynskii, in Origin of Life on the Earth, A.I. Oparin, ed., Pergamon Press, Inc., New York, 1959.

32. S.W. Fox, J.E. Johnson, and M. Middlebrook, J. Am. Chem. Soc., 77, 1048 (1955).

33. S.W. Fox, in Organic Geochemistry, Pergamon Press, Oxford, 1963.

34. R. Paschke, R.W.H. Chang, and D. Yound, Science, 125, 881 (1957).

35. K. Dose and B. Rajewsky, Biochim. Biophys. Acta, 25, 225 (1957).

36. A.N. Terenin, in Origin of Life on the Earth, A.I. Oparin, ed., Pergamon Press, Inc., New York, 1959.

37. T. Hasselstrom, M.C. Henry, and B. Murr, Science, 125, 350 (19570.

38. F. Master, M.S. Thesis, University of Houston, Houston, Texas, 1957.

39. A. Hickling and G.R. Newns, Proc. Chem. Soc., 368 (1959).

40. A.R. Deshreider, Nature, 182, 528 (1958).

41. G. Ferrari and R. Cultrera, Gazz. Chim. ital., 90, 1637 (1960).

42. J. Oro, A. Kimball, R. Fritz, and F. Master, Arch. Biochem. Biophys., 85, 115 (1959).

43. W.E. Groth and H.V. Weyssenhoff, Planet. Space Sci., 2, 79 (1960).

44. B. Franck, Ber., 93, 446 (1960).

45. K.S. Pitzer, J. Am. Chem. Soc., 70, 1140 (1948).

46. J. Oro, Nature, 197, 862 (1963).

47. J. Oro, Nature, 197, 971 (1963).

48. C. Palm and M. Calvin, J. Am. Chem. Soc., 84, 2115 (1962).

49. G.D. Steinman and H.A. Lillevik, Arch. Biochem. Biophys., 105, 303 (1964).

50. J. Oro and J.S. Kamat, Nature, 190, 442 (1961).

51. C.U. Lowe, M.W. Rees, and R. Markham, Nature, 199, 219 (1963).

52. M. Kotake, M. Nakagawa, T. Ohara, K. Harada, and M. Ninomia, J. Chem. Soc., Japan Ind. Chem. Sect., 59, 121 (1956).

53. K. Harada and S.W. Fox, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

54. J. Oro, Biochem. Biophys. Res. Comm., 2, 407 (1960).

55. J. Oro and A.P. Kimball, Arch. Biochem. Biophys., 94, 217 (1961).

56. J. Oro, Nature, 191, 1193 (1961).

57. J. Oro, Ann. N.Y. Acad. Sci., 201, 464 (1962).

58. C. Palm and M. Calvin, J. Am. Chem. Soc., 84, 2115 (1962).

59. C. Ponnamperuma, R.M. Lemmon, R. Mariner, and M. Calvin, Proc. Natl. Acad. Sci., 49, 737 (1963).

60. C. Ponnamperuma, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

61. S.W. Fox and K. Harada, Science, 133, 1923 (1961).

62. C. Ponnamperuma, R.S. Young, E. Munoz, and B. McCaw, Science, 143, 1449 (1964).

63. A. Butlerow, Ann., 120, 295 (1861).

64. E.C.C. Baly, Ind. Eng. Chem., 16, 1016 (1924).

65. J.C. Irvine and G.V. Francis, Ind. Eng. Chem., 16, 1019 (1924).

66. E. El'piner and A.V. Sokolskaya, in Origin of Life on the Earth, A.I. Oparin, ed., Pergamon Press, Inc., New York, 1959.

67. C. Ponnamperuma, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

68. G. Steinman, M.S. Thesis, Michigan State University, E. Lansing, Michigan, 1963.

69. J. Oro and A.C. Cox, Federation Proc., 25, 80 (1962).

70. C. Ponnamperuma, R. Mariner, and C. Sagan, Nature, 198, 1199 (1963).

71. C. Ponnamperuma and P. Kirk, Nature, 203, 400 (1964).

72. C. Ponnamperuma, C. Sagan, and R. Mariner, Nature, 199, 222 (1963).

73. C. Ponnamperuma and R. Mack, Science, 148, 1221 (1965).

74. A. Gulick, Am. Sci., 43, 479 (1955).

75. S.L. Miller and M. Parris, Nature, 204, 1248 (1964).

76. J.B.S. Haldane, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

77. F. Lipmann, Proc. Natl. Acad. Sci., 46, 1194 (1960).

78. A.L. Lehninger, in Biophysical Science - A Study Program, J.L. Oncley, ed., John Wiley and Sons, Inc., New York, 1959.

79. C.E. Stoops and C.L. Furrow, Science, 134, 839 (1961).

80. W.M. Garrison, D.C. Morrison, J.G. Hamilton, A.A. Benson, and M. Calvin, Science, 114, 416 (1951).

81. W.M. Garrison, et al., J. Am. Chem. Soc., 74, 4216 (1952).

82. T. Hasselstrom and M.C. Henry, Science, 123, 1038 (1956).

83. W.M. Garrison, et al., J. Am. Chem. Soc., 75, 2459 (1953).

84. N. Getoff, G. Scholes, and J. Weiss, Tetrahedron Letters, 18, 17 (1960).

85. J.E. Manton and A.W. Tickner, Canad. J. Chem., 38, 858 (1960).

86. F.W. Lampe, J. Am. Chem. Soc., 79, 1055 (1957).

87. C. Ponnamperuma and F.H. Woeller, Differences in the Character of $C_6$ to $C_9$ Hydrocarbons from Gaseous $CH_4$ in Low-frequency Electric Discharges, NASA, Moffett Field, California, 1963.

88. D.H. Kenyon and M.S. Blois, Photochem. and Photobiol., 4, 335 (1965).

89. A.R. Hochstim, Proc. Natl. Acad. Sci., 50, 200 (1963).

90. A. White, P. Handler, E.L. Smith, and D. Stetten, Principles of Biochemistry, McGraw-Hill Book Co., New York, 1959.

91. A. Szutka, Radiation Res., 19, 183 (1963).

92. S.W. Fox and K. Harada, Science, 128, 1214 (1958).

93. K. Otozai, S. Kume, S. Nagai, T. Yamamoto, and S. Fukushima, Bull. Chem. Soc. (Japan), 27, 476 (1954).

94. S.A. Barker, P.M. Grant, M. Stacey, and R.B. Ward, J. Chem. Soc. 2648 (1959).

95. K. Bahadur and S. Ranganayaki, Proc. Natl. Acad. Sci. (India), Sec. A, 27, 292 (1958).

96. S.W. Fox and K. Harada, J. Am. Chem. Soc., 82, 3745 (1960).

97. A. Vegotsky and S.W. Fox, Federation Proc., 18, 343 (1959).

98. K. Harada and S.W. Fox, 137th Meeting Am. Chem. Soc., Cleveland, Ohio, p. 28c, 1960.

99. A.M. Galinsky, J.E. Gearien, and E.E. Smissman, J. Am. Pharm. Assoc., 46, 391 (1957).

100. S.W. Fox, Science, 132, 200 (1960).

101. G. Krampitz, Naturwiss, 46, 558 (1959).

102. S.W. Fox and K. Harada, J. Am. Chem. Soc., 82, 3745 (1960).

103. C. Sagan, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

104. P.H. Abelson, Ann. N.Y. Acad. Sci., 69, 276 (1957).

105. K. Bahadur and S. Nanganayaki, Proc. Natl. Acad. Sci. (India), 27, 292 (1958).

106. J. Oro and L. Guidry, Arch. Biochem. Biophys., 93, 166 (1961).

107. S. Akabori, in Origin of Life on the Earth, A.I. Oparin, ed., Pergamon Press, Inc., New York, 1959.

108. S. Akabori, K. Okawa, and M. Sato, Bull. Chem. Soc. (Japan), 29, 608 (1956).

108a. J.D. Bernal, The Physical Basis of Life, Routledge and Kegan Paul, London, 1951.

109. J. Oro and C.L. Guidry, Nature, 186, 156 (1960).

110. E. Ellenberger, Abstr. Am. Chem. Soc. Meeting, Chicago, p. 47c, 1958.

111. K.A. Grossenbacker and C.A. Knight, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

112. A. Thompson, A. Kimika, M.L. Wolfrom, and M. Inatome, J. Am. Chem. Soc., 76, 1309 (1954).

113. P.T. Mora and J.W. Wood, J. Am. Chem. Soc., 80, 685 (1958).

114. E. Fischer and K. Delbruck, Ber., 42, 2776 (1909).

115. F. Klages and R. Niemann, Ann., 529, 185 (1937).

116. W.H. Emmons, G.A. Thiel, C.R. Stauffer, and I.S. Allison, Geology, McGraw-Hill Book Co., Inc., New York, 1955.

117. A.W. Schwartz, E. Bradley, and S.W. Fox, in Origin of Prebiological Systems, S.W. Fox, ed., Academic Press, New York, 1965.

118. G. Schramm, H. Groetsch, and W. Pullman, Angew. Chem., Intern. Ed. (England), 1, 1 (1962).

119. M. Calvin, AIBS Bulletin, 12, 29 (1962).

120. H.G. Khorana, Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest, John Wiley and Sons, Inc., New York, 1961.

121. J.H. Bradbury and D.C. Shaw, Australian J. Chem., 12, 300 (1959).

122. J.C. Sheehan and G.P. Hess, J. Am. Chem. Soc., 77, 1067 (1955).

123. G.W. Kenner, C.B. Reese, and A. Todd, J. Chem. Soc., 546 (1958).

124. G. Losse and H. Weddige, Ann. Chem., 636, 144 (1960).

124a. G. Losse and H. Weddige, Angew. Chem., 72, 323 (1960).

125. B. Bineau, Ann. Chim. Phys., 67, 368 (1838).

126. R. Klinger, Z. physiol. Chem., 155, 206 (1926).

127. V. Volhard, J. pr. Chem., 9, 25 (1836).

128. B. Baumann, Ber., 6, 1371 (1873).

129. K. Grube and G. Kruger, Z. physikal. Chem., 86, 65 (1913).

130. H.E. Williams, Cyanogen Compounds, Edward Arnold and Co., London, 1948.

131. A. Franssen, Bull. soc. chim. (4), 43, 177 (1928).

132. J. Werner, J. Chem. Soc., 107, 715 (1915).

133. V. Migrdichian, The Chemistry of Organic Cyanogen Compounds, Reinhold Publishing Corp., New York, 1947.

134. L.A. Pink and H.C. Hetherington, Ind. Eng. Chem., 27, 834 (1935).

135. D. Drechsel, J. pr. Chem., 11, 350 (1875).

136. J. Buchanan and B. Barsky, J. Am. Chem. Soc., 32, 198 (1930).

137. D. Drechsel, J. pr. Chem., 13, 331 (1876).

138. H. Hale and H. Vibrans, J. Am. Chem. Soc., 40, 1046 (1918).

139. W.D. Kumler, J. Org. Chem., 20, 700 (1955).

140. American Cyanamid Company, Cyanamid's Nitrogen Chemicals Digest, Vol. III, A.C.C., New York, 1949.

141. E.W. Hughes, J. Am. Chem. Soc., 62, 1258 (1940).

142. M.T. Dangyan, Proc. Acad. Sci. Armenian S.S.R., 1, 22 (1944).

143. F. Chastellin, Helv. Chim. Acta, 18, 1287 (1935).

144. K. Klason, J. pr. Chem., 33, 116 (1886).

145. L. Kahovec and K.W.F. Kohlrausch, Z. Physik. Chem., 193, 188 (1944).

146. B. Bamberger, Ber., 16, 1074 (1883).

147. S. Stolle and K. Krauch, J. prakt. Chem., 88, 306 (1913).

148. N. Pohl, J. prakt. Chem., 77, 533 (1908).

149. K. Taub and T. Kropp, C. A., 28, 2016 (1934).

150. G.D. Diana, E.S. Zalay, and R.A. Culter, J. Org. Chem., 30, 298 (1965).

151. E.H. Rodd, ed., Chemistry of Carbon Compounds, Elsevier Publishing Co., Amsterdam, Vol. IB, p. 915, 1951.

152. W. Madelung and E. Kern, Ann., 427, 1 (1922).

153. W.L. Burdick, J. Am. Chem. Soc., 47, 1485 (1925).

154. E. Baumann, Ber., 8, 708 (1875).

155. G.E.K. Branch and M. Calvin, Theory of Organic Chemistry, Prentince-Hall, Inc., New York, 1941.

156. A.A. Benson, et al., J. Am. Dhem. Soc., 72, 1710 (1950).

157. R.C. Weast, ed., Handbook of Chemistry and Physics, Chemical Robber Co., Cleveland, Ohio, 1964.

158. J.G. Buchanan, C.A. Dekker, and A.G. Long, J. Chem. Soc., 3162 (1950).

159. G. Steinman, R.M. Lemmon, and M. Calvin, Proc. Natl. Acad. Sci., 52, 27 (1964).

160. Pabst Laboratories, Ultraviolet Absorption Spectra of 5'-Ribonucleotides, Pabst Brewing Co., Milwaukee, Wisconsin, 1956.

161. E. Karl-Kroupa, Anal. Chem., 28, 1091 (1956).

162. R.S. Bandursky and B. Axelrod, J. Biol. Chem., 193, 405 (1951).

163. F.C. Norris and J.J.R. Campbell, Can. J. Research, C27, 253 (1949).

164. I. Smith, ed., Chromatographic and Electrophoretic Techniques, Vol. I, Interscience Publishers, Inc., New York, 1960.

164a. R.J. Block, E.L. Durrum, and G. Zweig, Paper Chromatography and Paper Electrophoresis, Academic Press, Inc., New York, 1958.

165. G. Steinman, D.H. Kenyon, and M. Calvin, Nature, 206, 707 (1965).

166. R.W. Cowgill, Anal. Chem., 27, 1519 (1955).

167. F. Lipmann, Biochem. Z., 262, 9 (1933).

168. G. Perlmann, Biochim. Biophys. Acta, 13, 452 (1954).

169. G. Perlmann, Nature, 174, 273 (1954).

170. F.M. Huennekens, D.J. Hanahan, and M. Uziel, J. Biol. Chem., 206, 443 (1954).

171. D.C. Mortimer, Can. J. Cham., 30, 653 (1952).

172. This standard was kindly supplied by Dr. Khorana of the University of Wisconsin.

173. G.W. Rushizky and H.A. Sober, J. Biol. Chem., 237, 834 (1962).

174. Private Communication from Dr. J. Fairley, Michigan State University.

175. P.W. Kent and M.W. Whitehouse, Biochemistry of the Amino Sugars, Buttersworth Scientific Publications, London, 1955.

176. M. Plumet, Bull. soc. chim. biol., 30, 129 (1949).

177. A.C. Giese, Cell Physiology, W.B. Saunders Co., Philadelphia, Pennsylvania, 1962.

178. Private Communication from Dr. L. Orgel, Salk Institute of Biological Sciences, La Jolla, California.

179. R.E. Grim, Clay Mineralogy, McGraw-Hill Publishing Co., New York, 1953.

180. G.W. Brindley and K. Robinson, Min. Mag., 27, 242 (1946).

181. R.H. Koloff, Anal. Chem., 32, 1687 (1960).

182. B.L. Strehler and J.R. Totter, Arch. Biochem. Biophys., 40, 28 (1952).

183. C. Ponnamperuma and E. Peterson, Science, 147, 1572 (1965).

184. C. Long, ed., Biochemists' Handbook, D. Van Nostrand Co., Inc., Princeton, New Jersey, 1961.

185. G. Steinman, R.M. Lemmon, and M. Calvin, Science, 147, 1574 (1965).

186. Biochemistry Division Staff, University of Illinois (Urbana), Experimental Biochemistry, Stipes Publishing Co., Champaign, Illinois, 1959.

187. J.W. McBain, Colloid Science, D.C. Heath and Co., Boston, 1950.

188. P.R. Carnegie and R.L.M. Synge, Biochem. J., 78, 692 (1961).

189. J.L. Oncley, Biophysical Science - A Study Program, John Wiley and Sons, Inc., New York, 1959.

190. E. Merck, Dyeing Reagents for Thin Layer and Paper Chromatography, E. Merck A G, Darmstadt, Germany

191. W. Pigman, The Carbohydrates, Academic Press, Inc., New York, 1957.

191a. L.F. Fieser and M. Fieser, Organic Chemistry, Heath, Boston, 1957.

192. T. Goda, U.S. Patent 3,066,086, November, 1962.

193. S. Moore and W.H. Stein, J. Biol. Chem., 192, 663 (1951).

194. G. Litwack, Experimental Biochemistry, John Wiley and Sons, Inc., New York, 1960.

195. F.G. Fischer and J.J. Nebel, Z. physiol. Chem., 302, 10 (1955).

196. S.T. Horowitz, S. Roseman, and J.J. Blumenthal, J. Am. Chem. Soc., 79, 5046 (1957).

197. D.F. DeTar, R. Silverstein, and F.F. Rogers, The Mechnaisms of the Reactions of Carbodiimides, Dept. of Chemistry, Florida State University, Tallahassee, Florida, 1965.

198. M. Calvin, Perspectives in Biology and Medicine, 5, 399 (1962).

199. A. Schimpl, R.M. Lemmon, and M. Calvin, Science, 147, 149 (1965).

200. R. Hayatsu, Science, 146, 1291 (1964).

201. D.D. Davis and R.J. Hanrahan, J. Am. Chem. Soc., 87, 3088 (1965).

202. M. Calvin, Science, 130, 1170 (1959).

203. J.A. Pople, W.G. Schneider, and J.J. Bernstein, High-resolution Nuclear Magnetic Resonance, McGraw-Hill Book Co., Inc., New York, 1959.

204. H.H. Jaffe and M. Orchin, Theory and Applications of Ultraviolet Spectroscopy, John Wiley and Sons, Inc., New York, 1962.

204a. M.A. Paul and F.A. Long, Chem. Revs., 57, 1 (1957).

205. A. Tabone, R. Robert, and R. Troestler, Bull. soc. chim. Biol., 30, 547 (1948).

206. W. Werner, J. Chem. Soc., 107, 721 (1915).

207. R.M.C. Dowson, ed., Data for Biochemical Research, Clarendon Press, Oxford, 1959.

208. W. Madelung and E. Kern, Annalen, 427, 1 (1922).

209. E.C. Franklin, Nitrogen System of Compounds, Reinhold Publishing Corp., New York, 1935.

210. American Cyanamid Company, New Product Bulletin - Collective Volume II, American Cyanamid Co., New York, 1950.

210a. Ibid., Collective Volume I.

211. G. Pratorius-Seidler, J. prakt. Chem. (2), 21, 129 (1880).

212. A. Jeanes et al., J. Am. Chem. Soc., 76, 5041 (1954).

213. S. A. Barker, E. J. Bourne, and M. Stacey, J. Chem. Soc., 3084 (1953).

214. J. C. Sowden and A. S. Spriggs, J. Am. Chem. Soc., 78, 2503 (1956).

215. S. Peat, W. J. Whelan, and K. A. Hinson, Chem. and Ind., 385 (1955).

216. W. R. Fetzer, E. K. Crosby, C. E. Engle, and L. C. Kirst, Ind. Eng. Chem., 45, 1075 (1953).

217. A. Thompson, M. L. Wolfrom, and E. J. Quinn, J. Am. Chem. Soc., 75, 3003 (1953).

218. E. Rischer, Ber., 23, 3687 (1890).

218a. K. V. Linderstrøm-Lang, in Lane Medical Lectures, 1951, Stanford Univ. Press, Stanford, 1952, Vol. VI.

219. J. R. Whitaker, Brewers Digest, 57, January 1965.

220. C. B. Anfinsen, Proc. Natl. Acad. Sci., 47, 1309 (1961).

221. J. N. Davidson, The Biochemistry of the Nucleic Acids, Methuen, London, 1960.

222. A. Kornberg, Science, 131, 1505 (1960).

223. F. Haurowitz, The Chemistry and Function of Proteins, Academic Press, New York, 1963.

223a. E. Schröder and K. Lubke, The Peptides, Academic Press, New York, 1965, Vol. I.

224. A. Dobry et al., J. Biol. Chem., 195, 149 (1952).

225. K. Hofmann, et al., J. Am. Chem. Soc., 79, 1641 6087 (1957).

226. E. Katchalski et al., Arch. Biochem. Biophys., 88, 361 (1960).

227. R. L. Hill and E. L. Smith, Biochim. Biophys. Acta, 19, 376 (1956).

228. H. Neurath, in Biophysical Science--A Study Program, J. L. Oncley, ed., John Wiley and Sons, Inc., New York, 1959.

228a. C. H. Wang and D. L. Willis, Radiotracer Methodology in Biological Science, Prentice-Hall, Inc., Englewood Cliffs, N. J., 1965.

229. H. Leuchs, Ber., 39, 857 (1906).

230. C. Tobias, Physics of Biological Systems, University of California, Berkeley, California, 1964.

231. R. W. Hegner and K. A. Stiles, College Zoology, MacMillan Co., New York, 1959.

232. R. B. Heslop and P. L. Robinson, Inorganic Chemistry, Elsevier Publishing Co., Amsterdam, 1960.

233. A. G. Ogston and O. Smithies, Physiol. Rev., 28, 283 (1948).

234. R. Lumry, in The Enzymes, P. D. Boyer, H. Lardy, and K. Myrback, eds., Vol. I, Academic Press, New York, 1959.

235. R. B. Roberts, in Biophysical Science--A Study Program, J. L. Oncley, ed., John Wiley and Sons, Inc., New York, 1959.

236. N. H. Horowitz, in The Origin of Life on the Earth, A. I. Oparin, ed., Pergamon Press, New York, 1959.

237. S. W. Fox, A. Vegotsky, K. Harada, and P. D. Hoagland, Ann. N. Y. Acad. Sci., 69, 328 (1957).

237a. F. A. Isherwood, in Glutathione, E. M. Crook, ed., Cambridge University Press, Cambridge, England, 1959.

238. W. Lovenberg, B. Buchanan, and J. C. Rabinowicz, J. Biol. Chem. 238, 3899 (1963).

239. J. H. Wang, J. Am. Chem. Soc., 80, 3168 (1958).

240. F. Haurowitz and P. Schwerin, Enzymologia, 9, 193 (1940).

241. S. W. Fox and G. Krampitz, Nature, 203, 1362 (1964).

242. S. W. Fox, K. Harada, and D. L. Rohlfing, in Polyamino Acids, Polypeptides, and Proteins, M. A. Stohman, ed., University of Wisconsin Press, Madison, Wis., 1964.

243. N. H. Horowitz, Proc. Natl. Acad. Sci., 31, 153 (1945).

244. R. E. Eakin, Proc. Natl. Acad. Sci., 49, 360 (1963).

245. A. I. Oparin, in Origin of Prebiological Systems, S. W. Fox, ed., Academic Press, New York, 1965.

245a. G. W. Wheland, Advanced Organic Chemistry, John Wiley and Sons, Inc., New York, 1949.

246. G. Wald, Proc. Natl. Acad. Sci., 52, 595 (1964).

247. F. S. Sjostrand, in Biophysical Science--A Study Program, J. L. Oncley, ed., John Wiley and Sons, Inc., New York, 1959.

248. R. J. Goldacre, Surface Phenomena in Chemistry and Biology, Pergamon Press, London, 1958.

249. M. Traube, Arch. Physiol., 87, 129 (1867).

250. S. W. Fox and S. Yuyama, Comp. Biochem. Physiol., 11, 317 (1964).

251. S. W. Fox, K. Harada, and J. Kendrick, Science, 129, 1221 (1959).

252. S. W. Fox, Origin of Prebiological Systems, Academic Press, New York, 1965.

253. E. R. Baylor and W. H. Sutcliffe, Limnology and Oceanography 8, 369 (1963).

254. E. R. Baylor, W. H. Sutcliffe, and D. S. Hirschfeld, Deep Sea Research, 9, 120 (1962).

254a. H. L. Booij and H. G. Bungenberg De Jong, Biocolloids and Their Interactions, Springer-Verlag, Vienna, 1956.

255. A. I. Oparin, Life, Its Nature, Origin and Development, Oliver and Boyd, Edinburgh, 1961.

256. M. M. Nass and S. Nass, J. Cell Biol., 19, 593, 613 (1963).

257. M. Calvin, Chemical Evolution, Condon Lectures, Eugene, Oregon, 1961.

258. H. Abelson, Ann. N. Y. Acad., 69, 276 (1957).

259. H. Abelson, Sci. Am., 193, 3 (1956).

260. H. Abelson, Carnegie Inst. Wash. Year Book, 53 (1954).

261. S. A. Tyler and E. S. Barghoorn, Science, 119, 606 (1954).

262. G. Eglinton, et al., Science, 145, 263 (1964).

263. W. G. Meinschein, E. S. Barghoorn, and J. W. Schopf, Science, 145, 262 (1964).

264. E. S. Barghoorn, W. G. Meinschein, and J. W. Schopf, Science, 148, 461 (1965).

264a. M. H. Studier, R. Hayatsu, and E. Anders, Science, 149, 1455 (1965).

265. T. Belsky, et al., Nature, 206, 446 (1965).

265a. E. S. Barghoorn, presented at Geological Society Meeting, Kansas, November 1965.

266. H. H. Hess, Chairman, Biology and the Exploration of Mars, Space Science Board, National Academy of Sciences, National Research Council, 1964.

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