

# NON-ENZYMATIC SYNTHESIS OF THE COENZYMES, URIDINE DIPHOSPHATE GLUCOSE AND CYTIDINE DIPHOSPHATE CHOLINE, AND OTHER PHOSPHORYLATED METABOLIC INTERMEDIATES

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**Abstract.** The synthesis of uridine diphosphate glucose (UDPG), cytidine diphosphate choline (CDP-choline), glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) has been accomplished under simulated prebiotic conditions using urea and cyanamide, two condensing agents considered to have been present on the primitive Earth. The synthesis of UDPG was carried out by reacting G1P and UTP at 70 °C for 24 hours in the presence of the condensing agents in an aqueous medium. CDP-choline was obtained under the same conditions by reacting choline phosphate and CTP. G1P and G6P were synthesized from glucose and inorganic phosphate at 70 °C for 16 hours.

Separation and identification of the reaction products have been performed by paper chromatography, thin layer chromatography, enzymatic analysis and ion pair reverse phase high performance liquid chromatography. These results suggest that metabolic intermediates could have been synthesized on the primitive Earth from simple precursors by means of prebiotic condensing agents.

**Abbreviations** UDPG, uridine diphosphate glucose; UTP, uridine triphosphate; UMP, uridine monophosphate; UDP, Uridine diphosphate; CDP-choline, cytidine diphosphate choline; CTP, cytosine triphosphate; CDP, cytosine diphosphate; CMP, cytosine monophosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; NADP, nicotinamide adenine dinucleotide phosphate; IP RP-HPLC, ion pair reverse phase high performance liquid chromatography; PEI-TLC, polyethyleneimine thin layer chromatography; UV, ultraviolet light.

## 1. Introduction

Studies on prebiotic chemistry have not given enough emphasis to the origin and evolution of metabolic pathways. Even though the role played by the several forms of high energy compounds in the primitive Earth is not entirely clear at the present time, the synthesis of metabolic intermediates should be helpful in understanding the way in which the first heterotrophic cells used up nutrients available in the prebiotic soup.

The chemical synthesis of CDP-choline has been reported by Kennedy (Kennedy, 1956) who obtained the compound by the condensation of choline phosphate with CMP in the presence of dicyclohexylcarbodiimide dissolved in water and pyridine. However, up to the present not much work has been done on the synthesis of phosphorylated coenzymes under simulated prebiotic conditions. The

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phosphorylation of sugars under various conditions has been previously described by several investigators (Steinman *et al.*, 1964; Degani and Halmann, 1971; Degani and Halmann, 1972, Degani *et al.*, 1975; Halmann *et al.*, 1969), suggesting that these reactions could have taken place with ease under presumed prebiological conditions.

Urea is formed, along with guanidine and cyanamide, when aqueous solutions of ammonium cyanide are exposed to sunlight or 254 nm UV radiation (Lohrmann, 1972), it is also obtained when an electrical discharge is passed through a mixture of methane, ammonia and water (Miller, 1957), and has been employed together with inorganic phosphate in the phosphorylation of compounds such as nucleosides (Lohrmann and Orgel, 1971; Bishop *et al.*, 1972). Cyanamide was first considered by Oró as a condensing agent for use in prebiotic chemistry (Oró, 1963) and can be synthesized from ammonium cyanide (Lohrmann, 1972), moreover it has also been detected in interstellar space (Turner *et al.*, 1976).

In the present study we have demonstrated the prebiotic synthesis of UDPG from UTP and G1P; CDP-choline from CTP and choline phosphate, and of G1P and G6P from an incubation of glucose and inorganic phosphate. The reactions were carried out under simulated prebiotic conditions, i.e. aqueous solutions, moderate temperatures, and mediated by condensing agents.

## 2. Experimental

*Materials:* Glucose, G1P, G6P, glucose-6-phosphate dehydrogenase (G6PDH), UTP, UDPG, choline phosphate, CTP, CDP-choline and  $K_2HPO_4$  were purchased from Sigma Chemical Co. Urea was obtained from Fisher and cyanamide from Eastman Chemicals. Snake Venom phosphodiesterase was from Worthington. 3MM filter paper was from Whatmann and the thin layer chromatographic plates were purchased from Merck. The solvents utilized were reagent grade. Anion exchange resin AG1 X-8 (200–400 mesh) was from Bio Rad Laboratories.

The HPLC was performed with an LDC/Milton Roy gradient system equipped with a 20  $\mu$ l Rheodine injection loop and a variable wavelength detector. The columns used were a Reverse Phase (RP)  $C_8$  (4.6 mm  $\times$  25 cm Hibar RT columns, 10  $\mu$ m Alltech Associates), a  $RPC_{18}$  (4.6 mm  $\times$  25 cm Milton Roy, 10  $\mu$ m Spherisorb) and a  $RPC_{18}$   $\mu$ Bondapak (3.9 mm  $\times$  30 cm Waters Assoc.). The solvents used for the HPLC runs were HPLC grade.

### CHROMATOGRAPHY

The following solvents were used for paper and thin layer chromatography (TLC): *Solvent I:* MeOH: (88%) HCOOH:  $H_2O$  (80:15:5), *Solvent II:* 20% HCOOH, *Solvent III:* 2%  $NH_4HCO_3$ , *Solvent IV:* Ethylene glycol monomethyl ether, pyridine, glacial acetic acid, water (80:40:10:10). *Solvent V:* MeOH:  $NH_4OH:H_2O$  (60:10:30). *Solvent VI:* HCOOH: $H_2O$ : $EtOH$  (1:29:70). *Solvent VII:* Stepwise elution with: 0.30 M LiCl (1 min.), 1.0 M LiCl (12 min.), and 1.6 M LiCl (47 min.).

## HPLC SEPARATIONS

The technique of ion pair reverse phase high performance liquid chromatography (IP RP-HPLC) was employed for the identification and separation of the prebiotically produced compounds. The solvents used were: *Solvent A*: 50 mM  $\text{KH}_2\text{PO}_4$  (pH 5.9), 2mM TBAHSO<sub>4</sub> (tetrabutyl ammonium hydrogen sulfate), *Solvent B*: 50 mM  $\text{KH}_2\text{PO}_4$  (pH 6.2), 2 mM TBAHSO<sub>4</sub>, 60%  $\text{CH}_3\text{CN}$ . The gradient of solvent B used for the identification of UDPG and phosphorylated sugars went from 15% to 80% in 60 min. For the identification of CDP-choline the gradient of solvent B was changed from 20 to 99% in 60 minutes.

## ENZYMATIC ANALYSIS WITH SNAKE VENOM PHOSPHODIESTERASE

The reaction mixture consisted of 10  $\mu\text{l}$  of 5 mg/ml of phosphodiesterase, 100  $\mu\text{l}$  of the UDPG-containing fraction and 0.9 ml of 0.11 M Tris-HCl (pH 8.9), 0.11 M NaCl and 15 mM  $\text{MgCl}_2$  and was incubated for 5 hours at 37 °C.

## GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY MIXTURE

The reaction mixture consisted of 0.05 ml of 1 M Tris HCL buffer (pH 7.6), 0.003 ml of 1 M  $\text{MgCl}_2$ , 0.01 ml of 100 mM NADP, 0.035 ml of 10 mM G6PDH, and 0.902 ml of water. The presence of G6P was measured by adding varying amounts of the G6P-containing fraction and following the rate of formation NADPH at 340 nm.

## ULTRAVIOLET SPECTRA ANALYSIS

The ultraviolet (UV) spectra of standards and of the fractions containing the product was determined by using a Beckman Scanning Spectrophotometer Model 25 attached to a recorder.

## 3. Synthetic Procedures

### SYNTHESIS OF UDPG

A reaction mixture (150  $\mu\text{l}$ , pH 6.5) containing 100  $\mu\text{mole}$  GIP, 100  $\mu\text{mole}$  UTP, 400  $\mu\text{mole}$  urea or cyanamide, and 200  $\mu\text{mole}$   $\text{NH}_4\text{Cl}$  was put into open glass tubes and placed in a heating block at a constant 70 °C for 24 hours during which time the reaction mixture dried. After the incubation was over, the reaction mixture was resuspended in 1 ml of triple distilled water and kept frozen until further analysis.

### SYNTHESIS OF CDP-CHOLINE

CDP-choline was synthesized by reacting 200  $\mu\text{mole}$  choline phosphate 200  $\mu\text{mole}$  CTP, 800  $\mu\text{mole}$  of urea or cyanamide, and 400  $\mu\text{mole}$   $\text{NH}_4\text{Cl}$  in 200  $\mu\text{l}$  of triple distilled water at 70 °C. The pH of the reaction was measured at 6.2. After 24 hours, the reaction was terminated by resuspending it in 1 ml of triple distilled water and freezing at -4 °C.

## SYNTHESIS OF G1P AND G6P

The synthesis of G1P and G6P was performed by reacting 0.05 mmoles of glucose with 1.0 mmole of urea or cyanamide and resuspended in 100  $\mu$ l of 5 M  $K_2HPO_4$ . The reaction mixture was maintained in open test tubes and kept in a heating block at 70°C for 16 hours. After 16 hours, the reaction mixture was resuspended in 1 ml of triple distilled water and kept frozen until further analysis.

## 4. Results

## CHROMATOGRAPHIC IDENTIFICATION OF UDPG

Product analysis of the reaction of UTP and G1P in the presence of either urea or cyanamide was initially carried out by TLC on PEI-cellulose plates and paper chromatography on 3MM Whatmann paper. Five  $\mu$ l of the reaction solution were spotted along with standards on the TLC plates and developed first in solvent II followed by solvent III (Sherwood *et al.*, 1977), (Table I). Ascending paper

TABLE I

$R_f$  values of the coenzymes standards and of the reaction products, detection done by the Hanes-Isherwood reagent

	PAPER CHROMATOGRAPHY	TLC ON PEI-CELLULOSE
UDPG (Std)	0.72 <sup>a</sup>	0.67 <sup>(b)</sup>
<i>Synthesized by:</i>		
Urea	0.73	0.67
Cyanamide	0.73	0.68
CDP-Choline (Std)	0.48 <sup>(c)</sup>	0.86 <sup>(d)</sup>
<i>Synthesized by:</i>		
Cyanamide	0.47	0.83
CDP-Choline (Std)	0.28 <sup>(e)</sup>	
<i>Synthesized by:</i>		
Cyanamide	0.28	
CDP-Choline (Std)	0.70 <sup>(f)</sup>	
<i>Synthesized by:</i>		
Cyanamide	0.71	
G1P (Std)		0.47 <sup>(e)(*)</sup>
<i>Synthesized by:</i>		
Urea		0.51
Cyanamide		0.52
G6P (Std)		0.70 <sup>(e)(*)</sup>
<i>Synthesized by:</i>		
Urea		0.72
Cyanamide		0.68

(a) Solvent IV.

(b) Solvents II and III.

(c) Solvent VI.

(d) Solvent VII.

(e) Solvent I.

(f) Solvent V.

(\*) On Silica Gel Plates.

chromatography was done by spotting 20  $\mu$ l of the sample along with standards in a sheet of 3 MM paper developed overnight in solvent IV (Runeckles and Krotkov, 1957), (Table I). The modified Hanes-Isherwood spray (Hanes and Isherwood, 1949), and UV light were used for the detection and identification of the reaction products and reactants in both paper and thin layer chromatography.

#### ION PAIR REVERSE PHASE HPLC OF UDPG

The technique of ion pair RP-HPLC on a  $C_8$  column (Alltech Assoc.), was used to separate the UDPG, UTP, UDP and UMP standards and to identify the UDPG produced under prebiological conditions by matching its retention time to that of the standard. Detection was done at 254 nm, with a flow rate of 1 ml/min. Solvents and gradient were as described in the methods section. Figure 1a shows the elution pattern of the fraction containing UDPG from the cyanamide mediated reaction. This fraction was obtained by repeatedly collecting and separating from the complex chromatographic mixture, the peak with the same retention time as that of the UDPG standard ( $R_t = 4.30$  min). The peak coming off at 4.28 min was therefore identified as UDPG.

Chromatography of the collected fraction from the reaction catalyzed by urea is shown in Figure 1b. The peak with a retention time of 4.04 min was identified as

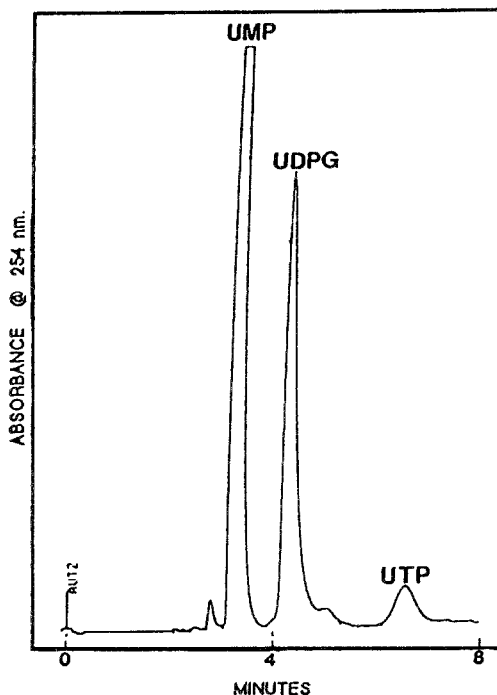


Fig. 1a. Ion pair RP-HPLC of the UTP and GIP reaction mediated by cyanamide. Solvents and gradient as described under methods. UDPG product was purified by repeatedly collecting the fraction corresponding to UDPG and rechromatographing in the same system. Traces of ATP and AMP can be seen, the shoulder after UDPG corresponds to UDP.

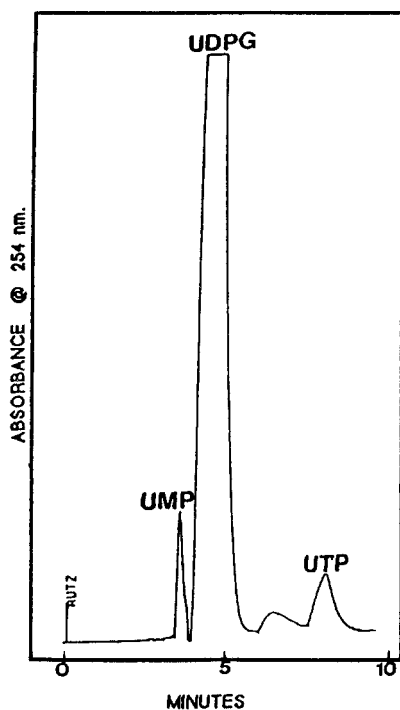


Fig. 1b. Ion pair RP-HPLC of the urea-mediated reaction of UTP and GIP. UDPG was purified as described in Figure 1.

TABLE II  
Percentage yield of reaction products

	Cyanamide-mediated reaction	Urea-mediated reaction
UDPG	6.02	10.02
G-1-P	11.11	5.92
G-6-P	1.65	2.2
CDP-choline	4.53	traces

UDPG. In both cases, even after repeated fractionations, UMP and UTP are detected. The increase in the concentration of UMP in both of the fractions can be explained by the fact that, when the unreacted UTP is heated at 70°C for 24 hours, it hydrolyses into 2Pi and UMP. The percentage yields of UDPG, based on initial UTP concentration, were calculated to be 6.02 in the cyanamide mediated reaction and 10.02 in the urea directed reaction as shown in Table II.

#### ACID HYDROLYSIS AND ENZYMATIC ANALYSIS OF UDPG

In order to further determine the exact nature of the coenzyme, the fraction corresponding to UDPG was collected from the HPLC and hydrolyzed in 1.0 N

H<sub>2</sub>SO<sub>4</sub> for 15 min at 100°C. Ion pair RP-HPLC analysis of the acid hydrolyzate reaction showed the disappearance of the UDPG peak and an increase in the UMP peak (data not shown). Similar results were obtained after treatment of the collected fraction with snake venom phosphodiesterase.

#### ULTRAVIOLET SPECTRUM OF UDPG

The identity of the synthesized UDPG was further confirmed by comparison of the UV spectra of UDPG standard and of the fractions identified as UDPG. The absorption maxima for both standard and synthesized was UDPG 262 nm (Figure 1c).

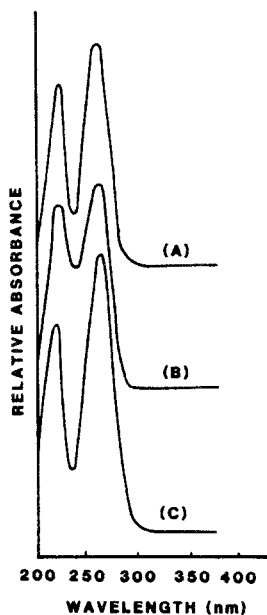


Fig. 1c. UV spectra of UDPG standard (A), UDPG synthesized by urea (B), and UDPG synthesized by cyanamide (C). All spectra were done at pH 7.

#### CHROMATOGRAPHIC IDENTIFICATION OF CDP-CHOLINE

Separation and identification of the prebiotically produced CDP-choline along with standards was done by paper and thin layer chromatography. In both cases the detection was carried out by UV light and the modified Hanes-Isherwood spray. For paper chromatography analysis, 20  $\mu$ l of the reaction mixtures were spotted, along with standards of CTP, CDP-choline and choline phosphate, on a long 3 MM paper and developed overnight in solvents I, V and VI.

Thin layer chromatography was done by spotting 3  $\mu$ l of the reaction mixture along with standards on a PEI-cellulose TLC plate with development on a LiCl gradient system (solvent VII). The cyanamide mediated reaction yields a spot, as

detected by UV light and the Hanes-Isherwood spray, with an  $R_f$  value corresponding to that of the CDP-choline standard by both paper and TLC (Table I). On the other hand, the urea mediated reaction gave only traces of a compound which had a different chromatographic behaviour than CDP-choline.

#### ION PAIR REVERSE PHASE CHROMATOGRAPHY OF CDP-CHOLINE

Additional supporting evidence for the prebiotic synthesis of CDP-choline by means of cyanamide was obtained by IP RP-HPLC analysis of the reaction products. Chromatography of 10  $\mu$ l of the cyanamide-mediated reaction shows a peak with a retention time of 3.34 min that corresponds to that of the authentic CDP-choline standard (Figure 2a), also seen are the residual peaks of CMP, CDP and CTP. The percentage of CDP-choline synthesized in the cyanamide-mediated reaction was estimated based on the initial concentration of CTP (Table II). This was confirmed by chromatographic isolation of the synthesized product and hydrolytic degradation to its constituent moieties as well as by the obtention of its UV spectrum.

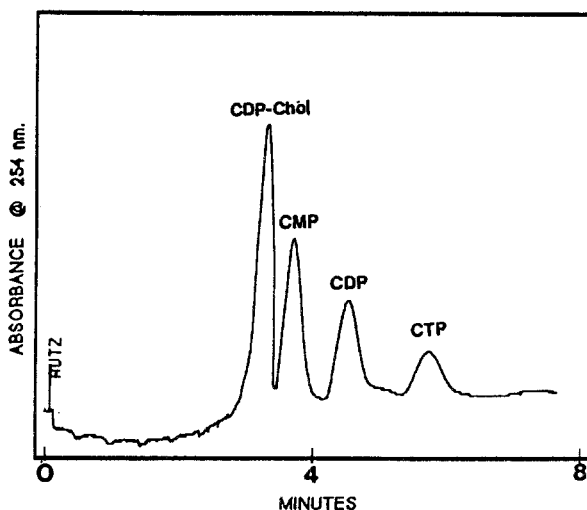


Fig. 2a. Ion pair chromatography of the cyanamide mediated reaction of CTP and choline phosphate on a  $C_{18}$  reverse phase column. The solvents and gradients are described under methods. Identification of the CDP-choline was done by comparing its retention time to that of the standard.

#### ACID HYDROLYSIS OF PREBIOTICALLY PRODUCED CDP-CHOLINE

Acid hydrolysis of the CDP-choline fraction from the cyanamide mediated reaction was performed in 1 N  $H_2SO_4$  for 15 min at 100°C. Ion pair RP-HPLC analysis showed the complete breakdown of the compound, and a corresponding increase in the concentration of CMP.

Additionally, TLC on PEI-cellulose plates developed on a LiCl gradient was performed on the hydrolysis product, and after detection by UV light and the Hanes



Isherwood spray only CMP and choline phosphate were detected, which are the hydrolysis products of CDP-choline. These results helped us to confirm the synthesis of the coenzyme under simulated prebiotic conditions, by means of cyanamide.

In the case of the product synthesized by means of urea, a small HPLC peak was obtained which had a different retention time (3.14 min) than that of CDP-choline ( $R_t = 3.37$  min). Acid hydrolysis of this reaction product did not alter the nature of the peak which provides further proof that this compound is other than CDP-choline.

#### ULTRAVIOLET SPECTRA OF CDP-CHOLINE

Comparison of the spectra of the CDP-choline standard and of the synthesized compound by cyanamide further confirmed the identity of the compound as CDP-choline (Figure 2b). The absorption maxima for both the standard as well as the reaction product was determined to be 268 nm.

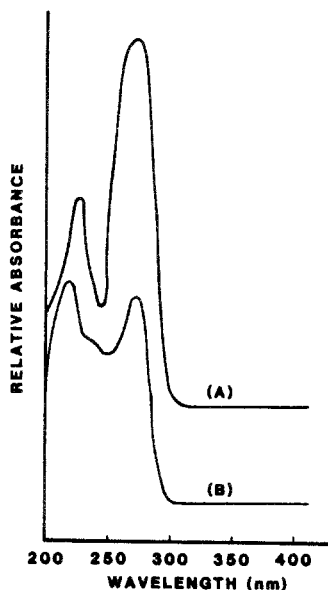


Fig. 2b. UV spectra of CDP-choline standard (A), and of CDP-choline synthesized by cyanamide (B). Spectra were done at pH 7.

#### IDENTIFICATION OF G1P AND G6P

After the reaction mixtures containing glucose and inorganic phosphate were incubated in the presence of urea or cyanamide, they were resuspended in 1 ml of water, and loaded into an anion exchange column packed with AG1-X8 ( $\text{Cl}^-$ ) (200–400 mesh) resin. The column was eluted with a NaCl gradient (0.1–0.5 M) and 5 ml fractions were collected. After determining, by paper chromatography on solvent IV, which fraction contained the phosphorylated sugars, the fractions were

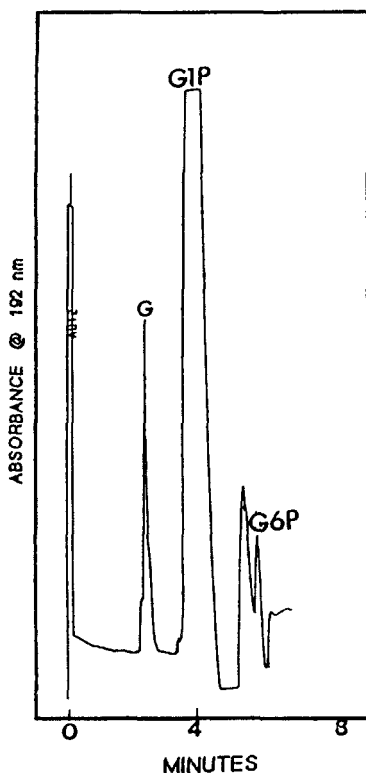


Fig. 3a. Elution pattern of the cyanamide mediated synthesis of G1P and G6P separated by ion-pair RP-HPLC. Detection was done at wavelength 192 nm by taking advantage that sugars absorb at this wavelength (Honda, 1984). Flow rate 1 ml/min. The solvents and gradient used are described under methods.

spotted on silica gel TLC plates and developed in solvent I. Spots were detected with the Hanes-Isherwood reagent and UV light. The  $R_f$  values for the standards and reaction products are shown in Table number I. G6P was further characterized by a highly specific enzymatic assay using G6P dehydrogenase in the reduction of NADP to NADPH. G1P and G6P were obtained in a 6–11% yield and a 2% yield respectively, as estimated by this enzymatic assay.

Ion pair RP-HPLC on a  $C_{18}$  reverse phase column was carried out for the separation of the products in the phosphorylation of glucose by inorganic phosphate, mediated by either urea or cyanamide. Chromatography of the cyanamide mediated phosphorylation reaction is shown in Figure 3a, the peak at 3.99 min corresponds to G1P, and the one at 5.99 min corresponds to G6P. The unreacted glucose elutes at 2.54 min. Figure 3b shows the chromatographic elution pattern of the urea mediated reaction.

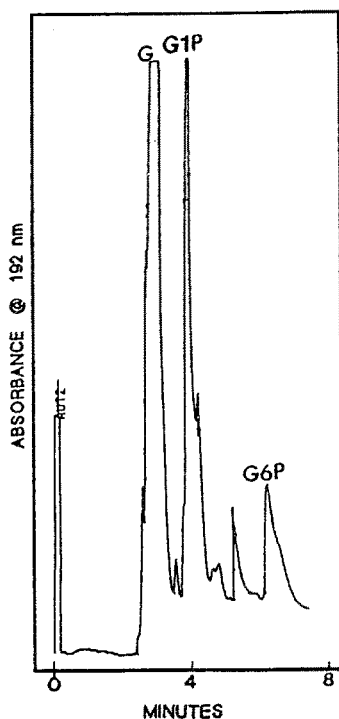


Fig. 3b. Ion pair RP-HPLC of the urea mediated synthesis of G1P and G6P. Solvent composition and detection are the same as Figure 3a.

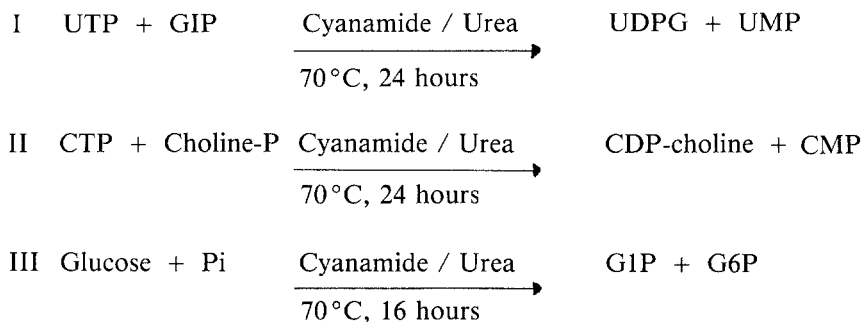
## 5. Discussion

In the context of the theory of chemical evolution, a useful notion is that modern metabolic pathways, which now require enzymes as catalysts, evolved from slower, less-specific chemical reactions that occurred on the prebiotic Earth and were enhanced by means of different forms of energy, UV, radiation, heat, shock waves, or by condensing agents considered to have been present in the primordial soup. Later, these chemical reactions were taken over by far more efficient protoenzymes and eventually gave rise to metabolism as it occurs today.

One crucial step in the utilization of nutrients by the primordial heterotrophic cells in the prebiotic Earth, must have been the synthesis of glucose phosphate esters, mediated by condensing agents, by single amino acids or functional groups of early enzymes. In this line of research, Stillwell *et al.*, have reported on the synthesis of glucose phosphates from glucose and inorganic phosphate catalyzed by histidine and some histidine derivatives (Stillwell *et al.*, 1972).

In the phosphorylation systems studied here we have attempted to closely mimic the conditions considered to have been prevalent in the primitive Earth; therefore, an aqueous system and moderate temperatures have been employed in our reactions

along with two plausible prebiotic condensing agents, cyanamide and urea, for the synthesis of two coenzymes, UDPG, the structure of which was first elucidated by Leloir and co-workers (Cardini *et al.*, 1950), CDP-choline, and of G1P and G6P, important intermediates in carbohydrate metabolism. These three reaction systems are briefly summarized below.



The plausibility of occurrence on the primitive Earth of these phosphorylating systems is based on the fact that all the reacting compounds plus condensing agents have been previously synthesized under prebiological conditions, and on the ease of the reaction. Moreover, inorganic phosphate is also considered to have been present on the primitive Earth. This abiotic synthesis of the coenzymes could provide an insight on the synthesis of other phosphorylated structural and metabolic compounds.

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