Prebiotic Synthesis: Phosphorylation in Aqueous Solution

Abstract. *Formation of uridine-5′-phosphate from uridine and inorganic phosphate in aqueous solution is effected by the following condensing agents: cyanogen, cyanoformamide, cyanate, cyanamide, thioformate, ethylisocyanide, and a water-soluble carbodiimide. The yields are always small, even when a large excess of condensing agent is used. The cyclization of uridine-3′-(2′)-phosphate occurs under the same conditions but in much greater yield.*

It is widely believed that the earliest stages in development of life involved the concurrent evolution of proteins and nucleic acids. If this is true, prebiotic mechanisms must have operated that effected condensation of amino acids to peptides and of sugars, nucleotide bases, and inorganic phosphate to polynucleotides.

Two widely different experimental approaches to this aspect of biochemical evolution have met with some success. Dry heating of certain mixtures of amino acids yields a mixture of polypeptides (*1*); dry heating of nucleosides with inorganic phosphates, particularly with acid salts such as $Ca(H_2PO_4)_2$, gives nucleotides (*2*). In aqueous solution very similar but less extensive condensations have been achieved with reagents such as cyanamide and cyanoguanidine (*3*). Here we report systematic experiments designed to study further phosphorylation with condensing agents in aqueous solution.

Various potentially prebiotic condensing agents bring about the reaction of a nucleoside (uridine or thymidine) with phosphate to give a nucleoside-5′-monophosphate in aqueous solution; terminal yields at different *p*H's and temperatures are summarized (Tables 1 and 2). These reactions have a common terminal step: the alcoholysis of an active phosphate $AO\sim PO_3^{2-}$ (*4*), where *A* symbolizes any phosphate-activating group:

$$\text{uridine-5′-phosphate} + AOH \quad (1)$$

In some instances phosphate is generated *in situ*:

$$AO\sim PO_3^{2-} + \text{uridine} \longrightarrow$$

$$X\text{—CN} + HPO_4^{2-} + H^+ \longrightarrow X\text{-}\overset{\overset{NH}{\|}}{C}\text{-}OPO_3H^- \quad (2)$$

$$RN{\equiv}C + HPO_4^{2-} + H^+ \longrightarrow RN=C\begin{matrix}H\\ \diagdown OPO_3H^-\end{matrix} \quad (3)$$

where *X* is the hydroxyl, amino, cyano, or guanidino group and *R* is an alkyl radical. In one instance we have taken advantage of the stability of the intermediate phosphate and added it directly to the nucleoside: acetylphosphate is readily prepared by addition of phosphate to aqueous solutions of ketene (*5*). Finally we believe that thioformic acid reacts with phosphate to give an active intermediate, formyl phosphate:

$$HC(=O)-OPO_3^{2-}$$

since thioformic acid is known to be a formylating agent (*6*).

There is good evidence that under the conditions of our experiments the condensing agents, if they are used directly, are converted almost completely to reactive phosphate adducts; this fact is already known for cyanate (*7*). We have shown that the hydrolysis of 1-ethyl-3-dimethylaminopropyl carbodiimide (Table 3), cyanamide (Tables 4 and 5), and cyanogen (*8*) is so strongly catalyzed by phosphate that it must proceed largely through a phosphate adduct.

Thus it is clear that under the conditions employed the inefficiency of the condensing agents largely reflects the competition of water with the nucleoside in reaction (*1*). If we take the concentration of water as 55*M*, we may use as a very rough measure of selectivity, in the hydrolysis of the intermediate active phosphate,

$$\epsilon = (\text{fractional conversion to nucleotide}) \times [55/(\text{molarity of condensing agent})]$$

This factor is small, ranging from 0.5 to 2, for all our reactions, including those with the carbodiimide. We conclude that, in homogeneous solution, reactions of this type are intrinsically inefficient as long as the water greatly exceeds the nucleoside in concentration.

To test these conclusions we have studied the cyclization of a mixture of the 2′-(3′)-phosphates of uridine. The activation of the phosphate group should be analogous to that of inorganic phosphate, but the intermediates can undergo an efficient intramolecular esterification to give the 2′,3′-cyclic phosphate. The yields (Tables 6 and 7) are much greater, lending support to our hypothesis; they suggest that, if we could align nucleotides correctly on a template so as to bring the phosphate of one molecule close to the 3′-hydroxyl group of another, efficient polynucleotide synthesis should be possible.

Since HCN is believed to be central to much prebiotic synthesis it was important to see whether it would function as a condensing agent. In a series of experiments uridine was kept in mixtures of 1*M* HCN with 1*M* potassium phosphate buffers (*p*H 6.0, 7.0, and 8.0) at room temperature or 60°C either alone or in the presence of acetate, thioacetate, dithioglycol, or metal ions (Cu^{2+},

Table 1. Reagents and conditions applied in the phosphorylation of a nucleoside with inorganic phosphate; *p*H 6.0, 7.0, and 8.0.

Condensing agent	Concentration (*M*)	
	Uridine	Phosphate
1.6*M* HCOSNa	0.16	1
1*M* NC • $CONH_2$	.16	1
1*M* NC • NH_2	.1	1
1*M* KOCN	.1	1
3.8*M* C_2H_5NC	.16*†	2†
1*M* 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride	0.1	1

*Thymidine used instead of uridine. †*p*H 5.0, 6.0, 7.0, and 8.0.

Table 2. Yields of nucleoside-5′-phosphate from a nucleoside and an inorganic phosphate under the conditions described (Table 1).

Reagent	UMP yield (%) at *p*H				Days to maximum yield
	5.0	6.0	7.0	8.0	
		At 37°C			
HCOSNa		1.5	1.2	0.9	6
NC • $CONH_2$		2.0	1.8	1.7	$\leqslant 1$
KOCN	1.2	0.9	0.5	< 0.5	$\leqslant 1$
Carbodiimide		3.1	3.4	2.4	$\leqslant 1$
		At 65°C			
HCOSNa		2.1	2.2	2.0	$\leqslant 1$
NC • $CONH_2$		2.9	3.3	4.0	1
NC • NH_2		2.8	2.0	< 0.5	30
EtNC*	12	10	3	1	$\leqslant 1$
Carbodiimide		3.5	3.6	3.2	$\leqslant 1$

* Thymidine used as nucleosidic component; yields are of thymidine monophosphate.

Zn^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+}), which we believed might act as catalysts. In these experiments, as in others with the trimers and tetramers of HCN, sodium thiocyanate, thioformamide, formamidine, and propiolamide, we could demonstrate no phosphorylation of uridine.

Our findings suggest that in discussion of the relative merits of condensing agents emphasis should be given to the ease with which they may have formed on prebiotic Earth. We believe that the most promising reagent from this point of view is cyanogen (*9*), since it may be produced readily from HCN in various ways; HCN itself is obtained in good yield from a mixture of N_2 or ammonia with methane by the action of electric discharges or strong heating. A second, equally plausible, reagent is cyanate, which is produced by hydrolysis of cyanogen in neutral or mildly alkaline solution. Of the remaining reagents we believe that cyanamide and its dimer cyanoguanidine are most plausible. Cyanamide is formed from cyanide and ammonia under the influence of ultraviolet or high-energy radiation (*10*); it dimerizes readily to cyanoguanidine, even in quite dilute solutions, so that the two may reasonably have occurred together.

There are reports (*11*) that subjection of mixtures of an olefin with HCN to a silent electric discharge yields isonitriles. We could not obtain appreciable yields in this way, nor could we obtain ketene at all easily from hydrocarbons with carbon monoxide; thus we see no obvious routes to isonitriles or acetyl phosphate.

A major obstacle to prebiotic synthesis is the difficulty in obtaining high enough concentrations of reactants. The availability of inorganic phosphate, at least under most contemporary terrestrial conditions, is limited by the insolubility of magnesium and calcium phosphates. Perhaps the reaction occurred heterogeneously on the surface of materials containing phosphate (*12*). Alternatively life may have begun in a very limited environment differing from the main water masses in that it contained much dissolved phosphate.

The organic materials taking part in these reactions may have been concentrated in various ways. Among the most plausible is evaporation—for example, of shallow lakes or of droplets on the shores of larger water masses. Adsorption and concentration in eutectics also may have been significant. Too little is yet known for selection among these possibilities.

Table 3. Hydrolysis half-life (τ) of 1-ethyl-3-dimethylaminopropyl carbodiimide at 29° ± 0.5°C.

*p*H	τ (hr) at phosphate conc. (*M*)			
	0.05	0.02	0.01	0.00
6.0 ± 0.1	0.23	0.60	1.15	21.5
7.0 ± .1	.58	1.25	2.47	78
8.0 ± .1	2.3	4.2	6.2	63

Our conclusion is that cyanogen and its hydrolysis products cyanoformamide and cyanate are the most plausible "prebiotic" condensing agents now available; cyanamide and its dimer also should be considered carefully. Appended are descriptions of our experiments:

General Methods: For paper chromatography we used the descending technique with Whatman 3-MM paper. The solvent systems were mixtures (by volume) of (A) 95-percent ethanol and 1*M* ammonium acetate, *p*H 7.5 (7:3); (B) *n*-propanol, concentrated ammonia, and water (55:10:35); (C) isopropanol, concentrated ammonia, and water (7 : 1 : 2); and (D) *n*-butanol, 95-percent ethanol, and water (4:1:1).

Compounds containing nucleoside bases were detected by viewing under an ultraviolet lamp, with a fluorescent screen to increase the sensitivity of detection. When unlabeled nucloesides were used, the yields were determined by elution of the ultraviolet-absorbing spots from the chromatography paper, and measurement of their optical densities against blanks in a Zeiss PMQ2 spectrophotometer.

For radioactively labeled material the papers were cut into strips and run through a Baird Atomic radiochromatogram scanner. Subsequently the radioactive zones were cut out and counted in a Beckman liquid-scintillation counter. The yield of nucleotide was calculated by taking the counts, from the zone containing the compound, as a percentage of the total number of counts on the paper; each background count was subtracted. Apparent yields of less than 0.5 percent were not considered significant.

For further characterization the reaction products were eluted from paper (solvent B) and subjected to electrophoresis on Whatman 3-MM paper (at 4000 volts) in a high-voltage tank (*13*). The paper was immersed in a cooled (30°C) high-boiling petroleum fraction; the buffer was 0.03*M* potassium phosphate (*p*H 7.1).

Formation of nucleotides from nucleosides and inorganic phosphate:

1) Uridine monophosphate (UMP) was obtained by heating, for 5 hours at 95°C in a well-stoppered tube, a mixture of sodium dihydrogenphosphate (0.5 mmole), uridine (0.05 mmole), and ethylisocyanide (*14*) (0.1 ml) in water (0.25 ml). The reaction mixture was chromatographed in solvent A, and the ultraviolet-absorbing spots were eluted. The yield of UMP was 12 percent.

A similar experiment with a mixture of 85-percent phosphoric acid (10 μl), uridine (0.05 mmole), and ethylisocyanide (0.1 ml) in 50-percent aqueous pyridine (0.25 ml) yielded 24 percent UMP; about 3 percent uridine cyclic phosphate also was formed.

Thymidine monophosphate was formed by heating (65°C) thymidine (0.05 mmole) and ethylisocyanide (0.080 ml) in 2*M* potassium phosphate buffers (0.2 ml; *p*H 5.0, 6.0, 7.0, and 8.0) in sealed ampules (2 ml) for 24 hours. Subsequently the reaction mixtures were analyzed by chromatography in solvent A (for yields see Table 2). A comparable experiment with 2*M* phosphoric acid in 50-percent aqueous pyridine (0.2 ml) yielded 12 percent thymidine phosphate after 24 hours.

2) In a series of experiments, mixtures of disilver acetylphosphate (0.5 mmole) and potassium chloride (1 mmole) in 1*M* potassium phosphate buffers (0.5 ml; *p*H 5.0, 6.0, 7.0, and 8.0) were shaken until all the AgCl had formed. After centrifugation, part of the supernatant (0.3 ml) was added to 2-C^{14}-uridine (0.05 mmole, 0.5 μc) dissolved in water (0.05 ml). The reaction mixtures were kept at room temperature or 65°C in stoppered tubes for various periods before portions were analyzed. After 1 day at room temperature the maximum yield was 2.5 percent UMP at all *p*H values investigated. In experiments at 65°C the optimum yield was 2.5 percent after 4 hours.

3) Solutions of 2-C^{14}-uridine (0.025 mmole, 0.5 μc) in 0.5, 0.1, and 0.02*M* potassium phosphate buffers (*p*H 6, 2 ml) were contained in rubber-sealed round-bottom flasks (2 liters) that were filled

Table 4. Cyanamide hydrolysis at different concentrations of cyanamide in 1*M* phosphate at 65°C.

Cyanamide conc. (*M*)	Hydrolysis (%) after					
	2 Days			1 Week		
	Cyanoguanidine	Urea	Cyanamide	Cyanoguanidine	Urea	Cyanamide
	*p*H 7.0					
1.0	42	13	45	58	28	14
0.2	22	17	61	29	43	18
.1	9	24	67	17	48	35
.02	3	28	69	4	53	43
.01	2	22	76	3	55	42
	*p*H 8.0					
1.0	77	10	13	84	13	3
0.2	49	19	32	63	29	8
.1	29	26	45	43	43	14
.02	9	32	59	18	58	24
.01	7	26	67	11	57	32

with a mixture of nitrogen and cyanogen (0.5 percent by volume). The solutions were stirred magnetically at room temperature. The cyanogen consumption was followed by analysis of samples of gas in a gas chromatograph (*15*) (column, 180 by 0.35 cm; oven temperature, 32°C).

When the cyanogen had fallen to about 20 percent of the original concentration, additional cyanogen (10 ml) was injected into each reaction flask containing 0.5*M* or 0.1*M* phosphate buffer. In each instance the reaction mixture slowly discolored and then gradually deposited brown polymers. Samples from the aqueous phase after 7 days showed UMP yields of 2.5, 1.1, and 0.1 to 0.2 percent at phosphate concentrations of 0.5, 0.1, and 0.02*M*.

4) Uridine was dissolved with the condensing agent (*16*) in potassium phosphate buffers (0.25 ml) (for concentrations, *p*H, and yields see Tables 1 and 2); 2-C^{14}-uridine (0.5 μc; specific activity, 25 mc/mmole) was then added to each reaction mixture, and the stoppered tubes were incubated at 37°C in a thermostated water bath or in an oven maintained at 65°C. Portions (0.03 ml) were withdrawn daily and chromatographed in solvent A or solvent B, with cold UMP as standard. Analysis in the radiochromatogram scanner and liquid-scintillation counter showed the yields listed (Table 2).

Formation of uridine-2′,3′-cyclic phosphate (UCP) from uridine-2′-(3′)-phosphate:

1) Ethylisocyanide (0.05 ml) was added to an aqueous solution (0.2 ml) of uridine-3′-(2′)-phosphoric acid (*17*) (7 mg). The mixture was kept in a small, well-stoppered test tube and shaken frequently. When it had stood for 11 hours at room temperature, concentrated ammonia was added and samples were analyzed by chromatography (solvent C) and paper electrophoresis. The yield of UCP, determined by the elution method, was 94 percent. A parallel experiment performed in 50-percent aqueous pyridine under otherwise identical conditions also gave a 94-percent yield.

2) Cyanogen gas (10 ml) was injected into an evacuated, rubber-stoppered tube (12 ml) already containing an aqueous solution of 2-C^{14}-uridine-3′-(2′)-phosphate (*18*) (0.05 mmole; 1 μc) in 1*M* potassium acetate (*p*H 5.7; 1 ml), and the mixture was kept at room temperature. Portions taken with a syringe after 24 and 48 hours showed, after chromatography in solvent C and further analysis, yields of 13.6 and 17 percent UCP, respectively.

3) Uridine-3′-(2′)-phosphate, 2-C^{14}-uridine 3′-(2′)-phosphate (0.5 μc), and the condensing agent were dissolved in 1*M* potassium acetate buffers at *p*H 5 or 6, or in tris-chloride buffer at *p*H 7.5 (for concentrations and yields see Tables 6 and 7). The cyclic uridine phosphate was characterized by paper chromatography and paper electrophoresis, with authentic material used as a standard. Yield was determined in the liquid-scintillation counter. The compound was further identified by incubation with pancreatic ribonuclease (50 μg) in tris-chloride (5 μmole) buffer at *p*H 7.5 and 37°C for 4 hours; total volume of the reaction mixture was about 0.1 ml. Chromatography of the incubation mixture in solvent A, followed by radiochromatographic scanning, showed that the cyclic uridine phosphate had been quantitatively degraded to uridine-3′-phosphate.

Hydrolysis of cyanamide: For determination of the effect of cyanamide concentration, 1, 0.2, 0.1, 0.02, and 0.01*M* solutions of C^{14}-cyanamide (*19*) (0.5 μc) were made up in 1*M* phosphate buffers at *p*H 7.0 and 8.0; they were incubated in stoppered tubes at 65°C. Portions were chromatographed daily in solvent D, with cyanamide, cyanoguanidine, and urea as standards. The cyanamide and cyanoguanidine zones were located by spraying of the standards with a mixture of 5-percent aqueous sodium nitroprusside (2 ml), 3-percent aqueous H_2O_2 (5 ml), and 10-percent aqueous sodium hydroxide (1 ml), diluted to 15 ml with water (*10*). The urea standard was located by spraying it with a solution of *p*-dimethylaminobenzaldehyde (1 g) in concentrated HCl (5 ml) and 95-percent ethanol (95 ml). The papers were then run through the radiochromatogram scanner, and the cyanamide, cyanoguanidine, and urea zones were cut out and counted in the scintillation counter. The amount of each product was calculated as a percentage of the total number of counts on the paper (Table 4).

The effect of phosphate concentration (Table 5) was determined by incubation at 65°C of 0.01*M* solutions of C^{14}-cyanamide (0.5 μc) in 1, 0.5, and 0.1*M* potassium phosphate buffers (*p*H 8). Samples were analyzed periodically.

Hydrolysis of 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (20):

1) Hydrolysis in the presence of phosphate (Table 3) was determined by shaking, in a water bath at 29°C, solutions of carbodiimide (1 mmole) in 10 ml of 0.01, 0.02, and 0.05*M* phosphate buffers at *p*H 6.0, 7.0, and 8.0. Periodically, portions (0.50 ml) were mixed with 0.10*M* oxalic acid (1.0 ml). After 1 hour, 10-percent sulfuric acid (1.5 ml) was added. The mixture was heated to about 60°C and titrated with 0.02*N* potassium permanganate (*21*).

2) Hydrolysis without phosphate (Table 3) was examined by incubation of aqueous 0.1*M* carbodiimide solutions at 29°C in a *p*H-stat; *p*H 6.0 and 7.0 were maintained with 0.01*N* hydrochloric acid, and *p*H 8.0 was maintained with 0.01*N* sodium hydroxide. Periodically, samples were analyzed as described in the preceding paragraph.

Table 5. Cyanamide hydrolysis at different phosphate concentrations, *p*H 8.0 and 65°C, with 0.01*M* cyanamide.

Phosphate conc. (*M*)	Cyanoguanidine (%)	Urea (%)	Cyanamide (%)
	After 2 days		
1.0	9	30	61
0.5	7	21	72
.1	6	12	84
	After 4 days		
1.0	8	42	50
0.5	10	33	57
.1	13	17	70

Table 6. Conditions applied for the cyclization reaction of 0.16*M* uridine-2′-(3′)-phosphate; *p*H 5.0, 6.0, and 7.5.

Reagent	Concentration (*M*)
NC • $CONH_2$	1.6
NC • NH_2	0.8
KOCN	1
Carbodiimide	0.8

Table 7. Yields of uridine-2′,3′-cyclic phosphate formed from uridine-2′,(3′)-phosphate under the conditions described (Table 6).

Reagent	*p*H 5.0	*p*H 6.0	*p*H 7.5	Days to max. yield
	37°C			
NC • $CONH_2$	38	23	12	$\leqslant 1$
NC • NH_2	40	45	1.5	10
KOCN	13	1.7	0.5	$\leqslant 1$
Carbodiimide	70			$\leqslant 1$
	65°C			
NC • $CONH_2$	14	13	11	$\leqslant 1$
NC • NH_2	73	39	1.2	6
Carbodiimide	51			$\leqslant 1$

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