

# PHOSPHORYLATION OF URIDINE WITH INORGANIC PHOSPHATES

SHERWOOD CHANG, JO ANN WILLIAMS,  
CYRIL PONNAMPERUMA, and JOSEPH RABINOWITZ\*

*Exobiology Division, Ames Research Center, NASA, Moffett Field, Calif., 94035, U.S.A.*

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**Abstract.** Phosphorylation of uridine by heating with inorganic orthophosphates can take place through reaction of nucleoside with thermally produced polyphosphate or direct reaction of nucleoside with orthophosphate or a combination of both. The relative importance of the two pathways varies with the orthophosphate and temperature.

## 1. Introduction

Phosphorylation of nucleosides with inorganic orthophosphates has been reported under hypohydrous conditions at temperatures ranging from 65° to 162° (Ponnamperuma and Mack, 1965; Skoda and Moravek, 1966; Moravek, 1967; Moravek and Skoda, 1967; Beck *et al.*, 1967). Previous work (Rabinowitz *et al.*, 1968a, b) indicated the approximate amounts of polyphosphates produced by heating a variety of orthophosphates under conditions similar to those used earlier to phosphorylate nucleosides (Ponnamperuma and Mack, 1965; Skoda and Moravek, 1966; Moravek and Skoda, 1967; Moravek, 1967). Notably no polyphosphates were detectable with dibasic and tribasic phosphates, all of which previously gave very low yields (less than 1 per cent) of nucleotides (Ponnamperuma and Mack, 1965). This report describes two different kinds of experiments conducted under thermal hypohydrous conditions: Polymerization of orthophosphates to polyphosphates and phosphorylation of uridine with ortho and polyphosphates. A comparison of the results makes it possible to assess the possible role of thermally produced polyphosphates as the phosphorylating agents.

## 2. Methods

Detailed data on polyphosphate formation at 162°, 126°, and 98° were obtained with radioactive [<sup>32</sup>P] orthophosphates. Aliquots of solutions (0.09M [<sup>32</sup>P] NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 2.12 μc/μl; 0.03M [<sup>32</sup>P] NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 2.43 μc/μl; 0.08M [<sup>32</sup>P] Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub> · H<sub>2</sub>O, 0.196 μc/μl) were lyophilized in small open test tubes and the tubes heated for various lengths of time at suitable temperatures. After cooling, each sample was dissolved in water and chromatographed on Eastman Chromagram Cellulose sheets using the solvent of Fuchs and Czeck (1963). Spots located by autoradiography were cut out and the radioactivity was measured in a liquid scintillation counter. Illustra-

\* National Research Council, Senior Research Associate. Present address: Department of Chemistry, University of California at Berkeley.

tive chromatograms showing typical separations are reproduced in Figure 1. Changes in phosphate composition which accompanied heating are present in Figure 2. The reproducibility of a typical experiment is illustrated in (a) and (b) of Figure 2. When the same experiment was performed on widely separated days, however, significant differences occurred in the overall rates of polyphosphate formation.\* Consequently, experiments providing data shown in Figure 2 were begun at the same time. Figure 2

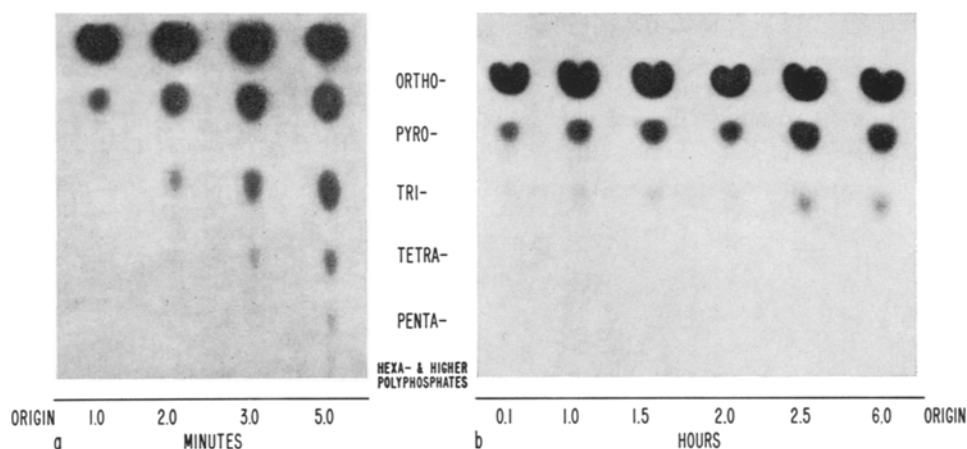


Fig. 1. Autoradiographs showing typical separation of various phosphates produced by heating  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  at (a)  $162^\circ$  and (b)  $126^\circ$ .

shows the ease of polyphosphate formation to be in the order  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} > \text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} > \text{NH}_4\text{H}_2\text{PO}_4$ . In separate experiments, detectable amounts of pyrophosphate formed when  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  were heated at temperatures as low as  $65^\circ$ . Cyclic polyphosphates were not detected in any experiment. The conversion of orthophosphates to polyphosphates at higher temperatures has been reported (Thilo and Grunze, 1955; Hill *et al.*, 1947; Edwards and Herzog, 1957), but no quantitative assessments of the rates of polyphosphate formation or polyphosphate composition were given.

Chromatographic interference from uridine phosphates precluded direct analysis of phosphorylation reaction mixtures for polyphosphate compositions. In separate experiments [ $^{32}\text{P}$ ] orthophosphates alone and mixtures of [ $^{32}\text{P}$ ] orthophosphates and uracil were heated at  $162^\circ$  and  $126^\circ$ . No significant differences in the rates or extents of polyphosphate formation were observed, indicating that they were not appreciably affected by admixture of orthophosphate and pyrimidine type compounds. It seems reasonable that data in Figure 2 could be used to represent polyphosphate contents in parallel phosphorylation experiments.

\* The importance of atmospheric water vapor pressure in the complex process of polyphosphate formation has been pointed out and may be a factor in causing such differences (see Thilo and Grunze, 1955).

Phosphorylation of uridine was carried out with various phosphates at 162°, 126°, and 98°. In general 12  $\mu$ moles each of phosphate and uridine in small test tubes were dissolved or suspended in 2 ml of water. The resulting solutions or suspensions were lyophilized, and the test tubes were heated at the desired temperature for appropriate times. Products were analyzed by chromatography on Brinkmann cellulose-F thin-layer chromatoplates in a mixture of isobutyric acid-0.5*N* ammonium hydroxide (60/36). All material in ultraviolet absorbing spots was extracted with 0.02*M* Tris-HCl buffer (pH 7.0) and analyzed by UV spectroscopy.

All experiments were performed at the same time in parallel with those represented in Figure 2. In some instances short reaction times were chosen to make it possible to determine the extent to which polyphosphate formation preceded or accompanied initial stages of phosphorylation. In experiments involving preheated phosphates, 12  $\mu$ moles of the orthophosphate were heated at 162° for 5 h, then dissolved in 2 ml of

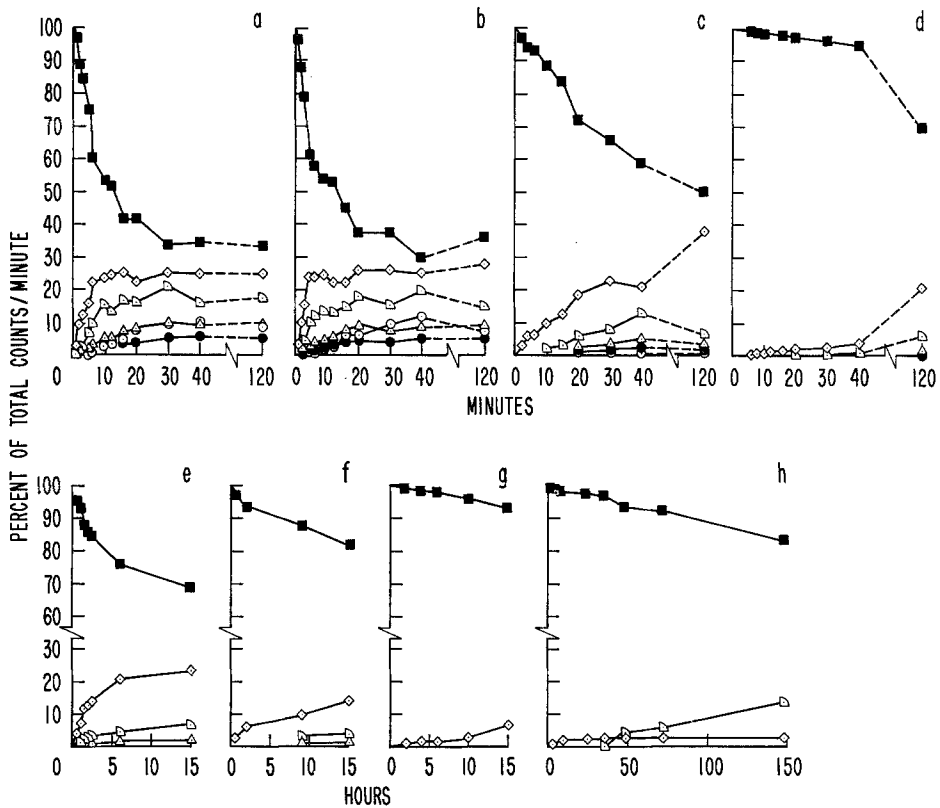


Fig. 2. Polyphosphate compositions produced after various times by heating (a) and (b) [ $^{32}\text{P}$ ]  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  at 162°; (c) [ $^{32}\text{P}$ ]  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  at 162°; (d) [ $^{32}\text{P}$ ]  $\text{NH}_4\text{H}_2\text{PO}_4$  at 162°; (e) [ $^{32}\text{P}$ ]  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  at 126°; (f) [ $^{32}\text{P}$ ]  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  at 126°; (g) [ $^{32}\text{P}$ ]  $\text{NH}_4\text{H}_2\text{PO}_4$  at 126°; and (h) [ $^{32}\text{P}$ ]  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  at 98°. Orthophosphate, —■—; pyrophosphate, —◇—; triphosphate, —△—; tetraphosphate, —▽—; pentaphosphate, —●—; hexa- and higher phosphates, —○—.

water containing 12  $\mu$ moles of uridine just prior to lyophilization. To determine the composition of preheated phosphates, the corresponding [ $^{32}\text{P}$ ] orthophosphates were heated in parallel experiments and analyzed as described above. In Table I are summarized the conditions of each experiment and the yields of individual products as percentages of the combined absorbance at 260 nm of all products. In order to facilitate comparison between total phosphorylation yields and polyphosphate contents in parallel experiments, the latter are included as the last column in the table.

In the solvent used for chromatography of the products, 2'(3')-UMP and 2'.3'-cyclic-UMP travel as a single spot as do 5'-UMP and diuridine phosphate. Because the products obtained by heating uridine with inorganic orthophosphates have been well characterized (Ponnamperuma and Mack, 1965; Skoda and Moravek, 1966; Moravek and Skoda, 1967; Moravek, 1967; Beck *et al.*, 1967) and our interests lay primarily in the total yield of phosphorylated uridine, no attempts were made to determine the presence of 2'.3'-cyclic-UMP and diuridine phosphate. Thus, material having  $R_f$ -values corresponding to 2'.3'-UMP and 5'-UMP were assumed to be only these species.

### 3. Results

Results in Table I obtained with various sodium phosphates show that much higher yields of uridine phosphates were obtained with polyphosphates than with orthophosphates. Notable differences occur in the reactivities of  $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ ,  $\text{Na}_5\text{P}_3\text{O}_{10}$ ,  $\text{Na}_3\text{P}_3\text{O}_9$  and Graham's salt, but no clear explanation for them is available. Apparently,  $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$  and Graham's salt (composed of linear polyphosphates with no species smaller than triphosphate) gave highest yields of phosphorylation, while cyclic trimetaphosphate gave lower yields than triphosphate. In aqueous medium, however, phosphorylation of alcohols and sugars was found to be most effective with trimetaphosphate (Feldmann, 1967).

Reaction of uridine with polyphosphates formed during the period of heating could account for the small amounts of phosphorylation observed with  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ . Similar conclusions can be drawn from experiments with  $\text{NH}_4\text{H}_2\text{PO}_4$  and the ortho- and polyphosphate mixtures obtained by preheating  $\text{NH}_4\text{H}_2\text{PO}_4$  and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ . In the case of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , no detectable polyphosphate formation occurred, and a very low phosphorylation yield was obtained. Reaction with  $\text{CaHPO}_4$  afforded neither detectable phosphorylation nor polyphosphate.

### 4. Discussion

The results indicate that heating uridine with  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  or  $\text{NH}_4\text{H}_2\text{PO}_4$  results in partial transformation of the orthophosphates to polyphosphates as well as in phosphorylation of the uridine. The resulting polyphosphates are effective phosphorylating agents and can phosphorylate nucleoside faster than the original orthophosphate. Therefore, strong circumstantial evidence exists for the intermediary role of polyphosphates in the phosphorylation of nucleosides with  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and

TABLE I  
Yields of uridine phosphates obtained by heating uridine with various inorganic phosphates

(°C)	t (hr)	Phosphate	Uracil %	2'(3')-UMP %	5'-UMP %	Uridine diphosphate %	Total yield of uridine phosphates %	Phosphorus as poly- phosphates %
162	0.1	Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	0.0	0.6	0.4	0.0	1.0	- <sup>b</sup>
162	0.1	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	0.0	1.6 ± 0.2 <sup>a</sup>	0.2 ± 0.1	0.2 ± 0.1	2.0 ± 0.4	6
162	0.1	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O (preheated)	0.4	0.1	1.2 ± 0.5	0.7 ± 0.2	5.5 ± 1.1	32 <sup>c</sup>
162	0.1	Na <sub>3</sub> P <sub>3</sub> O <sub>10</sub>	0.0	7.5 ± 1.2	6.0 ± 2.0	1.9 ± 1.0	15.4 ± 4.2	
162	0.1	Na <sub>3</sub> P <sub>3</sub> O <sub>9</sub>	0.7	3.9	1.7	1.4	7.0	
162	0.5	NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	0.0	1.7 ± 0.2	0.2 ± 0.1	0.0	1.9 ± 0.3	4
162	0.5	NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> (preheated)	0.0	3.7	2.4	0.0	6.1	64 <sup>c</sup>
162	2.0	KH <sub>2</sub> PO <sub>4</sub>	0.0	3.2 ± 0.4	1.7 ± 0.0	0.0	4.9 ± 0.4	-
126	6.0	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	0.0	2.8	2.6	0.0	5.4	8
126	6.0	Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	0.0	0.5 ± 0.1	0.5 ± 0.1	0.0	1.0 ± 0.2	-
126	6.0	Na <sub>2</sub> H <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	0.0	13.3	13.5	3.0	29.8	
126	6.0	Na <sub>5</sub> P <sub>3</sub> O <sub>10</sub>	0.0	11.3	7.5	2.3	21.1	
126	6.0	Na <sub>3</sub> P <sub>3</sub> O <sub>9</sub>	0.0	6.5	6.4	0.0	12.9	
126	6.0	Graham's salt	0.0	12.7	12.4	3.1	28.2	
126	6.0	CaHPO <sub>4</sub>	0.0	0.0	0.0	0.0	0.0	-
126	0.1	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O	0.8	2.6	3.8	3.3	9.7	1
98	48.0	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O	2.7 ± 1.0	5.6 ± 0.1	16.1 ± 1.2	7.5 ± 0.8	29.2 ± 2.1	7
98	336.0	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O	7.4 ± 1.8	3.8 ± 0.4	14.5 ± 1.0	8.5 ± 0.3	26.8 ± 1.7	

<sup>a</sup> Average and average deviation of two independent experiments indicated by ±.

<sup>b</sup> Undetectable amounts, less than 1% indicated by -, taken from Rabinowitz *et al.* (1968a).

<sup>c</sup> Content prior to heating with uridine.

$\text{NH}_4\text{H}_2\text{PO}_4$  reported earlier. Possibly, at the interface of the amorphous polyphosphate phase known to occur (Thilo and Grunze, 1955; Hill *et al.*, 1947; Edwards and Herzog, 1957), uridine reacts by nucleophilic attack of a hydroxyl group at a P-O-P bond in accordance with the mechanism presumably operating in phosphorylation of nucleosides by polyphosphoric acid (Hall and Khorana, 1955; Michelson, 1958; Weiss *et al.* 1958; Waehneltd and Fox, 1967). Present data do not eliminate a mechanism directly involving orthophosphate, and it is possible that such a pathway may also contribute to the formation of phosphorylated products.

Yields of uridine phosphates obtained by heating uridine with  $\text{KH}_2\text{PO}_4$  at  $162^\circ$  and  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  at  $126^\circ$  far exceed the amount of polyphosphate formed in parallel experiments without uridine during the same periods of heating. It is improbable that nucleoside admixed with these orthophosphates catalyzed formation of polyphosphates to serve as phosphorylating agents. A more reasonable conclusion is that a direct phosphorylation mechanism was available involving orthophosphate and uridine. At  $162^\circ$  calcium polyphosphates are readily formed so that some phosphorylation at that temperature (Ponnamperuma and Mack, 1965) may involve polyphosphates.

A relationship has been suggested (Newesley, 1967) between weakening of the crystal lattice of orthophosphates due to loss of water of hydration, or loss of ammonia (Thilo and Grunze, 1955) in the case of ammonium orthophosphates, and their tendency to form polyphosphates. The effectiveness of orthophosphates in direct phosphorylation may be related to the same phenomenon. Possibly, diffusion of uridine molecules and  $\text{H}_2\text{PO}_4^{-2}$  ions together in crystalline phases occurs followed by thermal dehydration. Why such a process should be so favorable in the case of  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  is not clear. Calcium ions are known to be catalysts for hydrolysis of phosphate esters; they may play a similar role in the phosphorylation reaction.

The thermal reaction between nucleosides and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  at  $160^\circ$  has recently been exploited as a means of synthesizing [ $^{14}\text{C}$ ] nucleotides (Skoda and Moravek, 1966; Moravek and Skoda, 1967; Moravek, 1967). Present results suggest that higher yields could be attained by using polyphosphates or  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  at lower temperatures. The last two entries in the table indicate that heating uridine with  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  beyond a certain time results in increased hydrolysis of the glycosidic linkage without improvement in phosphorylation yield.

## 5. Conclusion

The occurrence and formation of inorganic phosphates and polyphosphates in a prebiological context have been discussed. They also appear to be formed during electric discharge through methane, ammonia, phosphine and water atmospheres (Rabinowitz *et al.*, 1969a). In aqueous media inorganic polyphosphates phosphorylate alcohols, sugars (Feldmann, 1967) and nucleosides (Schwartz and Ponnamperuma, 1968) and promote the condensation of  $\alpha$ -amino acids to dipeptides (Rabinowitz *et al.*, 1969b). The ability of calcium and sodium phosphates to enhance the anhydrous

condensation polymerization of  $\alpha$ -amino acids has been demonstrated (Vegotsky, 1961). These results all point to a central role for inorganic phosphates in primordial chemical synthesis.

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