NUCLEOSIDE AND DEOXYNUCLEOSIDE PHOSPHORYLATION IN FORMAMIDE SOLUTIONS*

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Abstract. Nucleosides or deoxynucleosides were converted to a number of phosphorylated nucleotide and deoxynucleotide derivatives by ammonium or alkali dihydrogen phosphates in formamide. Conversions were smaller and slower at room temperature and greater and faster at elevated temperatures. Nucleotides afforded product mixtures similar to those obtained for nucleosides under the same conditions, indicating the occurrence of transphosphorylation processes. Products of reaction at elevated temperatures were cyclic nucleotides, nucleoside monophosphates, nucleoside diphosphates and cyclic nucleotide phosphates. The relative amounts of products formed were quite temperature dependent. Cyclic nucleotides were found to be in greatest abudance for reactions run at 125° or above. Relative yields of 2′, 3′ and 5′ nucleotides and 3′ and 5′ deoxynucleotides from several experiments are reported. 5′-Monophosphates were generally found to be present in larger quantities than 2′ or 3′ monophosphates. 2′-Deoxyadenosine showed a preference for phosphorylation at the 3′ position. Conclusions reached from mechanistic studies are that the phosphorylations are a series of equilibrium reactions, with cyclic nucleotides being formed irreversibly.

1. Introduction

Phosphorylations of nucleosides and deoxynucleosides in formamide have been reported previously (Schoffstall, 1976; Schoffstall and Kokko, 1978; Phillip and Seliger, 1977). These reactions took place between nucleosides and unactivated phosphates at room temperature and above. For reactions of a nucleoside at elevated temperatures, products were the 2′, 3′ and 5′-nucleotides, 2′, 5′ and 3,′5′-nucleoside diphosphates and 2′,3′-cyclic nucleotide 5′-phosphate. For a deoxynucleoside, the phosphorylation products were the 3′ and 5′-deoxynucleotides and 3′,5′-deoxynucleoside diphosphate. Products found in reaction mixtures kept at room temperature were mononucleotides or monodeoxynucleotides. It has been reported that deoxynucleosides afforded predominantly 5′-monodeoxynucleotides when phosphorylation was performed at 37° (Phillip and Seliger, 1977). Our previous reports did not consider relative percentages of individual mononucleotides or monodeoxynucleotides, but we noted qualitatively the appearance of about twice as much 5′ product as 2′(3′) product from adenosine phosphorylations. The report of nearly exclusive 5-deoxynucleotide formation prompted further investigation. Therefore, we developed chromatographic techniques which allowed for the separation and analysis of deoxynucleotides and nucleotides. We performed reactions at several different temperatures and time intervals.

Elevated temperature (140°) phosphorylations were not used in our previous work (Schoffstall, 1976; Schoffstall and Kokko, 1978) because such temperatures were

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deemed as having been less relevant in chemical evolution than temperatures below 100°. The possible relevance of formamide in chemical evolution has been discussed previously (Schoffstall, 1976). In the present work, we have included some reactions at higher temperatures in order to provide a more comprehensive analysis of the phosphorylation pathways. We have also formulated reaction mechanisms, which we believe to be a series of dehydration condensations. The reactions of nucleosides leading to nucleotides appear to be reversible transphosphorylations.

2. Experimental

Nucleosides and deoxynucleosides were purchased from Sigma Chemical Company and were used without purification. Formamide was obtained from Fisher Scientific Company. Formamide that was dried and vacuum distilled gave results similar to those obtained using formamide without purification. Thin layer chromatography was performed using Eastman chromatogram cellulose sheets (#13181). Quantitative TLC was done using Brinkman Cel 300–25 precoated (0.25 mm) cellulose plates or homemade cellulose plates. Ultraviolet spectra were determined using a Varian 634 Spectrometer.

3. Procedure

Nucleosides and phosphates were placed in open test tubes. Formamide (5 ml) was added to each tube. Tubes kept at 37 ± 3° were maintained using an oil bath. Other tubes were held at ±2° using a Koflac heating block. Aliquots of each reaction mixture were removed and analyzed qualitatively in at least three different solvent systems. Quantitative data were taken from plates developed in one or more solvent systems reported previously (Schoffstall, 1976). Sources of error were a 1–2° variation among tubes in the tube slots of the heating block, a 1° thermometer error and ambient air temperature variability. Error analysis gave a total error of 5–10% for reported percentages. In addition, presence of moisture and formic acid in the solvent gave lower yields of phosphorylated products.

4. Thin Layer Chromatography

Data for TLC standards and reaction products and procedures for quantitative plates have been reported previously (Schoffstall, 1976). All yield data in this paper are based upon quantitative TLC results except where HPLC data are specified.

5. High Pressure Liquid Chromatography

An analytical method for separating and quantifying nucleoside and nucleotides has been developed (Ramos and Schoffstall, unpublished results). Aliquots of reaction mixtures were analyzed using an Altex system, which consisted of two Model 110 pumps, a Supelco reverse phase column, variable wavelength detector, recorder and

solvent programmer. Analyses were performed by elution with 10% methanol-water containing 0.05 *M* $NH_4H_2PO_4$ (pH 5) at a flow rate of 1.9 ml/min with the detector at 254 nm. An example of typical results is shown in Table VI. The order of elution for adenosine derivatives was ADPs and 5′-AMP, followed by 2′-AMP, 2′3′-cAMP, 3′-AMP and adenosine.

Hydrates of nucleotides or their salts were heated in formamide affording results as shown in Table II. The amounts of diphosphates as originally determined were too high by HPLC as compared with TLC results. Evaporation of volatile impurities *in vacuo* from the reaction mixtures prior to HPLC analysis gave diphosphate readings that matched the TLC data. The corrected HPLC data are presented in Table III.

6. Results

Adenosine and potassium dihydrogen phosphate were kept in open tubes in formamide at 37° for several months and analyzed for the presence of phosphorylated products using quantitative thin layer chromatography. The results are given in Table I. The reactions analyzed after four months were examined for total nucleotides. Those after 6.5 months were examined for the relative amounts of mononucleotides as well as the combined total. There was a 2 to 1 preference for 5′-AMP for 2′(3′)-AMP at 37°. There was less than 0.2% of 2′,3′-cAMP formed according to analysis by HPLC. The presence of excess phosphate gave slightly higher yields of phosphorylated products. These results were consistent with our earlier qualitative observations.

Analysis of the reaction of adenosine (0.02 *M*) with KH_2PO_4 (0.10 *M*) for 12 hr at 140° showed 18% AMPs and 51% of 2′,3′-cAMP. The formation of cyclic nucleotide was very significant at high temperature. However, reaction mixtures kept at 150° for more than 12 hr turned brown and showed a brown precipitate, characteristic of the decomposition of sugars under these reaction conditions.

Phosphorylation of adenosine (0.05 *M*) with KH_2PO_4 (0.10 *M*) at 100° in formamide (5 mls) for 12 hr gave adenosine (50%), AMPs (32%), 2′,3′-cAMP (8%) and ADPs (6%) and some unidentified material. When 2′-AMP, 3′-AMP or 5′-AMP were placed individually in formamide with or without added KH_2PO_4 and heated for 2 hr at 100°, an array of products similar to the one above was observed. Heating 5′-GMP

SCHEME 1

$$\text{Adenosine or 2′, 3′ or 5′-AMP} \xrightarrow[KH_2PO_4]{\text{Formamide } 100^\circ} \text{Adenosine} + \text{2′, 3′ and 5′-AMPS} + \text{2′,3′-cAMP} + \text{ADPs}$$

(Na salt hydrate) or 2′(3′)-GMP (Na salt hydrate) also showed isomerization upon being heated in formamide. Data for equilibrations at 54° and 110° are shown in Table II. Thus, dephosphorylation of nucleotides also occurred in formamide. Our

TABLE I
Yields from Phosphorylation of Adenosine

Adenosine +	XH_2PO_4 $\xrightarrow{\text{Formamide}}$			5′ AMP + 2′(3′)-AMP +		2′,3′ cycAMP +	ADPS +	Adenosine
0.01 *M*	0.01 *M*[a]	90 days	25°	3% total nucleotides		0%	0%	97%
0.05 *M*	0.05 *M*[b]	4 months	37°	10% total nucleotides		0%	0%	90%
0.05 *M*	0.05 *M*[b]	6.5 months	37°	10%	5%	trace	0%	85%
0.05 *M*	0.50 *M*[b]	6.5 months	37°	12%	7%	trace	0%	81%
0.05 *M*	0.10 *M*[b]	12 hr	100°	24%	8%	8%	6%	50%[c]
0.05 *M*	0.10 *M*[b]	12 hr	130°	8%	20%	35%	6%	28%[c]
0.02 *M*	0.10 *M*[b]	12 hr	140°	4%	14%	51%	16%	12%[c]

[a] X was ammonium ion. [b] X was potassium ion. [c] There was 3–4% unidentified material.

TABLE II
Isomerization of nucleotides and deoxynucleotides

Nucleotide	$\xrightarrow[\Delta]{\text{Formamide}}$		Products					
Nucleotide	Temperature	Length of reaction	5′	2′	3′	2′, 3′ cyclic	Diphosphates	Nucleoside
2′(3′)-GMP Na salt, dihydrate	110°	12 hr	28%	4%	10%	4%	11%	43%
5′-AMP Na salt hydrate	110°	12 hr	14%	7%	4%	8%	16%	45%[a]
5′-dAMP free acid	54°	1 wk	74%	–	10%	–	3%	13%
2′(3′)-AMP free acid	54°	1 wk	30%	8%	18%	4%	8%	32%

[a] There was 6% adenine in this solution. All analyses were by HPLC.

conclusion is that nucleotide formation in formamide is reversible. When 2′,3′-cAMP was heated under the same conditions, 2′-AMP, 3′-AMP and 5′-AMP were not observed. Cyclic phosphate formation from 2′(3′)-AMP was irreversible within the time frame that is sufficient to equilibrate the individual nucleotides in formamide.

HPLC analysis of reactions of other nucleotides with KH_2PO_4 in formamide at 125° gave further evidence of the generality of the phosphorylation as shown in Table III. Phosphorylation at the 5′ position of guanosine was preferred over the combined percentages of attack at the 2′ and 3′ positions. Reactions of the pyrimidine nucleosides went further toward completion than guanosine. The HPLC analysis method gave considerable overlap of nucleotide peaks. Assignment of percentages of the individual 2′, 3′ and cyclic nucleotide products was not possible for cytidine. However, the high percentage of 5′-UMP gives an indication of preferential attack at the 5′ position for a pyrimidine nucleoside. Cytidine also gave a high amount of 5′-CMP.

TABLE III
Phosphorylation of nucleosides at 125°

	Nucleoside + KH_2PO_4 $\xrightarrow[125°]{\text{Formamide}}$				Products	
	0.05 *M*	0.10 *M*	0.5 day			
Nucleoside	Recovered nucleoside	5′	2′	3′	2′, 3′ cyclic	Diphosphates
Uridine	12%	43%[a]	–	45%[b]	–	NA
Cytidine	10%	45%[a]	–	45%[b]	–	NA[a]
Guanosine	26%	22%	3%	8%	23%	11%

[a] These totals include possibly as much as 5% of diphosphates which were coeluted with the 5′ nucleotides.
[b] These are combined totals of 2′, 3′ and 2′, 3′ cyclic products. All analyses were by HPLC.
Formamide

Phosphorylation of thymidine at 100° afforded a mixture of 3′-TMP and 5′-TMP along with 3′,5′-TDP. At 46°, a mixture of the 3′ and 5′ isomers was also observed. Thymidine showed a preference for phosphorylation at the 5′ position. The overall yields of phosphorylated products exceeded those observed for 2′-deoxyadenosine. Results are shown in Table IV.

2′-Deoxyadenosine gave more phosphorylation at the 3′ position in a number of experiments run at various temperatures. There were about equal amounts of 3′-dAMP and 5′-dAMP in a reaction mixture that was allowed to stand for 6 months. At 54° there was also a preference for phosphorylation at the 3′ position. Therefore, there was selectivity for phosphorylation at the 3′ position of 2′-deoxyadenosine at temperatures below 100°. 2′-Deoxyadenosine was exceptional in this respect. Results are shown in Table V.

TABLE IV
Phosphorylation of thymidine

Thymidine +	KH_2PO_4	Formamide →		5′-TMP + 3′-TMP		3′,5′-TDP +	Thymine +	Thymidine
		Time	Temp					
0.05 *M*	0.05 *M*	15 days	46°	15% total nucleotides		7%	0%	78
0.05 *M*	0.05 *M*	15 days[a]	54°	11%	9%	4%	0%	76
0.05 *M*	0.10 *M*	0.5 day[a]	100°	26%	18%	6%	trace[b]	47
0.05 *M*	0.10 *M*	0.5 day[a]	130°	29%	17%	18%	12%	24

[a] The reaction mixture was analyzed by HPLC.
[b] There was 3% unidentified material.

TABLE V
Phosphorylation of 2′-deoxyadenosine

2′-Deoxyadenosine +	KH_2PO_4	Formamide →		5′ dAMP +	3′ dAMP +	3′, 5′ dAMP +	Adenine
0.05 *M*	0.05 *M*	4 months	37°	7% total deoxynucleotides		0%	NA[a]
0.05 *M*	0.05 *M*	6 months	37°	3%	5%	0%	NA[a]
0.05 *M*	0.05 *M*	6 months[b]	37°	8%	8%	0%	21%
0.05 *M*	0.05 *M*	15 days[c]	54°	9%	15%	2%	1%
0.05 *M*	0.10 *M*	0.5 day[d]	110°	14%	14%	7%	2%

[a] Not analyzed.
[b] The reaction mixture was kept at 37° for 6 months, stored for an additional year and analyzed using HPLC to give the results shown.
[c] Analyzed by HPLC.
[d] This reaction mixture also contained some unidentified material.

7. Mechanism

Phosphorylation in formamide using KH_2PO_4 and other inorganic phosphates has been observed to occur under very mild conditions. The mechanism of reaction involves non-selective or partially selective phosphorylation of nucleosides at temperatures ranging from ambient to 37°. Nucleotide derivatives were formed reversibly at temperatures as low as 54° and possibly as low as room temperature. The reversibility in 20–53° range has not been tested. 2′,3′-cAMP has been observed in reaction mixtures held at 70° (Schoffstall, 1976). 2′,3′-cAMP was not observed in reactions kept at 20–37°.* High temperatures favored formation of cyclic nucleotide at the expense of mononucleotide. (See Tables I and III.)

$$\text{Adenosine} + KH_2PO_4 \overset{20\text{–}37^\circ}{\rightleftharpoons} 5'\text{-AMP} + 2'(3')\text{-AMP} + H_2O$$

$$5'\text{-AMP} \overset{54^\circ\text{–}150^\circ}{\rightleftharpoons} 2'(3')\text{-AMP}$$

$$2'(3')\text{-AMP} \xrightarrow{54\text{–}150^\circ} 2',3'\text{-cAMP} + H_2O.$$

Formamide served to solubilize both inorganic phosphates (Becker, 1970) and nucleosides. Formamide may serve as a catalyst for the esterification. The experimental results for hydrolysis of nucleotides and isomerization of the nucleotide isomers (Table II) are consistent with the hypothesis that formamide effects both phosphorylation and dephosphorylation.

8. Discussion

Formation of deoxynucleotides, nucleotides and certain of their derivatives under possible prebiotic conditions has been reported earlier (Schoffstall, 1976; Schoffstall and Kokko, 1978; Phillip and Seliger, 1977). Dihydrogen phosphates afforded 20–50 % phosphorylation of nucleosides at 70° after 15 days. Condensed phosphates and nucleosides in formamide gave higher yields. Some phosphorylation occurred at room temperature after several months. These results have some similarity to those done using urea (Lohrmann and Orgel, 1971). The conditions employed in formamide or urea are milder than those used to bring about nucleotide synthesis in the absence of solvent (160°) (Ponnamperuma and Mack, 1965). Amides other than formamide may also be used as phosphorylation media (Schoffstall and Kokko, 1978; and Schoffstall *et al.*, in preparation).

The effect of temperature on extent of phosphorylation and on product distribution is shown in the Tables. The AMPs are a mixture of isomers as presented in Table I. The 140° data are comparable with those obtained for the reaction of adenosine with H_3PO_4 in DMF at reflux (Ueda and Kawai, 1970; Honjo *et al.*, 1966). More of 2′ and 3′-AMPs were formed at the expence of 5′-AMP at 130–140°.

* HPLC results showed trace amounts of cyclic nucleotide were formed at 37° after 0.5 yr.

Selectivity of phosphorylation generally favors reaction at the 5′-position of nucleosides and deoxynucleosides, presumably because the 5′-position bears a primary hydroxyl group whereas the 2′ and 3′ hydroxyl groups are secondary. (See, however, Schwartz, 1969 and Saffhill, 1970). As shown in Table I, there was a preference for phosphorylation at the 5′-position of adenosine at 37°. While there was a preference, the attack was not exclusively at 5′. The ratio of attack at 5′, 2′, and 3′ was approximately 6:3:1. Specific amounts of each nucleotide derivative were determined by HPLC (Table VI). For the deoxynucleosides, thymidine showed a 3 to 2 preference

TABLE VI
HPLC Data

Adenosine + KH_2PO_4 $\xrightarrow{(1)\ 37°,\ 6.5\ \text{months}^*}$ Products

0.05 *M*	0.05 *M*
Component	Yield (%)
2′-AMP	3.4
3′-AMP	1.4
2′,3′-cAMP	0.1
ADP's	trace
5′-AMP	7.6
Adenosine	87.5
Adenine	0.0

* The reaction mixture was kept at 37° for 6.5 months, stored at room temperature for an additional year and was then analyzed by HPLC.

for the 5′ product. 2′-Deoxyadenosine gave preferential formation of the 3′ phosphate. These results differ from those of Phillip and Seliger, who reported that the products of such phosphorylations were predominantly 5′-monophosphates based upon analyses of products using snake venom followed by TLC. Our chromatographic results clearly show the presence of both 3′ and 5′ products at 37°, as well as the 3′,5′-diphosphate at higher temperature (Tables IV and V).

Phosphorylation of adenosine at high temperature also gave a distribution of mononucleotides as well as predominant conversion to 2′,3′-cAMP. Formation of cyclic nucleotide is postulated as proceeding as in the mechanism above. The 2′, 3′, and 5′ mononucleotides are present in equilibrium. At high temperature, where formation of 2′,3′-cAMP is favored the 2′ and 3′ nucleotides lose water and become cyclized. Reestablishment of the equilibrium furnishes new 2′ and 3′ nucleotide products for further conversion to cyclic product. Such interconversions of nucleotides and subsequent cyclization of the 2′ or 3′ isomers may have operated in systems reported previously by other workers (Lohrmann and Orgel, 1971; Ueda and Kawai, 1970; Honjo *et al.*, 1966; Saffhill, 1970).

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