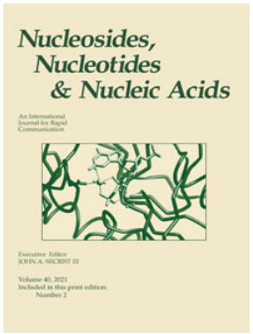

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Kinetic and NMR spectroscopic study of the chemical stability and reaction pathways of sugar nucleotides

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ABSTRACT

The alkaline cleavage of two types of sugar nucleotides has been studied by ^{1}H and ^{31}P NMR in order to obtain information on the stability and decomposition pathways in aqueous solutions under alkaline conditions. The reaction of glucose 1-UDP is straightforward, and products are easy to identify. The results obtained with ribose 5-UDP and ribose 5-phosphate reveal, in contrast, a more complex reaction system than expected, and the identification of individual intermediate species was not possible. Even though definite proof for the mechanisms previously proposed could not be obtained, all the spectroscopic evidence is consistent with them. Results also emphasise the significant effect of conditions, pH, ionic strength, and temperature, on the reactivity under chemical conditions.

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Introduction

Sugar nucleotides are biological phosphates where a monosaccharide and a nucleoside are linked by a phosphate or a pyrophosphate bridge. They can be divided into two structurally distinct categories: the phosphate is attached either to the anomeric carbon, or to any other carbon leaving the anomeric hydroxyl group unsubstituted and hence free for anomeric equilibration. The first category, glycosylic sugar nucleotides, such as glucose-1-UDP (**1** in Scheme 1) are well-known for their role as glycosyl donors in carbohydrate biosynthesis catalysed by Leloir glycosyl transferases.[1,2] Glycosylic sugar nucleotides serve also as substrates for phosphoglycosyl transferases in the biosynthesis of pyrophosphate linked glycolipids,[3,4] or of glycosyl-1-phosphate transferases in the synthesis phosphodiester-linked carbohydrates.[5] Ribose 5-ADP (**2**, in Scheme 2) is an example of a reducing sugar nucleotide, and it is found in post-translational modification

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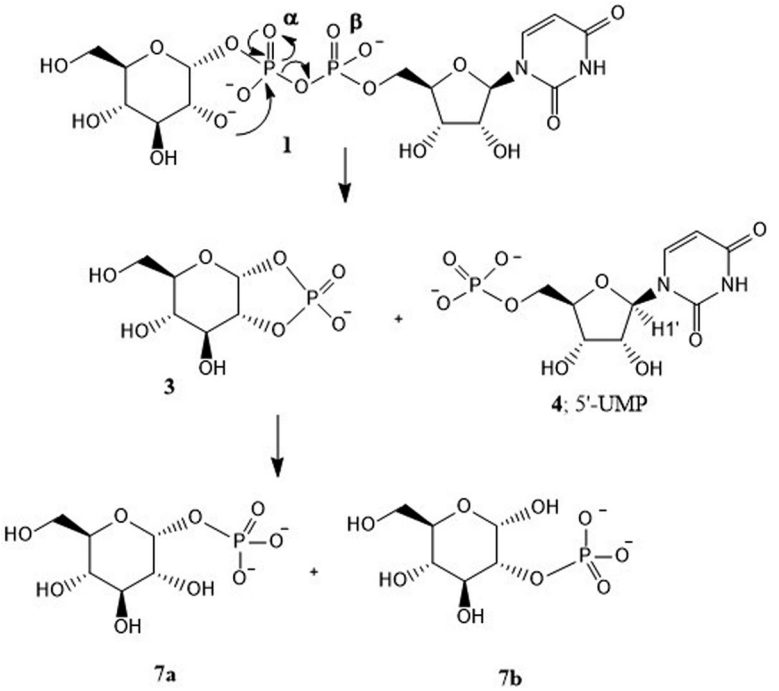
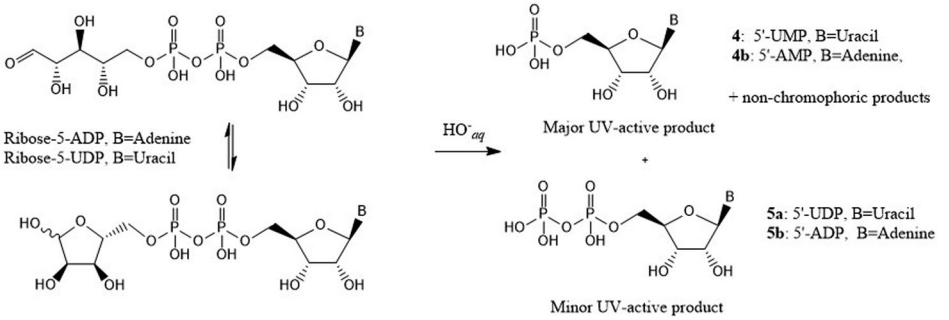


Scheme 1. Alkaline decomposition of glucose -1-UDP (**1**) and subsequent hydrolysis of glucose 1,2-cyclic monophosphate (**3**)



Scheme 2. Alkaline decomposition of ribose 5-nucleoside diphosphates **2** and **6.**

processes where a single ADP-ribosyl unit or an oligomeric structure is attached to a protein.[6,7] Unconventional naming of sugar nucleotides is applied in this work to emphasise the position of the dinucleotide moiety in the sugar ring.

The biosynthesis of carbohydrates and glycoconjugates is essential for metabolic processes as well as for communication, interactions, and defence mechanisms of cells. Sugar nucleotides hence serve in important roles in every living species, and their analogs are useful tools in research and medicine.[8–10] Furthermore, glycosylic sugar nucleotides are essential

as substrates in the chemoenzymatic synthesis of oligosaccharides,[2,11] or as labelling tools in glycobiology.[12] Efficient synthesis methods and information on the reactivity of both natural and modified structures is, therefore, important for the development of reliable diagnostic and therapeutic methods.

Enzymatic and chemical reactions of sugar nucleotides follow different reaction routes. Enzymatic reactions of glycosylic sugar nucleotides usually involve an intermolecular nucleophilic attack on the anomeric carbon, or on the phosphate.[1,2,5] In chemical reactions, an intramolecular nucleophilic attack occurs under neutral and alkaline conditions.[13–17] A neighbouring HO-group in *cis*-orientation attacks on the α-phosphate (in this context α refers to the sugar bound phosphate in the pyrophosphate moiety) (Scheme 1). The reaction of **1** results in the formation of glycopyranose 1,2-cyclic monophosphate (**3**) and uridine 5′-monophosphate (5′-UMP, **4**). Only under acidic conditions, the reaction takes place at the anomeric carbon.[14,18] The reaction is similar to that of the acid-catalysed hydrolysis of other glycosides: A nucleoside diphosphate is released as a leaving group and an oxocarbenium intermediate is formed.[18]

Biological processes of reducing sugar nucleotides utilise the carbonyl group of the acyclic form as a means to attach a monomeric or polymeric ADP-ribosyl structure to a protein.[6,7] In chemical reactions, the pyrophosphate moiety is the predominant reaction site, and an intramolecular displacement at the phosphate occurs.[15,16] As an evidence of such a reaction, a nucleoside monophosphate (**4,4b**) is released as the major product (Scheme 2). The neighbouring 4-OH group of the acyclic ribose moiety is believed to be the most probable nucleophile in the reaction.[15] However, under alkaline conditions, a nucleoside diphosphate (**5a,b**) is formed as a minor product (Scheme 2).[15,16] A reaction sequence involving a series of keto-enol equilibria, followed by an elimination of the phosphate, similarly to a reaction proposed for a reaction of ribose phosphates,[19] has been tentatively proposed as a mechanism for the formation nucleoside diphosphate.[15]

In the present article, we aim to gather further information on the chemical decomposition of sugar nucleotides. As we have previously utilised capillary zone electrophoresis (CZE) with UV-detection in the kinetic experiments,[15,16] we have been able to observe only the UV-active nucleic acid base containing products. In the present work, the alkaline cleavage of glucose 1-UDP (**1**) and ribose 5-UDP (**6**) were followed by ^{1}H and ^{31}P NMR experiments to obtain information on the processes taking place in the non-chromophoric sugar moiety. Nunez and Barker,[13] as well as Bedford et al.[14] have previously proved the formation of **3** in the presence of metal catalysts by experiments with authentic samples. More recently,

the formation of **3** in the presence of Mg^{2+} ions under neutral and slightly alkaline conditions has been shown by ^{31}P NMR experiments.[17]

As the alkaline cleavage of **1** is a well-established reaction and ^{31}P NMR experiments have been reported before,[17] it serves as a good reference point to start from. Proceeding in a systematic manner from well-known systems to more complicated reactions was hoped to shed light on the processes taking place in the non-chromophoric part of the molecules, particularly on the mechanism that produces a nucleoside diphosphate in the alkaline cleavage of reducing sugar nucleotides.

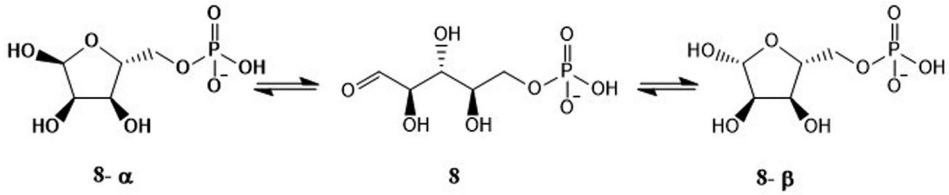
Results

NMR experiments

As discussed in the Introduction, the alkaline cleavage of **1** was chosen as the reference reaction, and therefore, we started by following its alkaline cleavage by ^{1}H and ^{31}P NMR experiments in NaOD-D_2O –solution. Consistent with previous reports, the reaction of **1** yielded two phosphate-containing products in an equal ratio, and ^{31}P NMR shifts of 3.9 and 10.8 ppm were observed (Fig. S1, Supporting material). The former was assigned to 5′-UMP (**4**) on basis of an independent experiment with an authentic sample. The latter was identified as glucose 1,2-cyclic monophosphate (**3**): According to ^{1}H-^{31}P-HMBC spectrum, the latter phosphorus is coupled to two protons, one of which is the anomeric H1 of the glucose moiety. This is fully consistent with the results reported by Hill et al.[17]

Decomposition of the 1,2-cyclic monophosphate **3** was also observed, but it was slow: When 80% of the starting material **1** had reacted, small signals were observed at 4.2 and 4.5 ppm (Fig. S1, Supporting material). They correspond to approximately 3% of total phosphate. Due to their low intensity, a clear coupling was not observed in the ^{1}H-^{31}P-HMBC spectrum, but most likely the cyclic monophosphate is opened to yield a mixture of glucose 1- and 2-monophosphates (**7a** and **7 b** in Scheme 1). The accumulation of **3** is somewhat surprising, for a rapid decomposition under alkaline conditions has been reported earlier.[20] On the other hand, accumulation of **3,** but no decomposition, in the presence of Mg^{2+} was reported by Hill et al.[17]

The most interesting and useful result obtained with glucose-1-UDP is that the reaction yielding the two monophosphate products **3** and **4** (Scheme 1) was clearly observed also in the ^{1}H spectrum (Fig. S2, Supporting material). The signals for the H6 of uracil base of **1** and **4** are observed separately, and as the reaction proceeds, a new doublet is formed at 7.9 ppm while that for the starting material at 7.7 ppm decreases. Both signals are doublets and the shape remains the same even on a prolonged reaction time. The percentage of the product calculated on basis of the H6



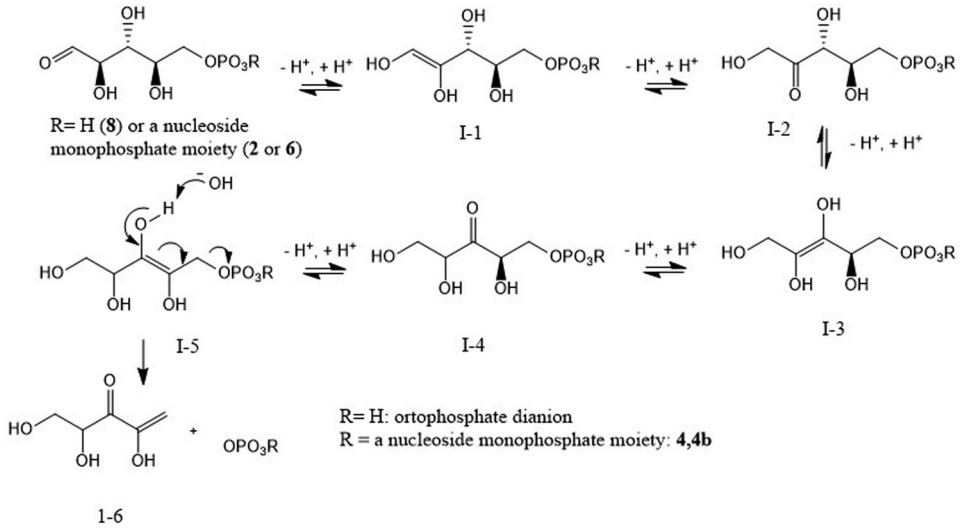
Scheme 3. Anomeric equilibrium of ribose 5-phosphate (**8**).

signals correlates well with the percentage calculated on the basis ^{31}P signals. Signals for the anomeric protons of glucose in **1** and **3** are also well resolved (Fig. S2, Supporting material).

The reaction of ribose 5-phosphate (**8** in Scheme 3) in NaOD solution both alone and in the presence of 5′-UMP was studied as another reference. As the anomeric OH is free, **8** exists as an equilibrium mixture of α- and β- anomers (**8-α** and **8-β**) with the acyclic form as the minor component (Scheme 3). The anomeric protons of the **8-α**- and **8-β** are seen separately in the ^{1}H NMR spectrum in D_2O (Fig. S3, Supporting material), and the ratio of integrals is roughly the same as that reported for ribose 5-phosphate at pH 4.5 and 6 °C.[21] When NaOD is added, only a single peak at 5.3 ppm was observed.

According to an earlier report,[22] **8** rapidly decomposes under alkaline conditions releasing free phosphate, and a mechanism that involves a series of keto-enol equilibria followed by elimination of the phosphate group, has been proposed (Scheme 4).[23] The results obtained by ^{31}P NMR are consistent with the proposed mechanism. As the reaction proceeds and the ribose 5-phosphate signal decreases, a number of small signals appear in the 4–5 ppm range (Fig. S4, Supporting material). These signals most likely refer to different phosphomonoesters, such as intermediates I-1 to 1-5 in Scheme 4. In ^{1}H-^{31}P-HMBC spectrum coupling to various sites within the ribose proton region was observed. On a longer reaction time, the signal for the inorganic phosphate at 3.3 ppm is the largest and the only well-defined one in the spectrum.

The ^{31}P signal for the free (inorganic) phosphate appears, however, to be clearly smaller than expected, and further experiments were carried out with a mixture of ribose 5-phosphate and 5′-UMP in NaOD solution, where 5′-UMP remains intact and serves, hence, as an internal standard. The results obtained with the mixture confirm the earlier observation that the signal for free phosphate is smaller than expected. Originally, the ratio of substrates is 2:1 ribose 5-phosphate to 5′-UMP. After 100 h, the signal for ribose 5-phosphate has almost completely disappeared, and the only clear product observed was free phosphate. The integral of the ^{31}P signal is approximately 60% of that of 5′-UMP. Several small signals, barely above the background noise, are also observed within the phosphomonoester region.



Scheme 4. Tautomerisation-elimination mechanism proposed for the alkaline decomposition of ribose 5-phosphate (**8**) and ribose 5-nucleoside diphosphates **2** and **6**. I1–I6 denote possible intermediates along the reaction route

To find out whether this phenomenon is of chemical or spectroscopic origin, a set of experiments with 5′-UMP and sodium phosphate as a substrate was carried out. ^{31}P NMR spectra were recorded with a longer relaxation delay time and by applying inverse gated proton decoupling. The results (Fig. S5, Supporting material) show that both increased relaxation delay time and the removal of 1H-31P NOE enhancements increase the integral for free phosphate relative to that for 5′-UMP. Hence it can be concluded that the discrepancy in the starting material and product peaks results from the different spectroscopic behaviour of organic and inorganic phosphates.

The ^{1}H NMR spectra offer little information on the alkaline decomposition of ribose 5-phosphate; ribose proton signals are quantitatively observed in the beginning, but as the reaction proceeds, they disappear into the increasingly noisy background (Fig. S6, Supporting material). In an experiment with 5′-UMP and ribose 5-phosphate mixture, the signals for 5′-UMP can be observed throughout the reaction, but signals from ribose 5-phosphate disappear as observed before (Fig. S7, Supporting material). It is also noteworthy that the H5, H6 and H1′ signals from 5′-UMP in the monophosphate mixture remain perfect doublets. This shows that any changes in these signals in the reactions of **1** or **6** reflect changes in sugar nucleotides rather than in free 5′-UMP.

Ribose-5-UDP (**6**) studied in the present work is similar to ribose 5-phosphate (**8**) in that the phosphate group is attached to the 5-position of ribose leaving the anomeric OH free for equilibration between α- and

β-anomers (Scheme 2). Similarly, to the situation with **8**, the anomeric equilibrium is observed as two separate signals for the anomeric H1-proton in approximately 1:2 ratio in neutral D_2O. After NaOD is added, only a single broad peak is observed.

In the case of **6**, the signals for ribose protons were broad and/or poorly resolved from the beginning. Only the H5 and H6 protons of uracil base, as well as the anomeric H1′ of uridine, were well defined. Similarly, to the situation with **1**, the cleavage of the diphosphate bridge of **6** is observed as the formation of two separate uracil H6-signals (Fig. S8a, Supporting material). In this case, the ratio of the two signals correlates roughly with the proportion of the unreacted sugar nucleotide, as judged on basis of ^{31}P NMR signals. Furthermore, the H6 signal for the intact sugar nucleotide was further divided on a longer reaction time, and a similar division was observed also with H5 and H1′ signals (Fig. S8b, Supporting material).

Apart from the lower field protons discussed above, the ^{1}H NMR spectra give little information on the details of the reaction. As the reaction proceeds, signals attributable to 5′-UMP start to prevail, while all other protons disappear into the noisy baseline. The ^{31}P NMR spectra are uninformative for a different reason: As the pyrophosphate signals of the starting material disappear, the only clear product signals are that of 5′-UMP and free phosphate (Fig. S8a,b, Supporting material). The two product signals are unequal in size as observed in the experiments with ribose 5-phosphate.

A few experiments were also carried out with fructose-6-UDP (**9**), but it was less extensively studied. The results obtained are rather similar to those obtained with **6** The progress of the intramolecular nucleophilic substitution at the phosphate can be seen in the development of an extra set of uracil C6 protons, and further signal sets are formed also around H5 and H1′ signals. The behaviour is even more pronounced than in the case of **6** (Fig. S9, Supporting material).

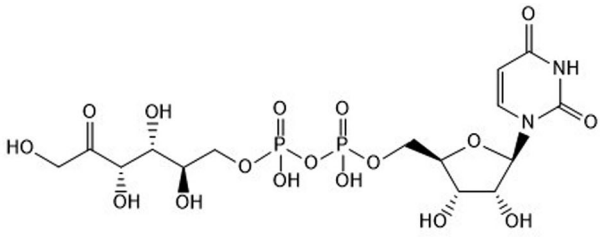
Kinetic experiments with capillary electrophoresis

Reactions of sugar nucleotides were studied in NaOH solutions and 5′,5′-UppU (**10**, $B^1 = B^2 =$ uracil, $n = 0$), that decomposes only very slowly under experimental conditions,[24] was used as an internal standard. The temperature was controlled with a water bath thermostated to the appropriate temperature. Reactions were followed for approximately two half –lives, whenever possible. Aliquots withdrawn were analysed by capillary zone electrophoresis, and pseudo first-order rate constants were calculated as described in the experimental section.

As we have reported earlier for ribose-5-ADP (**2**),[15] both a nucleoside monophosphate and diphosphate were observed as products in the alkaline

cleavage of ribose 5-UDP (**6**) and fructose-6-UDP (**9**). No other UV-active products were detected. The results collected in Fig. S10 (Supporting material) show, that the rate of the reactions is first-order in hydroxide ion concentration. It was also observed, that the proportion of UDP (**5a**) is independent of the base concentration. In the reaction of ribose-5-UDP the proportion of UDP is approximately 20%, whereas in the reaction of fructose-6-UDP the proportion is higher, approximately 30%. In an individual kinetic run, the proportion of UDP remains constant throughout the reaction.

In the kinetic studies, the reactions follow pseudo first-order kinetics under the experimental conditions and the half-lives of the decomposition of **6** and **9** in NaOH solutions at an elevated temperature range from 15 min to a few hours. Second-order rate constants of $(1.4 \pm 0.2)\cdot10^{-2}$ dm^3 mol^{-1} s^{-1} and $(3.1 \pm 0.1)\cdot10^{-3}$ dm^3 mol^{-1} s^{-1} at 50 °C were calculated as the slopes of *k(*obs*)* vs [HO^-] plots shown in Fig. S10 (Supporting material). As we have reported before,[16] glycosylic sugar nucleotides with an attacking HO-group in *cis*-position are generally more reactive: A second-order rate constant of $(1.6 \pm 0.2)\cdot10^{-2}$ dm^3 mol^{-1} s^{-1} at a lower temperature (25 °C) was determined for galactose-1-UDP (**11**).



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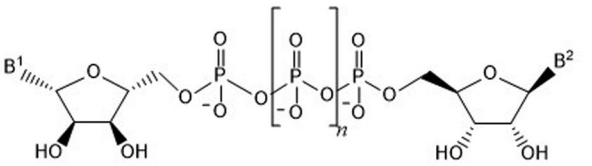

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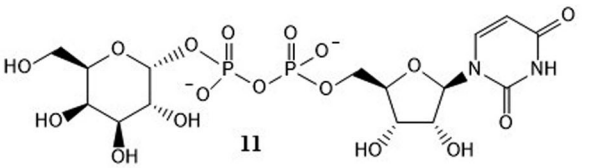

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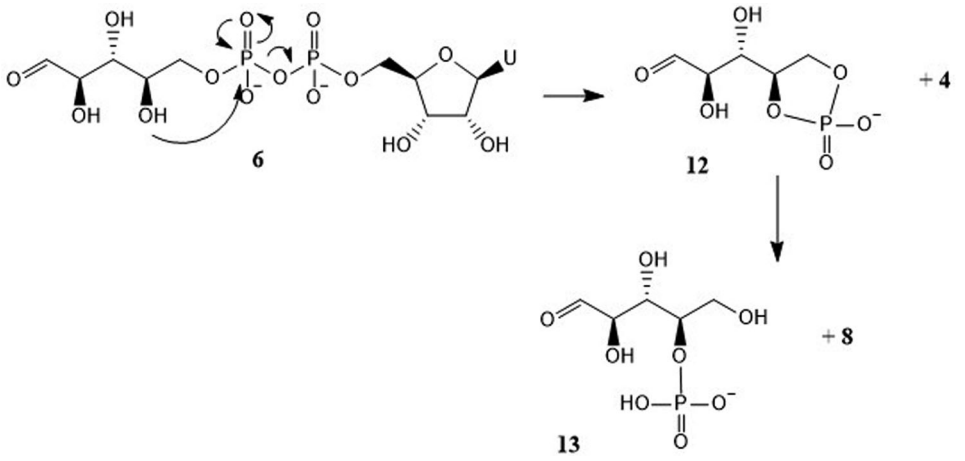

Scheme 5. Mechanism of the intramolecular transesterification of ribose 5-UDP (**6**).

The reactivities under the conditions of the NMR-experiments were much lower, which results from different conditions: temperature and deuterated solvent. The temperature dependence of the alkaline cleavage is, however, fairly modest: Rate constants determined at different temperatures for the alkaline cleavage of glucose-1-UDP (**1**) in 5 mM NaOH,[18] show that the rate increases two-fold for every 10 °C. The effect of the solvent is significant as well. Kinetic solvent isotope effect k_H/k_D of 4.94 has been reported for reactions involving a nucleophilic attack of an adjacent HO-group on the phosphate group of a phosphodiester with a good leaving group.[25] Furthermore, it was observed that in cases where the ionic strength was not adjusted, the reactions were slower than when it was kept constant at 50 mM. The reaction tended also to slow down on a longer reaction time if the ionic strength was not controlled. Finally, as the concentrations of the base and the substrates in the NMR experiments are of the same order, the reaction follows second-order kinetics, and the rate slows down as the reaction proceeds.

These results serve as a reminder: Even though the reactions under neutral and slightly alkaline conditions seem very slow,[15] the rates depend on several factors. Concentrations of the base and the substrate, temperature, and the composition of the reaction solution all affect the reactivity. Our previous results suggest that the configuration of the sugar may affect as well. Reducing sugar nucleotides with a more flexible structure tend to be less reactive, whereas within glycosylic substrates, the structure of D-galactose in **11** seems to favour the reaction more than that of D-glucose in **1**.[16]

Discussion

The results obtained with glucose-1-UDP (**1**) are fully consistent with those obtained in kinetic[14,15] and NMR studies[17] previously. In addition to the

clear results obtained by ^{31}P NMR, the results show that the progress of the reaction resulting in a formation of two monophosphates **3** and **4** can be seen also by ^{1}H NMR, as the H6 signals of **1** and **4** can be seen separately. While the results obtained with ribose-5-UDP (**6**) are less quantitative, a similar phenomenon is still observed, and a substitution at the pyrophosphate moiety is with no doubt the predominant reaction pathway. Formation of 5′-UMP was observed in both CE and NMR experiments. The efficiency of the reaction is also consistent with an intramolecular reaction; dinucleoside 5′,5′-oligophosphates **10** without an intramolecular nucleophile are significantly less reactive than **6**.[24] Furthermore, results obtained by ^{31}P NMR show that a free phosphate is released as the other phosphorous containing product, which means that a ribose phosphate must have been formed as an intermediate along the reaction route. Faint traces of such products can be seen in the spectra. Nucleotides dephosphorylate under alkaline conditions only very slowly, if at all.[26]

We have previously proposed,[15] that the nucleophile in the intramolecular displacement of **6** is the adjacent 4-OH of the open-chain ribose (Scheme 5). Such a reaction would result in a formation of a 4,5-cyclic ribose phosphate (**12**) and a release of 5′-UMP (**4**). Even though there are other potential nucleophilic OH-groups available, the 4-OH is the most likely nucleophile, since a five-membered cyclic phosphate is formed. A formation of a 4,5-cyclic phosphate has also been observed with ribitol containing carbohydrate phosphodiesters, while the formation of a 6-membered ring is less favoured.[27] However, no cyclic phosphates were detected in ^{31}P NMR experiments with **6** as a substrate.

Base catalysed hydrolysis of the cyclic phosphate **12** would yield ribose monophosphates **8** and **13** (Scheme 5) that decompose under alkaline conditions releasing free phosphate, as has been reported earlier,[22,23] and as we have seen in NMR-experiments with **8**. Decomposition of **13** has not been studied, but considering the mechanism proposed for degradation of sugar phosphates,[19,22] there is no reason to suspect that it is completely unreactive under alkaline conditions. The results of Khym et al.[22] show, however, that there are reactivity differences between ribose phosphate isomers, and according to Hauck et al.[19] ribulose-5-phosphate is clearly more reactive than **8**.

We do not, hence, see the whole picture, only pieces of it. Everything we see is consistent with the proposed mechanism, but essential pieces are missing. Onepiece we would have expected to see is the cyclic phosphate product **12** that was not observed in the ^{31}P NMR spectra. No signal was detected at 14–16 ppm where it could have been expected on the basis of the results obtained with different five-membered cyclic phosphates.[28–30] In contrast to this, accumulation corresponding 1,2-cyclic glucose

monophosphate **3** was clearly observed in the alkaline cleavage of **1**. These two results are inconsistent with each other considering that glucose 1,2-cyclic monophosphate has been reported to be rapidly cleaved under alkaline conditions,[20] whereas **12** with an acyclic sugar moiety could be expected to be less reactive. It is known that cyclic monophosphates that are attached to a cyclic structure, such as cyclic nucleoside monophosphates, are more reactive than their non-nucleosidic counterparts.[31] However, the structure of a 4,5-cyclic monophosphate formed as an intermediate in the reaction **6**, is much more complex than simple models studied by Oivanen et al.[31] As described below, a number of different forms are possible, and their reactivities may vary.

The conditions of the NMR experiments were not controlled strictly enough to allow kinetic analysis of the reactions. Consequently, rates of different reactions cannot be quantitatively compared. It seems, however, that the rates of decomposition of ribose 5-phosphate and ribose 5-UDP are of the same order. The reaction system of ribose 5-UDP is hence more complex than just two parallel and/or consecutive reactions described in Schemes 2, 4 and 5. The intramolecular transesterification may take place at various stages of the enediol equilibria. Intermediates I1–I4 depicted in Scheme 4 can all undergo intramolecular transterification. In addition, further tautomerization is possible after the 4,5-cyclic phosphate group is formed.

As different possible 4,5-cyclic phosphates are hydrolysed, a mixture of 4- and 5-phosphates may be formed. The fact that ribose monophosphates are not observed to any significant extent may result from the presence of a large number of structurally different forms. It is also possible that some of these forms are significantly more reactive than others and form a reaction pathway that results in an even more efficient release of the free phosphate than observed with ribose 5-phosphate.

The main aim of the NMR experiments was to obtain direct evidence to support the suggested mechanism for the formation of UDP in the alkaline cleavage of ribose-5-UDP, but this turned out to be impossible. Even though the formation of UDP is undeniably observed in CE-experiments, UDP-signals were not detected in ^{31}P spectra. In an independent ^{31}P NMR experiment with UDP in NaOD/D_2O signals at −10.9 and −6.5 ppm were detected (Fig. S11, Supporting material). It is possible that different conditions, a lower temperature, and deuterated solvent, may slow the reaction route in comparison to the intramolecular substitution, hence further decreasing the proportion of the elimination product.

It is, however, tempting to suggest that the further division of the H1′, H5 and H6 signals observed in ^{1}H NMR spectra (Figs. S8a,b, Supporting material) reflect the release of UDP. There is no direct proof for this suggestion, but it is clear, that another uracil base containing compound is

formed in addition to the starting material and UMP. As no extra signals were observed in experiments with 5′-UMP, no uridine was detected in CZE or NMR experiments, and as 5′-UMP is stable under basic conditions,[26] the extra signals must refer to a process that takes place in a substrate with an intact pyrophosphate bridge, and a release of 5′-UDP from ribose 5-UDP is the most potential explanation. Another piece of evidence supporting the suggestion is that the extra peaks are more pronounced in the reaction of fructose 6-UDP where the proportion of UDP observed by CE is higher than in the case of ribose 5-UDP. If this was the case, ^{31}P signals for UDP could be expected, but it can be speculated that as UDP still is a minor product, the detection is difficult.

Even if UDP was released as a result of enedieol -pathway similar to that in the decomposition of ribose-5-phosphate, it would be impossible to detect on the basis of ^{1}H NMR spectra. As was shown earlier, the intramolecular transesterification producing 5-UMP is accompanied by the formation of intermediates that rapidly decompose releasing free phosphate most probably through the enediol pathway. As a result of this, the ^{1}H NMR spectra were messy and uninformative to start with, and an additional reaction resulting in similar signals would be completely masked by the major pathway.

Experimental

Glucose 1-UDP and ribose 5-phosphate were commercial products of Sigma Aldrich, and the synthesis and full characterisation of ribose 5-UDP and fructose 6-UDP have been reported elsewhere.[16] Reactions solutions for kinetic experiments were prepared in de-ionised water. NaOH from a Titrisol ampoule was used to control the hydroxide ion concentration of the reaction solutions, and their ionic strength was adjusted to 0.1 M with NaCl.

Capillary electrophoretic experiments

Reactions were started by adding a few microlitres of the substrate and internal standard stock solutions in 1 ml of the reaction solution. The reaction temperature was controlled by a water bath thermostated to 50 °C. Ten to twelve 50 µl aliquots were withdrawn at appropriate intervals to cover two half-lives of the reaction and the reaction was quenched by adding 5–10 µl 0.25 M KH_2PO_4 solution into each aliquot. The aliquots were kept on ice or in a freezer until analysed.

Samples were analysed by capillary zone electrophoresis in a fused silica capillary of 75 µm i.d. and 57 cm effective length. The background

electrolyte was 25 mM phosphate buffer (pH 7.0). The voltage of 30 kV was applied. UV active reaction components were detected at 260 nm. Products were identified with authentic samples.

The peak areas were first normalised by dividing them by the migration time. The once normalised areas for reaction components were further divided by that for internal standard. Integrated first-order rate law was applied to determine the rate constant for the disappearance of the starting material.

NMR experiments

NMR spectra were recorded on a Bruker Avance 500 instrument (1H: 500.13 MHz) equipped with Bruker's broadband inverse (BBI) probe. ^{1}H NMR spectra were measured using 30° flip angle, 4.1 s acquisition time and 1.0 s relaxation delay. For ^{31}P NMR spectra, acquisition time of 1.6 s and relaxation delay of 2 s were applied in normal kinetic experiments. In studies for the reasons of the unexpected behaviour of inorganic phosphate the ^{31}P NMR spectra were recorded with 30° flip angle, 0.40 s acquisition time and either 2.0 or 20.0 s relaxation delay with regular or inverse-gated proton decoupling using the waltz16 sequence. Reactions were studied in 7.4 mM NaOD solution in D_2O, and the concentration of the substrates were 1.5 mM. The measurements were carried out at 298 K, and the samples were stored at room temperature between the analyses. Chemical shifts are reported on the δ scale (ppm) relative to TSP (trimethylsilylpropanoic acid) or 90% phosphoric acid.

Conclusions

The results obtained with glucose 1-UDP and with ribose 5-phosphate are fully consistent with the mechanisms proposed, and studies on those reactions yielded results that serve as good references to evaluate the results obtained with ribose 5-UDP. The reaction of ribose-5-UDP is a very complex system, and both ^{1}H and ^{31}P spectra were fairly uninformative. The fact that the cleavage of the pyrophosphate bridge can be seen as separate H6 signals in ^{1}H NMR is very helpful in the analysis of these results.

Even though we found no definite proof for the mechanisms we have previously proposed[15] for the reactions of ribose 5-UDP under alkaline conditions, we found nothing that argues against them, either. It is, however, evident that the ribose part of the starting material **6** and various intermediates, such as I1-I6 in Scheme 4, exist in different forms, and their reactivities may be different. Therefore, it is impossible to determine the

exact details of the reaction mechanisms on the basis of the current data, or even at all.

The large difference between the reactivity of observed in kinetic and NMR experiments underlines the importance of various factors that determine the reactivity of sugar nucleotides, and need to be taken into account when a reactivity under given conditions is estimated.

Disclosure statement

The authors declare that there are no conflicts of interest

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References

[1] Lairson, L. L.; Henrissat, B.; Davies, G. J.; Withers, S. G. Glycosyltransferases: Structures, Functions, and Mechanisms. *Annu. Rev. Biochem.* **2008**, *77*, 521–555. DOI: 10.1146/annurev.biochem.76.061005.092322.

[2] Nidetzky, B.; Gutman, A.; Zhong, G. Leloir Glycosyltransferases as Biocatalysts for Chemical Production. *ACS Catal.* **2018**, *8*, 6283–6300. DOI: 10.1021/acscatal.8b00710.

[3] Lukose, V.; Walvoort, M. T. C.; Imperiali, B. Bacterial Phosphoglycosyl Transferases: Initiators of Glycan Biosynthesis at the Membrane Interface. *Glycobiology* **2017**, *27*, 820–833. DOI: 10.1093/glycob/cwx064.

[4] Allen, K. A.; Imperiali, B. Structural and Mechanistic Themes in Glycoconjugate Biosynthesis at Membrane Interfaces. *Curr. Opin. Struct. Biol.* **2019**, *59*, 81–90. DOI: 10.1016/j.sbi.2019.03.013.

[5] Litschko, C.; Romano, M. R.; Pinto, V.; Claus, H.; Vogel, U.; Berti, F.; Gerardy-Schahn, R.; Fiebig, T. The Capsule Polymerase CslB of *Neisseria meningitidis* serogroup L catalyzes the synthesis of a complex trimeric repeating unit comprising glycosidic and phosphodiester linkages. *J. Biol. Chem.* **2015**, *290*, 24355–24366. DOI: 10.1074/jbc.M115.678094.

[6] Kim, M. Y.; Zhang, T.; Kraus, W. L. Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD + into a nuclear signal. *Genes Dev.* **2005**, *19*, 1951–1967. DOI: 10.1101/gad.1331805.

[7] Di Girolamo, M.; Fabrizio, G. Overview of the Mammalian ADP-Ribosyl-Transferases Clostridia Toxin-Like (ARTCs) Family. *Biochem. Pharmacol.* **2019**, *167*, 86–96. DOI: 10.1016/j.bcp.2019.07.004.

[8] Ko, H.; Fricks, I.; Ivanov, A. A.; Harden, T. K.; Jacobson, K. A. Structure-Activity Relationship of Uridine 5′-Diphosphoglucose Analogues as Agonists of the Human P2Y14 Receptor. *J. Med. Chem.* **2007**, *50*, 2030–2039. DOI: 10.1021/jm061222w.

[9] Merino, P.; Delso, I.; Tejero, T.; Ghirardello, M.; Juste-Navarro, V. Nucleoside Diphosphate Sugar Analogues That Target Glycosyltransferases. *Asian J. Org. Chem.* **2016**, *5*, 1413–1427. DOI: 10.1002/ajoc.201600396.

[10] Madec, A. G. E.; Schocker, N. S.; Sanchini, S.; Myratgeldiyev, G.; Das, D.; Imperiali, B. Facile Solid-Phase Synthesis and Assessment of Nucleoside Analogs as Inhibitors of Bacterial UDP-Sugar Processing Enzymes. *ACS Chem. Biol.* **2018**, *13*, 2542–2550. DOI: 10.1021/acschembio.8b00477.

[11] Mestrom, L.; Przypis, M.; Kowalczykiewicz, D.; Pollender, A.; Kumpf, A.; Marsden, S. R.; Bento, I.; Jarzebski, A. B.; Szymanska, K.; Chrusciel, A.; et al. Leloir Glycosyltransferases in Applied Biocatalysis: A Multidisciplinary Approach. Int. *J. Mol. Sci.* **2019**, *20*, DOI: 10.3390/ijms20215263.

[12] Wen, L.; Gadi, M. R.; Zheng, Y.; Gibbons, C.; Kondengaden, S. M.; Zhang, J.; Wang, P. G. Chemoenzymatic Synthesis of Unnatural Nucleotide Sugars for Enzymatic Bio-Orthogonal Labeling. *ACS Catal.* **2018**, *8*, 7659–7666. DOI: 10.1021/acscatal.8b02081.

[13] Nunez, H. A.; Barker, R. The Metal Ion Catalyzed Decomposition of Nucleoside Diphosphate Sugars. *Biochemistry* **1976**, *15*, 3843–3847. DOI: 10.1021/bi00662a030.

[14] Bedford, C. T.; Hickman, A. D.; Logan, C. J. Structure-Activity Studies of Glucose Transfer: Determination of the Spontaneous Rates of Hydrolysis of Uridine 5′-diphospho-alpha-D-glucose (UDPG) and uridine 5′-diphospho-alpha-D-glucuronic acid (UDPGA). *Bioorg. Med. Chem.* **2003**, *11*, 2339–2345. DOI: 10.1016/S0968-0896(03)00065-8.

[15] Huhta, E.; Parjanen, A.; Mikkola, S. A Kinetic Study on the Chemical Cleavage of Nucleoside Diphosphate Sugars. *Carbohydr. Res.* **2010**, *345*, 696–703. DOI: 10.1016/j.carres.2009.12.026.

[16] Dano, M.; Elmeranta, M.; Hodgson, D. R. W.; Jaakkola, J.; Korhonen, H.; Mikkola, S. Metal ion-promoted cleavage of nucleoside diphosphosugars: a model for reactions of phosphodiester bonds in carbohydrates. *J. Biol. Inorg. Chem.* **2015**, *20*, 1299–1306. DOI: 10.1007/s00775-015-1308-9.

[17] Hill, B. L.; Figueroa, C. M.; Asencion Diez, M. D.; Lunn, J. E.; Iglesias, A. A.; Ballicora, M. A. On the Stability of Nucleoside Diphosphate Glucose Metabolites: Implications for Studies of Plant Carbohydrate Metabolism. *J. Exp. Bot.* **2017**, *68*, 3331–3337. DOI: 10.1093/jxb/erx190.

[18] Mikkola, S. Hydrolysis and Isomerization of Sugar Phosphates and Carbohydrate Phosphodiesters. *Curr. Org. Chem.* **2013**, *17*, 1525–1544. DOI: 10.2174/1385272811317140008.

[19] Hauck, T.; Hubner, Y.; Bruhlman, F.; Schwab, W. Alternative Pathway for the Formation of 4,5-Dihydroxy-2,3-Pentanedione, the Proposed Precursor of 4-Hydroxy-5-Methyl-3(2*H*)-Furanone as Well as Autoinducer-2, and Its Detection as Natural Constituent of Tomato Fruit *Biochim. Biochim. Biophys. Acta.* **2003**, *1623*, 109–119. DOI: 10.1016/j.bbagen.2003.08.002.

[20] Kokesh, F. C.; Cameron, D. A.; Kakuda, Y.; Kuras, P. V. Hydrolysis of α-D-Glucospyranose 1,2-Cyclic Phosphate: The Effect of pH and Temperature on the Product Distribution, and the Position of Opening of the Phosphate Diester Ring in Formation of d-Glucose 2-Phosphate. *Carbohydr. Res.* **1978**, *62*, 289–300. DOI: 10.1016/S0008-6215(00)80876-9.

[21] Pierce, J.; Serianni, A. S.; Barker, R. Anomerisation od Furanose Sugars and Sugar Phosphates. *J. Am. Chem. Soc.* **1985**, *27*, 289–296.

[22] Khym, J. X.; Doherty, D. G.; Cohn, W. E. Ribose Phosphates: Production from Nucleotides, Ion Exchange Separation and Characterisation. *J. Am. Chem. Soc.* **1954**, *76*, 5523–5530. DOI: 10.1016/S0008-6215(00)8089.

[23] Munanairi, A.; O'Banion, S. K.; Gamble, R.; Breuer, E.; Harris, A. W.; Sandwick, R. K. The Multiple Maillard Reactions of Ribose and Deoxyribose Sugars and Sugar Phosphates. *Carbohydr. Res.* **2007**, *342*, 2575–2592. DOI: 10.1016/j.carres.2007.08.003.

[24] Mikkola, S. Hydrolytic Reactions of Diadenosine 5',5'-triphosphate. *Org. Biomol. Chem.* **2004**, *2*, 770–776. DOI: 10.1039/b313200e.

[25] Virtanen, N.; Polari, L.; Välilä, M.; Mikkola, S. Kinetic Solvent Deuterium Isotope Effect in Transesterification of RNA Models. *J. Phys. Org. Chem.* **2005**, *18*, 385–397. DOI: 10.1002/poc.883.

[26] Oivanen, M.; Lönnberg, H.; Karagiannis, K.; Francis, G. W.; Brekke, T.; Aksnes, D. W.; Tokii, T. Kinetics of Reactions of Pyrimidine Nucleoside 2′-and 3′-Monophosphates under Acidic and Neutral Conditions: Concurrent Phosphate Migration, Dephosphorylation and Deamination. *Acta Chem. Scand.* **1990**, *44*, 239–242. DOI: 10.3891/acta.chem.scand.44-0239.

[27] Zon, G.; Szu, S. C.; Egan, W.; Robbins, J. D.; Robbins, J. B. Hydrolytic Stability of Pneumococcal Group 6 (type 6A and 6B) Capsular Polysaccharides. *Infect. Immun.* **1982**, *37*, 89–103. DOI: 10.1128/IAI.37.1.89-103.1982.

[28] Kluger, R.; Thatcher, G. R. J. Exocyclic Cleavage in the Alkaline-Hydrolysis of Methyl Ethylene Phosphate. Evidence against the Significance of Stereoelectronic Acceleration in Reactions of Cyclic Phosphates. *J. Am. Chem. Soc.* **1985**, *107*, 6006–6011. DOI: 10.1021/ja00307a027.

[29] Ostrovsky, D. N.; Dyomina, G. R.; Deryabina, Y. I.; Goncharenko, A. V.; Eberl, M.; Shumaev, K. B.; Shashkov, A. S. Properties of 2-C-Methyl-D-Erythritol 2,4-Cyclopyrophosphate, an Intermediate in Nonmevalonate Isoprenoid Biosynthesis. *Appl. Biochem. Microbiol* **2003**, *39*, 497–502. DOI: 10.1023/A:1025452804046.

[30] Takeuchi, T.; Kuramochi, K.; Kobayashi, S.; Sugawara, T. Synthesis and Stereochemistry of the Antitumor Antibiotic PD 113. *Org. Lett.* **2006**, *8*, 5307–5310. DOI: 10.1021/ol062111u.

[31] Oivanen, M.; Mikhailov, S. N.; Florentiev, V. L.; Vihanto, P.; Lönnberg, H.; Led, J. J.; MIlanova, R. K.; Nakata, H.; Nasiri, A.; Tsuda, T. Interconversion and Hydrolysis of 1-[(s′S)-2′,3′-Dihydroxypropyl]Cytosine Analogs of Isomeric Dinucleoside Monophosphates, 2′,5′-CpA and 3′,5′-CpA. *Acta Chem. Scand.* **1993**, *47*, 622–625. DOI: 10.3891/acta.chem.scand.47-0622.