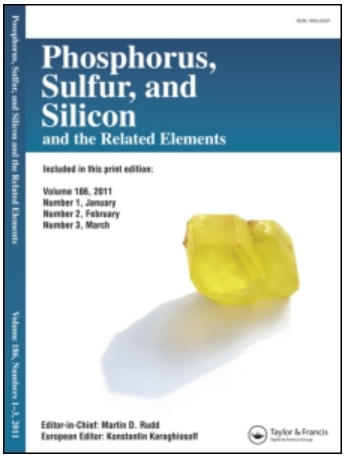

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THE INFLUENCE OF METAL ION COMPLEXATION ON ^{31}P-NMR PARAMETERS OF PHOSPHOROTHIOATES OF GUANOSINE NUCLEOTIDES

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An extension of methods already used for the preparation of adenosine phosphorothioates was used to prepare guanosine phosphorothioates in quantities sufficient for extensive ^{31}P-NMR studies. Guanosine phosphorothioates were used to exemplify the influence of Cd^{2+} and Mg^{2+} complexation on ^{31}P-NMR parameters as pK-values, chemical shifts, and P—O—P coupling constants. pK_a values decrease in general in the order $pK_a^{(\alpha\text{-S})} > pK_a^{(\beta\text{-S})} > pK_a^{(\gamma\text{-S})}$; the magnitude of the pK_a decrease on metal complexation is also in this order. The chemical shifts for the P—S resonances are changed downfield on Mg^{2+} complexation and upfield on Cd^{2+} complexation. P—O—P coupling constants are increased by about 7 Hz on sulfur substitution at one of the contributing phosphorus atoms; this difference is at least partially ineffective in the Cd^{2+} complexes.

INTRODUCTION

The interactions of nucleotides and enzymes are of fundamental importance in living organisms. Cleavage of adenosine-triphosphate (ATP) at the terminal phosphate group, for example, is a very common process for energy transfer from the nucleotide to an enzyme for the purpose of some physical or chemical action of the enzyme. Other enzymes catalyse the transfer of a phosphate group between a nucleotide and another organic molecule, thereby speeding up the establishment of equilibrium states in living systems. A common feature of these phosphotransfer reactions is their requirement of a divalent metal ion.

Unfortunately, little is understood about the chemical and physical steps of enzyme/nucleotide/metal ion interactions. Even worse, not even the seemingly simpler interaction between divalent metal ions and free nucleotides is understood at the level of groups acting as chelating agents of the metal ion. Recent developments suggest that the former question can be answered for a few enzymes by the combination of chemical procedures and magnetic resonance experiments on labelled nucleotides.[1-3] For electron paramagnetic resonance (EPR) experiments ^{17}O labelled nucleotides were used, whereas nuclear magnetic resonance (NMR) experiments made use of the changes in chemical shift induced by sulfur substitution of one of the oxygen atoms of adenosine nucleotides by metal chelation and by binding to enzymes. An important feature of these nucleotide phosphorothioates is their different complexation of Cd^{2+} and Mg^{2+} ions: Cd^{2+} is preferentially coordinated by the sulfur atom, Mg^{2+} by oxygen atoms, so that these ions yield two stereochemically different metal complexes for each one of the diastereomers of the phosphorothioates which can be distinguished by ^{31}P-NMR. This has been demonstrated for phosphorothioates of ATP and ADP by ^{31}P-NMR.[4]

The interactions of the phosphate chain of free nucleotides with metal ions, especially Mg^{2+}, has also aroused much interest. A notable NMR approach was used recently by Huang and Tsai, who use ^{17}O-NMR as method of detecting the interaction of metal ions with the phosphate chain of ATP.[5] However, neither this method nor any other could so far give a clear demonstration of the type of complex magnesium ions form with ATP. Indeed, nearly all types of conceivable complexes have been suggested [Ref. 5, and literature cited therein].

In the present paper we should like to restrict ourselves to the mere presentation of some useful ^{31}P-NMR data which originated in titration studies of phosphorothioates of guanosine nucleotides and their magnesium and cadmium complexes, therewith completing data earlier presented on guanosine nucleotides and adenosine nucleotides and their phosphorothioates.[6,4] In addition, we include simple procedures for the preparation of pure diastereomers of the phosphorothioates.

RESULTS AND DISCUSSION

The synthesis of the diastereomers of GDP(α-S) has been reported previously.[8] The preparation of GTP(α-S)(S_p) was also reported in the same publication. All of the diastereomers of GTP(α-S) and GTP(β-S) could be synthesized by simple extension of enzymatic reactions first reported for the corresponding adenosine phosphorothioates in Ref. 7. It was of particular interest to note that the nucleoside diphosphate kinase-catalysed phosphorylation of GDP(α-S) to give GTP(α-S)(S_p)

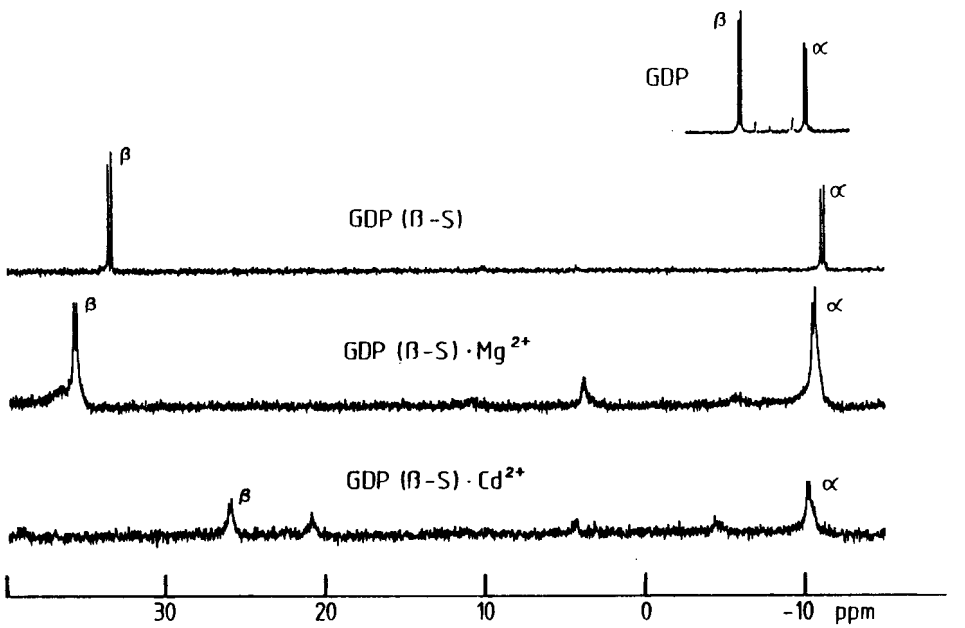

FIGURE 1: ^{31}P-NMR spectra at neutral pH of (top to bottom): (a) [GDP], 15 mM; (b) [GDP(β-S)], 7 mM; (c) [GDP(β-S)], 15 mM; [$MgCl_2$], 42 mM; (d) [GDP(β-S)], 12 mM; [$CdCl_2$], 12 mM (additional peaks are caused by products showing up during the measurement time). Buffer: [Hepes], 50 mM; [EDTA], .1 mM.

was highly stereospecific, and that hexokinase was suitable for the preparation of GTP(α-S)(R_p) from the mixed triphosphate diastereomers, although the enzyme is considered to be specific for adenosine nucleotides. Hexokinase was much less effective in removing traces of GTP(β-S)(S_p) from GTP(β-S)(R_p) than with the corresponding adenosine derivatives, but at sufficiently high enzyme levels this could be achieved. A procedure for the enzymatic synthesis of GDP(α-S) and GTP(α-S) from GMP(S) could not be found, since guanosine monophosphate kinase did not accept the thiophosphate as a substrate. Alternative procedures for the preparation of the diastereomers have recently been published.[9]

Figures 1 and 2 show as an example for the ^{31}P-NMR titration experiments we performed with all guanosine nucleotides and their phosphorothioates the spectra of GDP(β-S) and its Mg^{2+} and Cd^{2+} complexes and the titration curves for GDP, its analogs, and their metal complexes. The numerical values for the titration parameters we obtained are collected in Tables I through III.

Basically very similar effects can be seen for all compounds: Specific substitution of oxygen with sulfur causes a downfield shift of the resonance of the phosphorus atom in question by about 50 ppm, whereas the other phosphorus resonances are hardly influenced by the substitution. The titration experiments show that the titration step for the secondary protonation of the resonance of a phosphate group is reversed in sign on sulfur substitution at this group, whereas the other resonances remain practically unaffected. pK_a values shift to lower pH as the substituted sulfur atom approaches the terminal phosphate, i.e. the relation holds $pK_a^{\alpha\text{-S}} > pK_a^{\beta\text{-S}} > pK_a^{\gamma\text{-S}}$, $pK_a^{\alpha\text{-S}}$ being very close to the pK of the unmodified compound.

The PO_3—O—PO_2S coupling constants are increased as compared to the PO_3—O—PO_3 coupling constants of the parent compound by 2 Hz to 10 Hz, the largest difference being observed for the compounds with a modified terminal phosphate.

The complexation of Mg^{2+} causes a downfield shift of the resonance of the modified phosphate group in the deprotonated form of the nucleotide, the value of the shift varying from 2 ppm to 7 ppm. The largest influence is observed on the β-resonance of GTP(β-S). Even for this resonance, the change in chemical shift in the protonated form is only 2 ppm, correspondingly less for the other resonances.

The pK value drops by about .5 for the monophosphates, by about 1 for the diphosphates, and by about 2 for the triphosphates on Mg^{2+} complexation, leaving

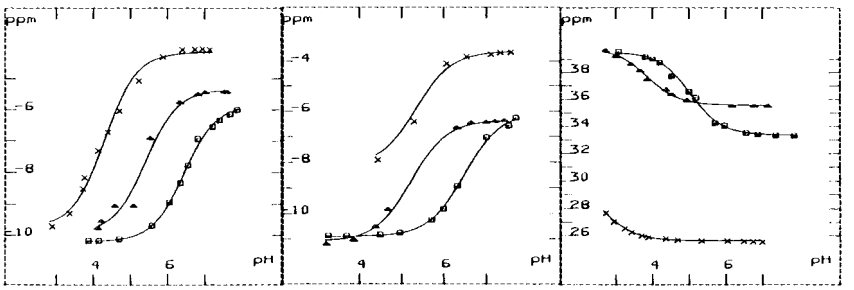

FIGURE 2: pH-titration curves of GDP, GDP(α-S), GDP(β-S), and their Mg^{2+} and Cd^{2+} complexes. Titration of the β-phosphate resonance is shown. Buffer: [Hepes], 50 mM; [EDTA], .1 mM. Left to right: (a) [GDP], 15 mM; [GDP], 10 mM; [$MgCl_2$], 20 mM; [GDP], 13 mM; [$CdCl_2$], 17 mM; (b) [GDP(α-S)], 7 mM; [GDP(α-S)], 5 mM; [$MgCl_2$], 20 mM; [GDP(α-S)], 4 mM; [$CdCl_2$], 4 mM; (c) [GDP(β-S)], 7 mM; [GDP(β-S)], 15 mM; [$MgCl_2$], 42 mM; [GDP(β-S)], 12 mM, [$CdCl_2$], 12 mM.

TABLE I

^{31}P-NMR parameters for free nucleotides and their phosphorothioates

Nucleotide	pK	δ_{AH}	δ_{A^-}	$\delta_{AH}-\delta_{A^-}$	J_{AH}	J_{A^-}	$J_{AH}-J_{A^-}$
GMP	6.3	.85	4.40	−3.55			
GMP(S)	5.0	48.31	40.52	7.79			
GDP	6.4	−10.73	−10.18	−.55	20.4	22.4	−2.0
		−10.20	−5.81	−4.39			
GDP(α-S)	6.5	43.73	41.85	1.88	26.9	30.1	−3.2
		−10.90	−6.01	−4.89			
GDP(β-S)	5.0	−11.64	−11.31	−.33	28.0	31.8	−3.8
		39.55	33.35	6.20			
GTP	6.7	−10.84	−10.56	−.28	19.4	19.4	.0
		−22.58	−21.28	−1.30	19.6	19.6	.0
		−10.24	−5.57	−4.67			
GTP(α-S)	6.6	44.25	43.66	.59	27.0	27.0	.0
		−23.39	−22.40	−.99	19.6	21.2	−1.6
		−10.20	−5.67	−4.53			
GTP(β-S)	6.4	−11.56	−11.31	−.25	26.7	26.7	.0
		30.42	29.50	.92	27.1	27.1	.0
		−11.06	−5.95	−5.21			
GTP(γ-S)	5.5	−10.75	−10.54	−.21	20.0	20.0	.0
		−23.43	−22.69	−.74	28.0	29.7	−1.7
		39.90	34.00	−5.90			

TABLE II

^{31}P-NMR parameters for Mg^{2+} complexes of nucleotides and their phosphorothioates

Nucleotide Mg^{2+}	pK	δ_{AH}	δ_{A^-}	$\delta_{AH}-\delta_{A^-}$	J_{AH}
GMP	6.0	.88	4.28	−3.40	
GMP(S)	4.5	51.38	43.84	7.54	
GDP	5.3	−10.67	−9.49	−1.18	20
		−9.92	−5.32	−4.60	
GDP(α-S)	5.3	43.80	44.80	−1.00	27
		−11.09	−6.31	−4.78	
GDP(β-S)	3.9	−11.76	−10.86	−.90	27
		39.87	35.56	4.31	
GTP	4.6	−10.82	−10.37	−.45	15
		−21.70	−19.02	−2.68	15
		−10.09	−5.37	−4.72	
GTP(α-S)	4.9	45.01	45.77	−.76	27
		−22.99	−20.42	−2.57	17
		−10.09	−5.23	−4.86	
GTP(β-S)	4.8	−11.61	−11.03	−.58	27
		32.49	36.97	−4.48	27
		−10.83	−5.65	−5.18	
GTP(γ-S)	3.5	−10.75	−10.20	−.55	16
		−23.25	−20.08	−3.17	25
		40.74	37.26	3.48	

TABLE III

^{31}P-NMR parameters of Cd^{2+} complexes of nucleotides and their phosphorothioates

Nucleotide Cd^{2+}	pK	δ_{AH}	δ_{A^-}	δ_{AH}–δ_{A^-}	J_{AH}
GDP	4.3	−10.44	−8.63	−1.81	18
		−9.72	−4.11	−5.61	
GDP(α-S)	4.3	40.66	37.32	3.34	24
		−8.17	−3.58	−4.59	
GDP(β-S)	2.7	−10.26	−9.96	−.30	24
		29.99	25.59	−4.40	
GTP	4.8	−10.25	−9.85	−.40	17
		−20.92	−18.98	−1.94	16
		−8.54	−3.91	−4.63	
GTP(α-S)	5.0	42.47	41.48	.99	24
		−21.33	−19.46	−1.87	24
		−8.90	−4.74	−4.16	
GTP(β-S)	3.4	−11.17	−11.81	.64	27
		25.34	26.17	−.83	27
		−10.91	−4.94	−5.97	
GTP(γ-S)	< 1.0		−9.7		19
			−20.7		19
			−31.9		

the above mentioned relation for the pK_a values intact. This lowering of the pK_a values may of course be explained by the partial compensation of the negative charge on the terminal phosphate by the positive metal ion.

The same argument applies to the Cd^{2+}/nucleotide complexes. Here the pK_a values drop even more pronouncedly than for the Mg^{2+} complexes, the decrease ranging from 2 units for the diphosphates to over 4.5 units for GTP(γ-S). The magnitude of the pK_a values still follows the usual pattern.

Complexation of Cd^{2+} also leads to changes in the chemical shift values, but in contrast to the Mg^{2+} complexes Cd^{2+} induces an upfield shift for the ^{31}P-resonances of the phosphate groups at which substitution took place. A change of chemical shift in the opposite direction is observed on complexation of Cd^{2+} to the unmodified compounds. This effect is explained by the preferential binding of Cd^{2+} to the sulfur atom, which is in contrast to the Mg^{2+}, which preferentially binds to the oxygen of the phosphates.

A somewhat curious phenomenon is observed if one compares the coupling constants of GTP(S) Cd^{2+} complexes: The coupling constants of the PO_3—O—PO_3 and the PO_3—O—PO_2S bonds differ for the phosphorothioates by about 7 Hz (GTP(α-S)) and 10 Hz (GTP(γ-S)). Similarly, the GTP(α-S) Mg^{2+} and GTP(γ-S)Mg^{2+} complexes show a difference of the two coupling constants of about 10 Hz. These compounds therefore yield a clearly resolved doublet of doublets for the β-P resonance. This difference between the coupling constants vanishes on Cd^{2+} complexation. Therefore, this complex yields the usual apparent triplet resonance for the β-phosphate. A comparison of the spectra is shown in Figure 3.

It should be mentioned at this point that an accurate determination of J-values was not possible for the Cd^{2+} complexes due to their increased linewidth especially in the pK_a-region.

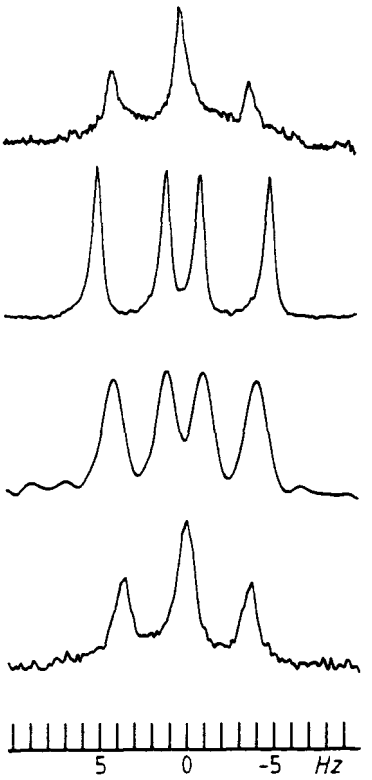

FIGURE 3: *J*-splitting of the β-phosphate resonance of GTP(γ-S) and its Mg^{2+} and Cd^{2+} complexes as compared to GTP. Buffer: [Hepes], 50 mM; [EDTA], .1 mM. Center of multiplets arbitrarily chosen as reference point of frequency scale. (Part c) resolution enhanced by Lorentzian-to-Gaussian lineshape conversion.) Top to bottom: (a) [GTP], 14 mM; (b) [GTP(γ-S)], 9 mM; (c) [GTP(γ-S)], 9 mM; [$MgCl_2$], 40 mM; (d) [GTP(γ-S)], 9 mM; [$CdCl_2$], 15 mM.

Finally it must be stressed that the set of data presented here for the guanosine nucleotides, their phosphorothioates, and the Cd^{2+} and Mg^{2+} complexes of these compounds is in generally good agreement with data so far available for the corresponding adenosine compounds.[4,10] It may therefore be inferred that data presented here, which are currently not available for the adenosine compounds, may also be used as references for the latter ones.

MATERIALS AND METHODS

All materials were commercially available. The diastereomers of GDP(α-S) were prepared according to Ref. 8 as was GDP(β-S). Guanosine monophosphate (GMP), guanosine diphosphate (GDP), and guanosine triphosphate were obtained from Pharma Waldhof.

Preparation of the diastereomers of GTP(α-S). A solution of mixed isomers of GTP(α-S) (90 mM), glucose (50 mM), $MgCl_2$ (10 mM) and dithiothreitol (10 mM) at pH 7.0, total volume 12.5 ml, was treated with hexokinase (2 mg) at room temperature for 8 h. The resulting mixture was separated on a column of QAE-Sephadex which was eluted with a linear gradient of triethylammonium bicarbonate (pH 7.5) (2 × 1C, 0.3–0.6 *M*). The last peak to be eluted was GTP(α-S)(R_p) (35 mM) containing ca. 2% of the S_p diastereomer, as judged by HPLC (see Table IV). GDP(α-S)(S_p) (37 mM) was eluted prior to GTP(α-S)(S_p)

TABLE IV

HPLC of guanosine phosphorothioates using a C18 5 reversed phase column (250 4.6 mm) at 23 C. Buffer was 50 mM potassium, pH 6.0, ( ) at a flow rate of 2.0 ml/min

Substance	Retention time (min)
GDP(α-S)(S_p)	1.68
GDP(α-S)(R_p)	2.19
GTP(α-S)(S_p)	1.51
GTP(α-S)(R_p)	2.01
GDP(β-S)	1.31
GTP(β-S)(S_p)	1.27
GTP(β-S)(R_p)	1.27

and could be phosphorylated quantitatively using pyruvate kinase (as for ADP(α-S); see Ref. 7) to GTP(α-S)(S_p) containing 3–4% of the R_p diastereomer.

GTP(α-S)(S_p) could also be obtained directly as described in Ref. 8 using nucleoside diphosphate kinase catalyzed transfer of the phosphate of ATP to GDP(α-S) (mixed diastereomers). No trace of the R_p-diastereomer could be detected in this preparation.

Preparation of the diastereomers of GTP(β-S). GTP(β-S)(S_p) was prepared as described for ATP(β-S)(S_p).[8] Traces of the R_p isomer could be removed by treatment with hexokinase and glucose, as described above for the preparation of GTP(α-S)(R_p). However, in this case much larger amounts of hexokinase are needed, since GTP(β-S)(R_p) is a very poor substrate. The product could be completely degraded with myosin subfragment 1 in the presence of Mg^{2+}, indicating negligible contamination with the R_p isomer.

GTP(β-S)(R_p) was prepared as described for ATP(β-S)(R_p).[8] Treatment of the product with myosin subfragment 1 indicated that negligible amounts of the A isomer were present.

Exclusively the R_p forms of the derivatives were used for the NMR measurements. All nucleotides and analogs were passed over a Chelex 100 column in order to remove divalent metal ions before the NMR measurements. The lyophilised substances were redissolved in 50 mM HEPES, .1 mM EDTA buffer and the solution adjusted to pH 7.

$MgCl_2$ and $CdCl_2$ were analytical grade reagents. The metal chlorides were dried in an oven at 120°C overnight, dissolved to a 500 mM stock solution in 50 mM HEPES, .1 mM EDTA buffer and the solution adjusted to pH 7. All enzymes were obtained from Boehringer, Mannheim.

All NMR measurements were carried out on a commercial Bruker HX 360 instrument working in the Fourier mode with a ^{31}P resonance frequency of 145.7 MHz. The instrument was equipped with an Aspect 2000 data system. The sample temperature was regulated to 288 $\pm$ 1 K with a Bruker SV 200 temperature control unit making use of a precooled stream of dry air. Chemical shifts are expressed with respect to 85% H_3PO_4 as external reference and given with increasing values in the direction of decreasing field. 10 mm sample tubes from Wilmad, New Jersey, were used. All spectra were obtained under complete proton decoupled conditions. The samples contained 10% to 20% deuterium oxide to provide a signal for the lock unit. The spectral width has always been chosen to result in a spectral resolution better than .5 Hertz. This allowed the use of $\pi/2$ pulses (about 27 μsec).

Much care was taken to ensure complete saturation of the nucleotides with magnesium ions in the experiments with nucleotide/magnesium ion complexes. The nucleotide level was always kept below 15 mM to avoid any mutual interaction. $MgCl_2$ was added from the stock solution until the ^{31}P-NMR spectrum did not change by further addition. Unfortunately, the nucleotide/$CdCl_2$ solutions tended to precipitate in an upredictable fashion as soon as [$CdCl_2$] > [nucleotide]. Therefore these experiments had to be restricted to equimolar concentrations of $CdCl_2$ and nucleotide or analog in these experiments, facing the danger of incomplete saturation. The situation was worst with GMP and GMP(S) solutions where a set of reliable data could not be acquired for the cadmium complexes due to early precipitation of the sample even with [$CdCl_2$] $\ll$ [GMP], [GMP(S)].

pH-titrations were performed with KOH or HCl. All pH values were measured with a Knick pH meter equipped with a custommade 3 mm Ingold electrode and uncorrected for isotope effects. pH values were determined before and after each single spectrum was taken. The spectrum was discarded if the difference of these measurements was more than .05 pH units. pK values and values for the chemical shift in the protonated (δ_{AH}) and deprotonated (δ_{A^-}) states were calculated using a least squares fit to the Henderson

Hasselbalch equation

$$\delta = \delta_{A^-} + \delta_{AH}\frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$

with δ_{A^-}, δ_{AH}, and pK as free parameters.

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