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Fluoride, beryllium and ADP combine as a ternary complex in aqueous solution as revealed by a multinuclear NMR study

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Abstract. Different kinds of nucleotide binding enzymes are sensitive to fluoroberyllate complexes (BeF_x) and fluoroaluminate complexes (AlF_y). It has been hypothesized that the effects of these fluorometals are related to the generation at a nucleotide binding site of a pseudo nucleoside triphosphate, consisting of a fluorometal moiety bound to the β phosphate group of a molecule of nucleoside diphosphate (Bigay et al. 1985; Lunardi et al. 1985). In order to establish whether ternary complexes comprising ADP, beryllium and fluoride can exist in slightly alkaline solution in the absence of enzyme, we have carried out a multinuclear (^{31}P, ^{9}Be and ^{19}F) NMR study. In preliminary experiments, pyrophosphate (PPi) was substituted for ADP and taken as a simpler analog of nucleoside diphosphate. In the absence of fluoride, three types of PPi-Be complexes were generated: two of these were bidentate chelates with either one or two pyrophosphate molecules bound per beryllium; the third one was a monodentate complex. It is probable that the same types of combination exist between the polyphosphate chain of ADP and Be. In the presence of fluoride, both ADP and PPi combined with beryllium to form ternary complexes. These complexes consisted of monofluoroberyllate(-BeF) or difluoroberyllate(-BeF_2) bound to the two phosphates of one molecule of ADP or PPi as a bidentate chelate. We failed to observe the formation of complexes between ADP and trifluoroberyllate(-BeF_3). The relevance of this study to the biological effects of fluoride and beryllium on various enzymic reactions is discussed.

Key words: Fluoroberyllates – NMR – ADP – Phosphate analogs

Abbreviations: PPi, pyrophosphate; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ADPβS, adenosine-5′-O-(2-thiodiphosphate); Ap_2A, P^1,P^2-di(adenosine-5′)pyrophosphate; F_1-ATPase, catalytic sector (soluble) of the beef heart mitochondrial ATPase complex; *Tris*,*tris*(hydroxymethyl)aminomethane

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Introduction

A number of enzymes or proteins involved in nucleotide binding or phosphate group transfer reactions have proved to be sensitive to fluoride anions. The biological effects (either enhancement or inhibition of the enzymic activity) occur in the presence of millimolar concentrations of this anion. Furthermore, the presence of micromolar concentrations of aluminum or beryllium ions is also frequently required (Sternweis and Gilman 1982). Al^{3+} and Be^{2+} are known to combine with up to 6 and 4 fluoride anions respectively, generating very stable complexes (Goldstein 1964; Mesmer and Baes 1969). In aqueous solutions, hydrated beryllium and fluoroberyllate complexes are all tetracoordinated. AlF_4^- is frequently assumed to be the active tetracoordinated species for fluoroaluminates. AlF_4^- and fluoroberyllate complexes exhibit shape, geometry and charge very similar to inorganic phosphate (Sternweis and Gilman 1982; Lange et al. 1986). These observations have led to the proposal that AlF_4^- and fluoroberyllates could bind to the enzymes as phosphate analogs in nucleotide binding sites, particularly at the level of the subsite responsible for the binding of the γ phosphate of a nucleoside triphosphate. The binding of one molecule of nucleoside diphosphate and one molecule of fluorometal in the nucleoside triphosphate binding site might facilitate a reversible interaction between the fluorometal and the β phosphate of the bound nucleoside diphosphate. This interaction would generate a complex structurally analogous to a nucleoside triphosphate (Bigay et al. 1985; Bigay et al. 1987). However, to date, the reality of such a ternary complex (combining nucleoside diphosphate, fluoride and metal) bound to the enzyme has not been directly proved. In addition, the role of AlF_4^- as an active inhibitory species has been questionned (Martin 1988; Jackson 1988). It has been claimed that nucleotides might behave as strong chelators for Al^{3+} and consequently, fluoroaluminates could no longer exist in solution whenever nucleotides are present (Jackson 1988). On the other hand, one must keep in mind that fluoride alone (Froede and Wilson 1985) or beryllium

alone (Robinson et al. 1986) might be responsible for the inhibition of different enzymes.

The question concerning the ability of nucleoside diphosphate, beryllium (or aluminum) and fluoride atoms to associate in a complex structurally analogous to a nucleoside triphosphate was addressed in a multinuclear NMR study. In this approach, we have studied the structure of the complexes generated in aqueous solution by mixing fluoride, beryllium and ADP, in the absence of enzyme. This study was focussed on the beryllium complexes owing to the better resolution of the NMR spectra of these complexes compared to that of fluoroaluminates. Furthermore, based on the assumption that pyrophosphate (PPi) behaves as an analog of the polyphosphate chain of ADP, we have carried out a parallel study in which ADP was substituted for by PPi. This study proved to be very useful in the elucidation of interactions between the polyphosphate chain of ADP and fluoroberyllates. Simpler spectra were obtained with PPi since, in this compound, the two phosphorus nuclei are equivalent. We obtained some evidence indicating that the following ternary complexes: ADP-BeF and ADP-BeF_2, or PPi-BeF and PPi-BeF_2 (in which two phosphates interact with Be) are generated spontaneously in solution. On the other hand, no ADP-BeF_3 complex was observed in the solution. Furthermore, the addition of Mg^{2+} in the medium favours the formation of ADP-Mg and free BeF_x species. These results are discussed according to a model in which the biological activity of fluorometals is related to the coordinated binding of a nucleoside diphosphate and the BeF_3 species in the nucleotide binding site of an enzyme to generate a pseudo-nucleoside triphosphate.

Experimental section

Materials

Nucleotides were from Boehringer, Ap_2A and pyrophosphate from Sigma, $BeCl_2$ from Fluka and NaF from Riedel de Haen. All samples contained 25% D_2O; the pH of all samples was carefully adjusted to 8.0 (uncorrected for D_2O) with *Tris* base. Free fluoride was measured by a potentiometric method (Dupuis et al. 1989) in the absence of masking buffer. Beryllium fluoride species distributions were calculated with a TOT simulation program (Rosset et al. 1985) using equilibrium constants compiled by Martin (1988).

NMR spectroscopy

Nuclear Magnetic Resonance was performed at 27 °C on Brüker AM 400 (or AM 300) spectrometers equipped with a process controller. D_2O was used as internal lock.

^{31}P-NMR spectra were run at 161.98 MHz on a multinuclear (VSP, 10 mm) probe. Typical acquisition parameters were: pulse width (PW) = 15 μs (ca. 90°), relaxation delay (RD) = 2.5 s, acquisition size (TD) = 8 or 16 K, spectral width (SW) = 3 000 Hz. For proton decoupling (ADP studies), Waltz sequence with ca. 0.5 W (10 H) was used. Before Fourier transformation, zero filling and Gaussian multiplication with enhancement parameters compatible with signal to noise ratio were performed. Chemical shifts are given relative to 85% orthophosphoric acid. 2D ^{31}P NOE experiments were performed by running the Brüker NOESYX.AU pulse sequence with the following parameters: RD = 2 s, mixing time from 1 to 3 s (depending on the exchange sites), number of scans (NS) = 96, SW = 945 Hz, TD = 512 W, number of increments (NE) = 256. Processing parameters: zero filling to 1 K with Gaussian apodisation on both dimensions. ^{31}P longitudinal relaxation times were measured with the inversion-recovery method. The selective saturation transfer experiment was obtaind by a selective presaturation of the Be-bound ADP signal (peak P_2) for 5 s, followed by a 90° non-selective pulse and recording the resulting spectrum. The quantification of the different species was obtained by peak integration.

^{9}Be NMR spectra were run either at 56.23 MHz, or at 42.17 MHz, on VSP probes. Typical acquisition parameters were: PW = corresponding to 90°, RD = 0.5 s, SW corresponding to 20 ppm, TD = 1 K. Prior to Fourier transform, zero filling to 16 K and Gaussian multiplication were performed. Chemical shifts are given relative to a 10 m*M* acidic aqueous solution of beryllium nitrate.

^{19}F NMR spectra were run at 376.50 MHz on a ^{19}F specific probe (5 mm). Typical acquisition parameters were: PW = 7 μs (ca. 60°), RD = 2 s, SW = 16.2 kHz, TD = 16 K. Prior to Fourier transform, zero filling to 32 K and Gaussian multiplication were performed. Chemical shifts are relative to trifluoroacetic acid. The quantification of the different species was obtained by peak integration.

Results

^{31}P *NMR*

A. Interactions of ADP with beryllium. Effect of fluoride anions. At pH 8.0, the α and β phosphorus atoms of ADP were revealed as two sharp doublets ($^2J_{P-P}$ = 22.2 Hz) resonating at −10.4 and −6.0 ppm respectively (Fig. 1 a) (in agreement with previous reports (Cohn and Hughes 1960; Cozzone and Jardetzky 1976; Tran-Dinh and Neumann 1977; Jaffe and Cohn 1978)). Additional broad signals ($\Delta\nu_{1/2}$ ca. 25 Hz), located between the two abovementioned doublets appeared when $BeCl_2$ was added to the solution (Fig. 1 b). Increasing the beryllium concentration led to an increase of the intensity of these signals and a concomitant decrease of the intensity of both the original α-P and β-P doublets. As the ratio of the intensities of α-P to β-P peaks remained unchanged, this was taken as evidence that the broad signals were related to ADP-Be complexes, whereas the two sharp doublets corresponded to the remaining free ADP. Furthermore, the absence of coalescence of the two types of signals indicated that the exchange rate between ADP-Be complexes and free ADP was slow on the NMR time scale. Total ADP-Be complexes amounted to a maximum value of about 60% of the total ADP content when the total concentrations of ADP and beryllium were both equal to

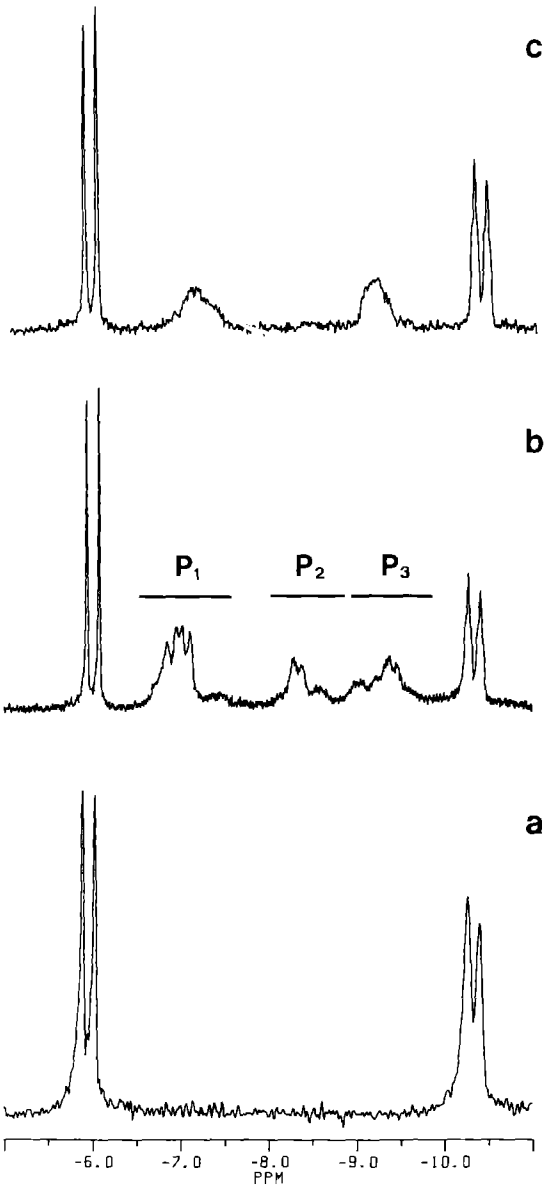


Fig. 1 a–c. ^{31}P NMR spectra of ADP in the presence of Be^{2+} or Be^{2+} and F^-. **a** Control spectra: 10 m*M* ADP; **b** 10 m*M* ADP + 10 m*M* $BeCl_2$; **c** 10 m*M* ADP + 10 m*M* $BeCl_2$ + 50 m*M* NaF. Experiments were performed in the absence of ^1H decoupling device

10 m*M*. When the concentration of beryllium was increased beyond this value, a precipitate of hydroxylated beryllium appeared, impairing total complexation of ADP and preventing the complete disappearance of the two doublets corresponding to free ADP. The linewidths of the signals assigned to ADP-Be complexes can be accounted for by the partial overlapping of different signals related to Be-bound α-P and Be-bound β-P phosphorus nuclei exhibiting $^2J_{P\text{-}P}$ and $^2J_{P\text{-}Be}$ couplings ($^2J_{P\text{-}Be}$ = ca. 3 Hz for pyrophosphate, as measured by ^9Be NMR; see below). However, the complexity of these signals greatly impeded a detailed identification of the different ADP-Be complexes. Consequently, we grouped these broad signals as three main large sets called P_1, P_2 and P_3, characterized by chemical shifts ranging between −6.5 and −7.6 ppm, −8.0 and −8.8 ppm and −8.9 and −9.8 ppm respectively (Fig. 1 b). 2D NOESY exchange NMR experiments were carried out to determine the relevance of these three sets of signals to the free α-P and β-P nuclei of ADP. As demonstrated by Fig. 2 a, the broad signal P_1 was correlated with the β phosphate, whereas the P_3 set was correlated with the α-P. The broad signal P_2 was correlated with the P_3 set but not with the free α-P, nor the free β-P. However, a one dimension saturation transfer experiment on the P_2 set unambiguously showed exchange between the broad signal P_2 and the free α-P nucleus (not shown). As deduced from the optimum mixing time value of the NOESY experiment the exchange rates were in the 1 s^{-1} range.

When fluoride was progressively added to an equimolar solution of ADP and Be^{2+}, the broad signal P_2 decreased and completely disappeared for a fluoride concentration close to 50 m*M* (Fig. 1 c and Fig. 3). A more complex situation was found for the P_1 and P_3 signals. The intensities of these signals increased upon addition of fluoride, reached a maximum for a total fluoride concentration of about 20 m*M* and then decreased and almost disappeared when the fluoride concentration was higher than 100 m*M* (Fig. 3). The disappearance of the broad signals was associated with the recovery of the signals corresponding to free ADP (Fig. 3). The total absence of ADP-Be complexes in the presence of high concentrations of fluoride can be explained by the fact that Be^{2+}

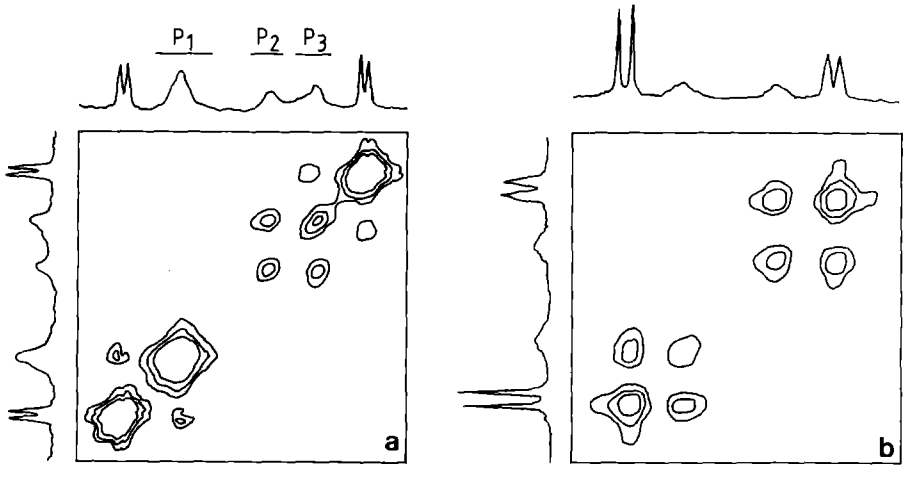


Fig. 2. Contour plot of 2D-^{31}P NOESY experiments for **a** 50 m*M* ADP, 50 m*M* $BeCl_2$; mixing time: 1 s. **b** 10 m*M* ADP, 10 m*M* $BeCl_2$, 50 m*M* NaF; mixing time: 2 s

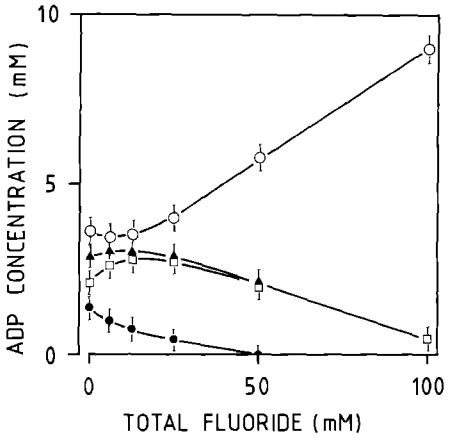


Fig. 3. Fluoride concentration dependence of ^{31}P NMR signals of free ADP and Be-bound ADP complexes. Samples were 10 m*M* ADP, 10 m*M* $BeCl_2$ and increasing concentrations of NaF. Free ADP: ○ = α-P + β-P; Be-bound ADP: ▲ = broad signals assigned to β-P (P_1); ● = broad signals assigned to α-P (P_2); □ = broad signals assigned to α-P (P_3). P_1, P_2 and P_3 signals are defined in the text and refer to Fig. 1 b

combines with fluoride anion to form highly stable fluoroberyllate complexes (Goldstein 1964; Mesmer and Baes 1969) (see below ^9Be and ^{19}F NMR). Consequently, increasing the concentration of fluoride led to a decrease of the free Be^{2+} concentration, hence, a decrease of the concentration of ADP-Be complexes. There was no evidence for the appearance of new resonance lines on the ^{31}P spectra upon addition of fluoride to the ADP-Be complexes. Nevertheless, the two broad signals remaining in the presence of 50 m*M* fluoride (Fig. 1 c) did not exhibit the same shape as the signals attributed to ADP-Be complexes in the absence of fluoride (Fig. 1 b). This probably indicates that the chemical structures of the ADP-Be complexes existing in the presence of fluoride are different from those in the absence of fluoride. The precise structure of these complexes could not be inferred from the ^{31}P NMR study; however, 2D NOESY exchange experiments carried out in the presence of fluoride unambiguously indicated that the downfield and upfield broad signals (Fig. 1 c) were correlated with the β and α phosphates of free ADP respectively (Fig. 2 b). The corresponding exchange rates deduced from the optimum mixing time value were both estimated to be approximately 0.5 s^{-1}.

B. Interactions of pyrophosphate with beryllium. Effect of fluoride anions. When increasing concentrations of $BeCl_2$ were added to a 10 m*M* PPi solution at pH 8, the sharp resonance line ($\Delta\nu_{1/2}$ ca. 3 Hz) of the pyrophosphate (−6.9 ppm at pH 8.0) (Fig. 4 a) progressively disappeared and concomitantly, new resonance lines were observed: two single lines (−6.1 ppm and −6.65 ppm, $\Delta\nu_{1/2}$ ca. 10 Hz) and two coupled doublets (−6.45 ppm and −9.05 ppm; $J = 14.3$ Hz, $\Delta\nu_{1/2}$ ca. 20 Hz) (Fig. 4 b). The analysis of the quantitative distribution of the phosphorus nuclei in these different resonance lines as a function of the total concentration of beryllium in the solution was carried out. In this study, the maximum value of the Be/PPi molar ratio was set at 1 to avoid the precipitation

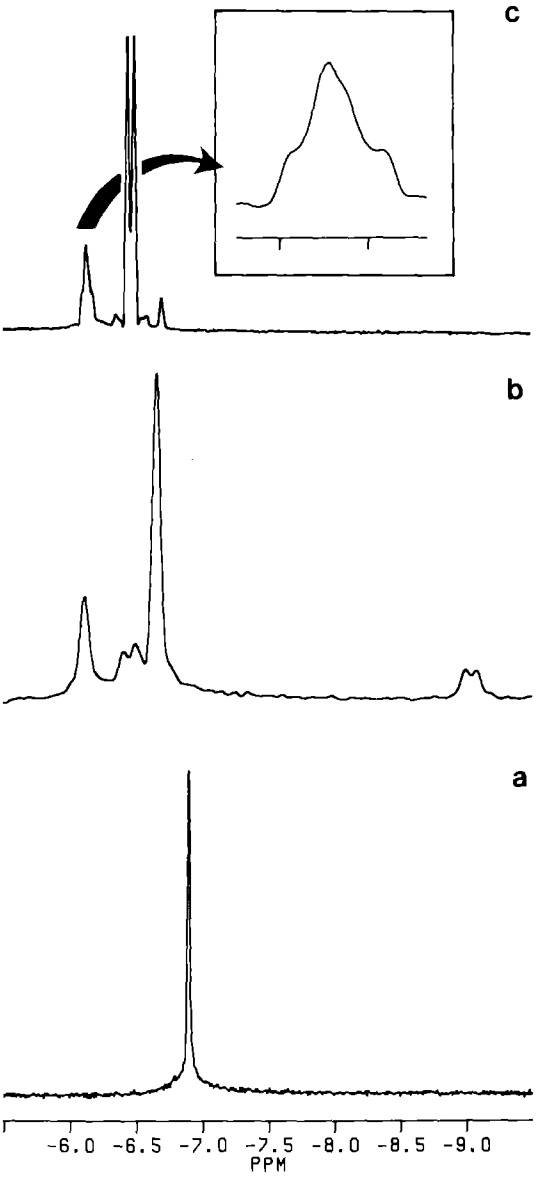


Fig. 4. ^{31}P NMR spectra of 10 m*M* pyrophosphate in the presence of Be^{2+} or Be^{2+} and F^-. **a** Control spectra: 10 m*M* PPi, **b** 10 m*M* PPi + 10 m*M* $BeCl_2$, **c** 10 m*M* PPi + 30 m*M* $BeCl_2$ + 100 m*M* NaF (this latter experimental condition was chosen to favor the observation of the triplet at −6.13 ppm). **c** Inset: enlargement of the downfield area around −6.1 ppm. Scale: one unit = 16.2 Hz

that occurred for a higher ratio. The intensity of the single line at −6.1 ppm reached a maximum at a pyrophosphate concentration of about 8 m*M* and a concentration of beryllium close to 4 m*M*, then decreased at higher Be^{2+} concentrations (Fig. 5). A ratio of two bound PPi per Be, and the absence of any observed coupling (indicating equivalent phosphorus nuclei) allowed us to assign this line to the $(PPi)_2$-Be complex as shown in Scheme 1 (**Ia**) in which the four phosphorus nuclei were equivalently bridged to Be^{2+} via an oxygen. The second line at −6.65 ppm was related to the predominant species of PPi-Be complex existing in an equi-stoichiometric solution of pyrophosphate and beryllium. This resonance line has therefore been attributed to the bidentate PPi-Be complex (**Ib**). The presence of a single resonance line

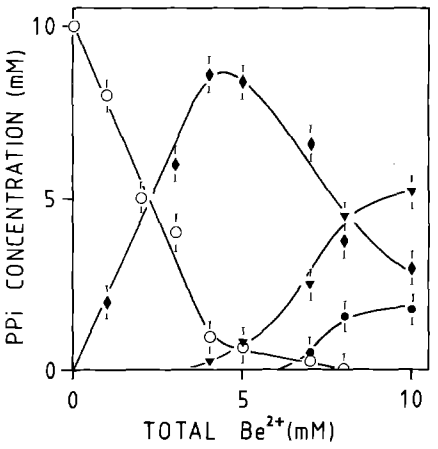


Fig. 5. Beryllium concentration dependence of the free PPi and Be-bound PPi complexes determined from the ^{31}P NMR. The relative amounts of each compound are expressed in PPi concentration vs total Be^{2+} concentration. ○ = Free PPi ($\delta = -6.9$ ppm); ♦ = $(PPi)_2$-Be ($\delta = -6.1$ ppm, formula **Ia**); ▼ = bidentate PPi-Be ($\delta = -6.65$ ppm, formula **Ib**); ● = monodentate PPi-Be ($\delta = -6.45$ ppm and -9.05 ppm, formula **Ic**)

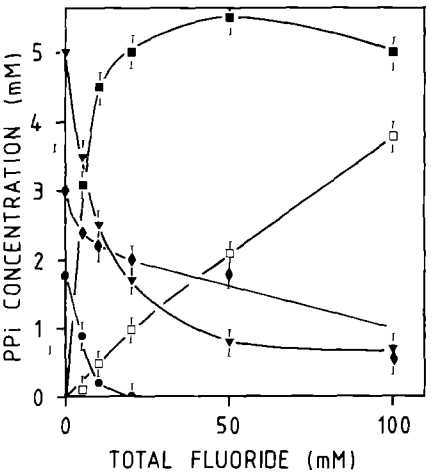
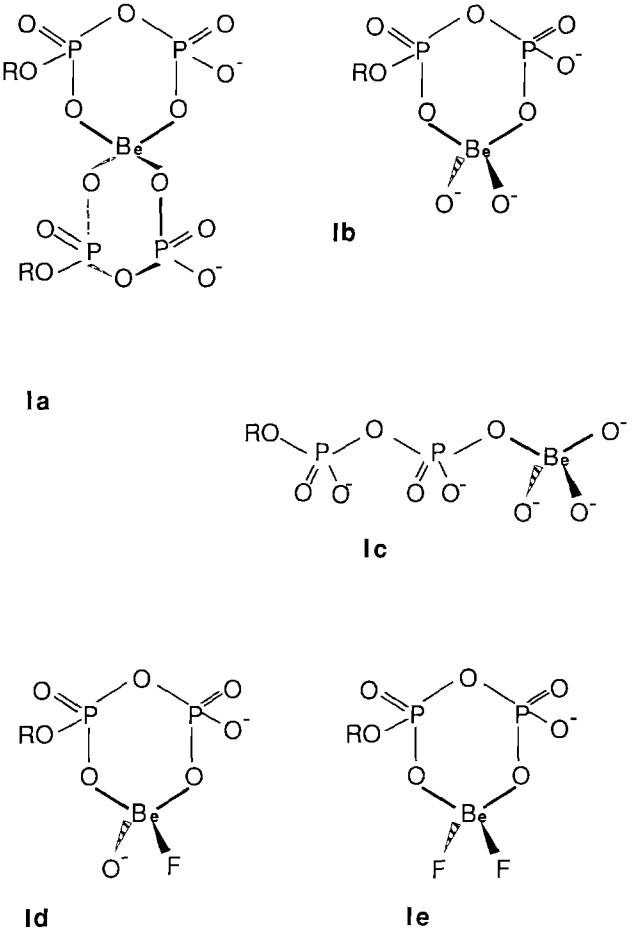


Fig. 6. Fluoride concentration dependence of the PPi-Be and PPi-fluoroberyllate complexes. The relative amounts of the complexes are expressed in PPi conconcentration vs total fluoride concentration. The concentrations of the PPi-BeF and PPi-BeF_2 complexes were ascertained by ^{19}F NMR. Samples were 10 m*M* PPi and 10 m*M* $BeCl_2$ with increasing concentrations of fluoride. ▼ = Bidentate PPi-Be; ♦ = $(PPi)_2$-Be; ● = monodentate PPi-Be; ■ = PPi-BeF; □ = PPi-BeF_2

corresponding to the phosphorus nuclei in the two above-mentioned complexes pointed to the symmetrical structure of these compounds. The similar linewidth of ca. 10 Hz measured for the resonance lines of these PPi-Be and $(PPi)_2$-Be complexes could be accounted for by a ca. 3 Hz coupling between the phosphorus nuclei and ^9Be of nuclear spin $S = 3/2$ (this is in agreement with the value of 3 Hz measured for $^2J_{P\text{-}Be}$ by ^9Be NMR, see below). Finally, the two doublets of similar intensities ($\delta = -6.45$ and -9.05 ppm) and coupling constants of 14.3 Hz were assigned to the monodentate PPi-Be complex (**Ic**) in which the two phosphorus atoms were non-equivalent, hence coupled. The coexistence of the three different pyrophosphate-beryllium complexes shows that the exchange rates between these compounds are slow on the NMR time scale.

When fluoride was progressively added to a solution containing equimolar amounts of pyrophosphate and beryllium, two types of changes were observed. Firstly, the intensities of the two single lines ($\delta = -6.1$ and -6.65 ppm) and of the two doublets ($\delta = -6.45$ and -9.05 ppm) decreased. Secondly, additional resonance lines appeared, (as illustrated by Fig. 4c): a multiplet which can be decomposed as a triplet ($\delta = -6.13$ ppm, J = ca. 7.2 Hz) and a doublet ($\delta = -6.49$ ppm, $J = 7.1$ Hz), the latter one overlapped the low field doublet assigned to the monodentate PPi-Be complex (**Ic**) whereas the former one overlapped the single line corresponding to $(PPi)_2$-Be (**Ia**) (see inset Fig. 4c). These changes indicated that the PPi-Be complexes, namely the monodentate PPi-Be complex, the bidentate complex and the $(PPi)_2$-Be complex, disappeared when fluoride was added to the medium. The monodentate PPi-Be complex even disappeared when the fluoride concentration was as low as 20 m*M* (Fig. 6). The disappearance of the PPi-Be complexes was not correlated with the release of free pyrophosphate, since its corresponding resonance line ($\delta = -6.9$ ppm) was not recov-



Scheme 1. Chemical formulae of the binary complexes of Be^{2+} with ADP or PPi (**Ia**, **Ib** and **Ic**) and of the ternary complexes of Be^{2+}, F and ADP or PPi (**Id** and **Ie**). **R** = H in pyrophosphate complexes or **R** = adenosine for the ADP complexes. Owing to the interconversion of the 6-heteroatomic rings in the complexes, these rings are simply represented as being planar (only the Be nucleus was sterically drawn)

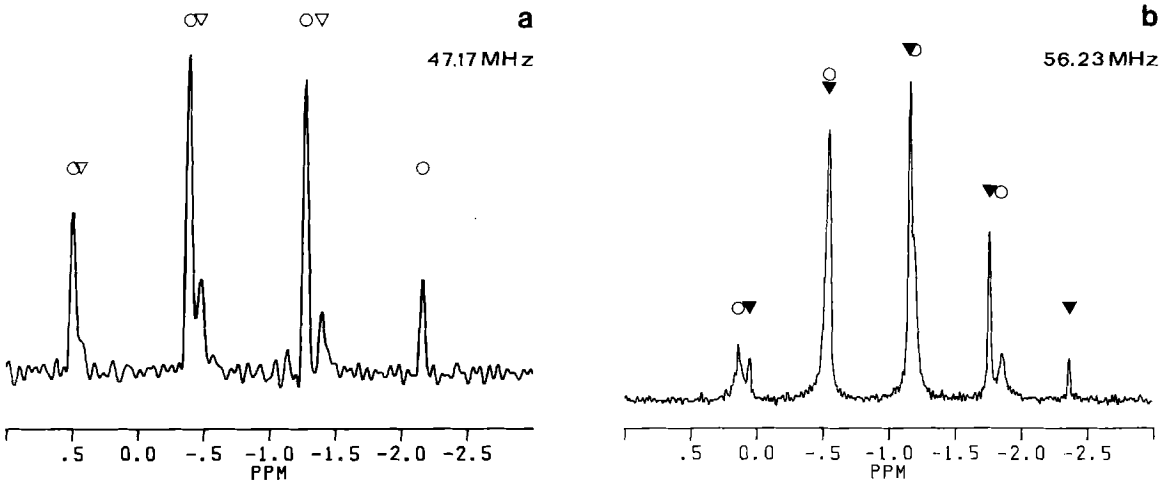


Fig. 7 a, b. ^{9}Be NMR of the BeF_2, BeF_3^- and BeF_4^{2-} complexes. **a** 10 m*M* $BeCl_2$ and 10 m*M* NaF (spectrum recorded at 47.17 MHz in order to differentiate the resonance lines of BeF_2 and BeF_3^-); **b** 10 m*M* $BeCl_2$ and 100 m*M* NaF (spectrum recorded at 56.23 MHz to differentiate BeF_3^- and BeF_4^{2-}). ▽ = BeF_2, ○ = BeF_3^- and ▼ = BeF_4^{2-}

ered (Fig. 4c). The two new signals (a doublet and a triplet) generated by the addition of fluoride indicated that they corresponded to new complexes formed from pyrophosphate, beryllium and fluoride. As stated above, the signals of these ternary complexes (a doublet and a triplet) exhibited coupling constant values of about 7 Hz. These coupling constants were assigned to $^{3}J_{P\text{-}F}$ couplings as confirmed below by ^{19}F NMR. Therefore, the structures of the ternary complexes have to meet the following criteria: *i)* to be bound to one or two fluorine atoms, as shown by the doublet or triplet structures of the signals, *ii)* to possess equivalent phosphorus nuclei, since there was no observable $^{2}J_{P\text{-}P}$ coupling. As further ascertained by ^{19}F NMR (see below), likely structures for these two ternary complexes are cyclic compounds (**Ib**) in which one or two Be-bound OH were replaced by fluorine leading to the complexes **Id** (assigned to the doublet, $\delta = -6.49$ ppm) and **Ie** (assigned to the triplet, $\delta = -6.13$ ppm). Phosphorus nuclei of those complexes are chemically equivalent because of the tetrahedral structure of the Be-complexes. The fluoride-concentration dependence of these two compounds, reported in Fig. 6, was also consistent with the stoichiometry of bound fluoride.

Table 1. Chemical shifts and coupling constants of the signals assigned to various fluoroberyllate compounds. Chemical shifts being somewhat fluoride concentration dependent, the values are measured in the presence of the following fluoride concentrations: [a] 40 m*M* NaF, [b] 200 m*M* NaF. The latter condition was chosen to reduce the overlapping between BeF_4^{2-} and PPi-BeF quadruplets. [c] $^{1}J_{F\text{-}Be}$ and $^{3}J_{F\text{-}P}$ are deduced from the ^{19}F NMR spectra. $\Delta\delta$ corresponds to the shift induced to BeF_2 signal when bound to ADP or PPi

Fluoroberyllate complexes	^{9}Be NMR		^{19}F NMR		
	δ (ppm)	$\Delta\delta$ (ppm)	δ (ppm)	$^{1}J_{F\text{-}Be}$ [c] (Hz)	$^{3}J_{F\text{-}P}$ [c] (Hz)
ADP-BeF	−0.54		−91.3 [a]		
PPi-BeF	−0.47		−91.1 [b]	38.5	7.1
BeF_2	−0.47		−94.5 [a]	38.8	
ADP-BeF_2	−0.85	−0.38	−88.0 [a]	ca. 34	
PPi-BeF_2	−0.81	−0.34	−88.2 [b]	34.3	7.2
BeF_3	−0.86		−92.5 [a]	37.1	
BeF_4	−1.16		−90.6 [b]	33.9	

^{9}*Be NMR*

Fluoroberyllate complexes. In solution, the Be^{2+} cation associates with fluoride anion in the form of stable $(BeF_x)^{2-x}$ complexes with x ranging from 1 to 4 depending on the free fluoride concentration (Goldstein 1964; Mesmer and Baes 1969; Martin 1988). The ^{9}Be NMR data concerning these fluoroberyllates are rather scarce and, to our knowledge, only the chemical shifts for the BeF_3^- and BeF_4^{2-} complexes have been reported (Kotz et al. 1967; Kovar and Morgan 1970; Wehrli 1978). We therefore carefully analyzed the ^{9}Be NMR spectra (at two different ^{9}Be frequencies) of slightly alkaline solutions (pH 8.0) containing Be^{2+} cations and different fluoride concentrations (up to 200 m*M*), and assigned to resonances corresponding to BeF_2 (triplet), BeF_3^- (quadruplet) and BeF_4^{2-} (quintuplet) (Figs. 7a and b). We did not observe the lines (doublet) corresponding to BeF^+, probably as a consequence of an unfavorable signal to noise ratio. Our study has shown that the chemical shifts of the resonances corresponding to BeF_2, BeF_3^- and BeF_4^{2-} were in a very narrow range (Table 1). From downfield to upfield, the signals of these fluoroberyllates were in the following order: BeF_2, BeF_3^-, BeF_4^{2-}. In other words, when the stoichiometry of bound fluorine per Be increased, the ^{9}Be corresponding resonances were upfield shifted, probably in accordance with a shielding effect on Be nuclei induced by the fluorine atoms. It should be noted that, in spite of the quadrupolar moment of ^{9}Be (nuclear spin = 3/2) the lines were relatively sharp (ca. 3 Hz). This fact could be explained by the axial symmetry of the BeF_x complexes (x = 2, 3 or 4). A quantitative analysis of the distribution of the complexes as a function of the fluoride concentration was impaired by the partial

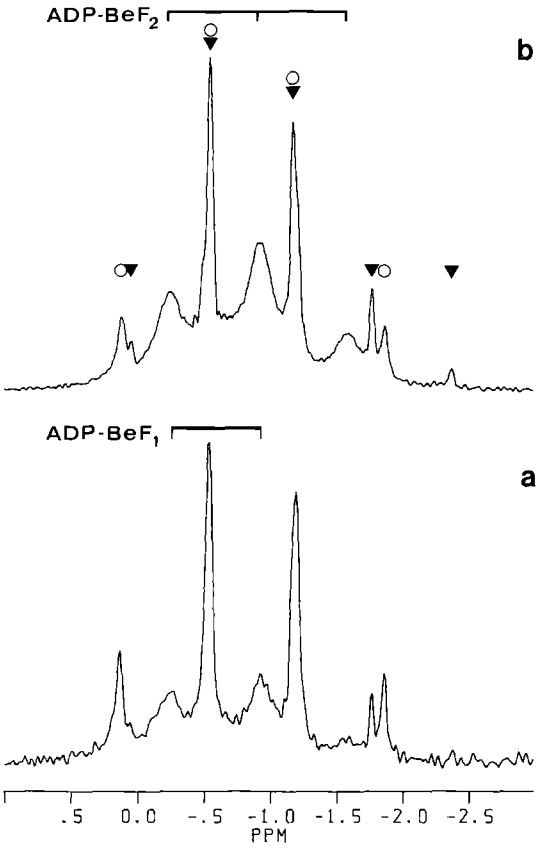


Fig. 8a, b. ^{9}Be NMR (56.23 MHz) of the ADP-BeF_x complexes. **a** 10 m*M* ADP, 10 m*M* $BeCl_2$ and 40 m*M* NaF; **b** 40 m*M* ADP, 20 m*M* $BeCl_2$ and 80 m*M* NaF. Resonance lines for ADP-BeF (doublet) and ADP-BeF_2 (triplet) are arrowed. In **b**, ADP-BeF_2 and ADP-BeF lines partially overlapped. Resonance lines for BeF_3^- (o) and BeF_4^{2-} (▾) were assigned from data of Fig. 7b

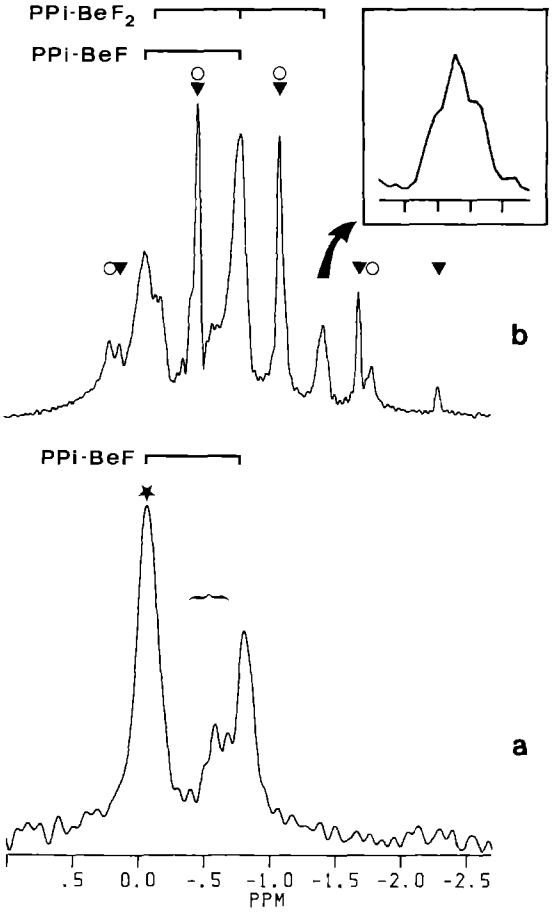


Fig. 9a, b. ^{9}Be NMR (56.23 MHz) of the PPi-BeF_x complexes. **a** 10 m*M* PPi, 10 m*M* $BeCl_2$ and 10 m*M* NaF; **b** 10 m*M* PPi, 15 m*M* $BeCl_2$ and 100 m*M* NaF. Assignments of the PPi-Be complexes (⌒) and beryllium hydrates (★) were deduced from the spectra recorded in the absence of fluoride. Resonance lines for PPi-BeF (doublet) and PPi-BeF_2 (triplet) are arrowed. In **b**, PPi-BeF_2 and PPi-BeF lines partially overlapped. Resonance lines for BeF_3^- (o) and BeF_4^{2-} (▾) were assigned in agreement with Fig. 7b. **b** Inset: enlargement of the upfield line ($\delta = -1.37$ ppm) belonging to the PPi-BeF_2 signal, showing the triplet structure due to $^{2}J_{Be\text{-}P}$ coupling (ca. 3 Hz). Scale: one unit = 5.8 Hz

overlapping of several resonance lines (Fig. 7a, b). We were able to perform such a quantitative analysis by using ^{19}F NMR (see below).

Fluoroberyllate complexes in the presence of ADP or PPi. Addition of ADP or PPi to the fluoroberyllate solutions led to the appearance of two types of signals: a doublet in the presence of low fluoride concentrations (Figs. 8a and 9a) and a triplet in the presence of high fluoride concentrations (Figs. 8b and 9b) (Table 1). No other resonance lines were observed when the concentration of fluoride was increased up to 200 m*M* either in the presence of ADP or PPi. These observations supported the conclusion that ADP as well as PPi was associated with fluoroberyllates containing either one or two fluorines (**Id** and **Ie**), depending on the fluoride concentration. The linewidths of approximately 6 Hz for the signals assigned to PPi-BeF (**Id**) and PPi-BeF_2 (**Ie**) could be due to the ^{2}J coupling between the Be nucleus and the two equivalent phosphorus nuclei. This $^{2}J_{Be\text{-}P}$ coupling could be estimated at 3 Hz (see the enlargement of the triplet structure of the upfield line from the PPi-BeF_2 signal in Fig. 9b). Similar values for $^{2}J_{P\text{-}Be}$ (from 4 to 6 Hz) have been observed for phosphate compounds by Delpuech et al. (1977). Thus, it is clear that in the case of PPi, the conclusions drawn from both ^{31}P NMR and ^{9}Be NMR studies are in perfect agreement. On the other hand, in contrast with ^{31}P NMR, ^{9}Be NMR revealed unambiguously that ternary complexes containing ADP, Be and F do exist in solution. Moreover, the parallelism between the results obtained with ADP and PPi validated a posteriori the choice of PPi as a diphosphate analog to study the interactions between F, Be and ADP. However, signals assigned to the ADP-BeF_x ternary complexes were so broad that no $^{2}J_{Be\text{-}P}$ coupling could be observed. This could be explained by the fact that in the ADP-BeF and ADP-BeF_2 complexes (**Id** and **Ie**), Be is differently coupled with the α-P and β-P of ADP giving more complicated spectra than similar complexes with pyrophosphate. Furthermore, the coexistence of diastereoisomers due to the chirality of the α-P bound to the Be nucleus and the absence of axial symmetry in these complexes contributed to the broadening of the ^{9}Be lines.

It is also noteworthy that the binding of ADP or PPi to BeF_2 to generate ADP-BeF_2 or PPi-BeF_2 (**Ie**) promoted the upfield shift of the resonance of this fluoroberyllate

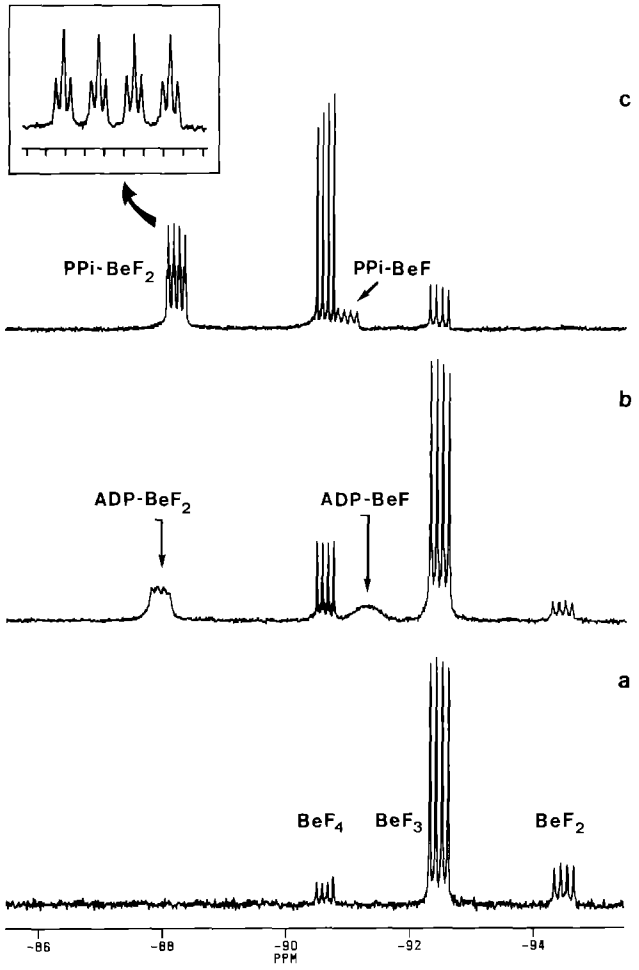


Fig. 10a–c. ^{19}F NMR of the fluoroberyllate, ADP-fluoroberyllate and PPi-fluoroberyllate complexes. **a** Signals of the BeF_2, BeF_3^- and BeF_4^{2-} complexes: 20 m*M* $BeCl_2$ and 50 m*M* NaF; **b** signals of the ADP-BeF_x complexes: 10 m*M* ADP, 10 m*M* $BeCl_2$ and 40 m*M* NaF; **c** signals of the PPi-BeF_x complexes: 10 m*M* PPi, 10 m*M* $BeCl_2$ and 200 m*M* NaF. Inset: enlargement of the PPi-BeF_2 quadruplet signal, showing the hyperfine triplet structure ($^3J_{F\text{-}P}=7.2$ Hz). Scale: one unit = 18.8 Hz

complex ($\Delta\delta = -0.38$ and -0.34 ppm with ADP and PPi respectively). This can be accounted for by an electron releasing effect of phosphate groups. A shielding effect of the Be nuclei by phosphorylated compounds has already been observed in a non-aqueous solvent (Delpuech et al. 1977).

^{19}F NMR

Fluoroberyllate complexes. When studied by ^{19}F NMR, the fluoroberyllate species are characterized by well resolved quadruplets as a consequence of the coupling with the ^9Be nucleus (S = 3/2) (Fig. 10a). Each fluoroberyllate species was unambiguously assigned on the basis of the $^1J_{Be\text{-}F}$ values measured by ^9Be NMR (Table 1). This assignment agreed with previously reported results (Gutowsky and Hoffman 1951; Feeney et al. 1968; Lincoln et al. 1977).

It is noteworthy that increasing the stoichiometry of bound fluorine in the fluoroberyllates led to a downfield

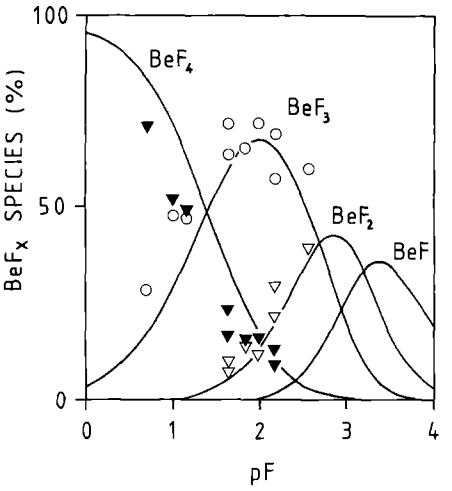


Fig. 11. Distribution of the fluoroberyllate complexes as a function of pF at pH 8.0 estimated by ^{19}F NMR. Solid lines are theoretical distributions calculated on the basis of the stability constants given by Martin (1988). ▽ = BeF_2; ○ = BeF_3^-; ▼ = BeF_4^{2-}

shift of the corresponding signals (see Table 1). This observation may be compared with the observation of the shielding of the Be nucleus induced by the increase of the bound fluorine atoms (see above). In contrast with the ^9Be NMR study, the resonances of the fluoroberyllate species were very well resolved, allowing a confident quantitative study of the distribution of these complexes vs the free fluoride concentration. The concentrations of the fluoroberyllate species and of the free fluoride were determined by measuring the intensity of each line and taking into account the total content of fluorine in the solutions. In addition the concentrations of free fluoride were also estimated by a potentiometric determination of the samples (see Experimental Section). In Fig. 11, the percentage of each fluoroberyllate species was plotted vs the colog of free fluoride concentrations (pF). The theoretical curves calculated using the equilibrium constants reported by Martin (1988) were also plotted. Taking into account an experimental error of about 10 to 20% for the quantitative measurements obtained from the NMR spectra, it appeared that our results reasonably fitted the theoretical distribution curves of the different fluoroberyllate species.

Fluoroberyllate complexes in the presence of ADP or PPi. The effect of the addition of ADP or PPi to solutions containing fluoroberyllates was investigated. We noticed the following changes in the respective spectra: *i)* addition of ADP led to the appearance of two broad signals ($\Delta\nu_{1/2}$ ca. 110 Hz and 160 Hz respectively) characterized by chemical shifts of -88 ppm and -91.3 ppm (Fig. 10b and Table 1); *ii)* addition of PPi led to the appearance of two new quadruplets located at -88.2 ppm and -91.1 ppm (Fig. 10c and Table 1).

In both cases, the new signals corresponded to the ternary complexes of ADP or PPi with fluoroberyllates (**Id** and **Ie**).

In the case of the pyrophosphate complexes, the quadruplet structures of the resonances located at $\delta = -91.1$ ppm ($J = 38.5$ Hz) and $\delta = -88.2$ ppm ($J = 34.3$ Hz)

further established that fluorine atoms were directly bound to beryllium. Furthermore, each line due to the coupling with Be was split into a triplet, with a coupling constant of 7.1 and 7.2 Hz respectively (Fig. 10c, inset and Table 1). These triplets were due to a ^3J coupling with the two equivalent phosphorus nuclei of the pyrophosphate. This coupling constant value was identical to that observed in the ^{31}P spectra recorded on the same samples (Fig. 4c). These results strengthened the conclusion previously drawn from the ^{31}P and ^9Be NMR studies indicating that pyrophosphate, beryllium and fluorine were associated in ternary bidentate complexes in which one molecule of pyrophosphate was complexed with one molecule of fluoroberyllium.

In the case of the ADP complexes, the signal at −88 ppm exhibited a poorly resolved quadruplet and no hyperfine structure could be distinguished from the resonance signal at −91.3 ppm. The broadening of these signals could be explained by: *i)* the existence of two different $^3J_{F\text{-}P}$ coupling constants ($^3J_{F\text{-}\alpha P}$ and $^3J_{F\text{-}\beta P}$) as a consequence of the chemical non-equivalence of the phosphorus nuclei of ADP, *ii)* the presence of possible diastereoisomers resulting from the chemical asymmetry of both α-P and Be nuclei. On the other hand, it is also possible that the broadening was related to an enhancement of the exchange rate between free fluoride and bound fluoride in the ADP-BeF_2 and ADP-BeF complexes in comparison to the exchange rate between free and bound fluoride in fluoroberyllate binary complexes. Indeed, a thermally induced increase of the exchange rate of the bound fluorine atom of BeF_4^{2-} species has been observed to promote the broadening of the quadruplet signal (Lincoln et al. 1977).

Finally, the ADP-BeF_2 (**Ie**) and ADP-BeF (**Id**) complexes were respectively assigned to the low field signal (−88 ppm) and to the high field signal (−91.3 ppm), and similarly, the PPi-BeF_2 (**Ie**) and PPi-BeF (**Id**) complexes were attributed to the signals at −88.2 ppm and −91.1 ppm respectively. These assignments are based on the following data: *i)* the most deshielded signals are expected to correspond to the complexes containing the higher stoichiometry of bound fluorine (consistent with the deshielding effect noticed above in the case of fluoroberyllates), *ii)* the possible 4 diastereoisomers of the ADP-BeF complex give rise to a less resolved spectrum than the 2 diastereoisomers of the ADP-BeF_2 complex, *iii)* the quantitative distribution of these signals is under the control of the free fluoride concentration. Indeed, experiments performed with PPi in which the fluoride concentration was varied indicated that the increase of the intensity of the upfield quadruplet required a NaF concentration lower than for the downfield quadruplet. The intensity of the upfield quadruplet was maximum when the fluoride concentration was close to 50 m*M*, then decreased at higher concentrations of fluoride (Fig. 6) whereas the intensity of the downfield quadruplet progressively increased. When the same study was conducted in the presence of ADP instead of pyrophosphate, a similar correlation between the fluoride concentration and the intensity of the signals corresponding to the two ternary complexes associating fluoroberyllates and ADP was obtained, i.e., the intensity of the upfield signal was higher than that of the downfield signal when the fluoride concentration was low and the reverse was true for high fluoride concentrations (not shown). However, unlike the ternary complexes containing pyrophosphate, the total concentration of the ternary complexes containing ADP gradually decreased when the concentration of fluoride was higher than 20 m*M*. This could be attributed to a lower affinity of fluoroberyllates for ADP than for PPi.

Effects of inorganic phosphate and magnesium on the fluoroberyllate species. The study of the biological effects of the fluoroberyllates on enzymic systems was performed with media containing different ligands. For example, F_1-ATPase inhibition by fluoroberyllates was observed in the presence of magnesium (Dupuis et al. 1989); with other enzymic systems, inorganic phosphate (Pi) was added to the media (Missiaen et al. 1988; Combeau and Carlier 1988; Carlier et al. 1988). Such ligands were predicted to dramatically alter the concentration of fluorometal complexes in the solution (Lange et al. 1986; Jackson 1988) which could question the biochemical relevance of these complexes. We have analyzed the effects of magnesium or Pi on the existence of the fluoroberyllates or ADP-fluoroberyllate complexes in solution.

The addition of magnesium induced the rapid decrease of the ^{19}F NMR signals assigned to the ADP-fluoroberyllate complexes (**Id** and **Ie**), but did not modify the signals of the fluoroberyllates: when 5 m*M* $MgCl_2$ was added to a solution containing 10 m*M* ADP, 10 m*M* $BeCl_2$ and 50 m*M* NaF, the amount of ternary complex decreased by about 50%. In ^{31}P NMR, both the α-P and β-P doublets corresponding to free ADP (−10.4 and −6.0 ppm respectively, $^2J_{P\text{-}P} = 22.2$ Hz) were replaced, under the same conditions, by the doublets corresponding to the α-P and β-P of ADP-Mg complexes (−9.8 and −5.6 ppm respectively, $^2J_{P\text{-}P} = 17.9$ Hz)(Cohn and Hughes 1962; Tran-Dinh and Neumann 1977; Ramirez and Marecek 1980). These results were consistent with the assumption that the addition of magnesium to ADP-fluoroberyllates gives rise to the dissociation of the ADP-BeF and ADP-BeF_2 ternary complexes (**Id** and **Ie**) and formation of ADP-Mg complexes.

On the other hand, increasing the concentration of Pi led to the disappearance of the fluoroberyllate species and the concomitant increase of the intensity of the resonance line of the free fluoride: the addition of 10 m*M* Pi to a solution containing 500 μ*M* $BeCl_2$ and 6.5 m*M* NaF, pH 8 induced the disappearance of the ^{19}F resonance lines of all the fluoroberyllate complexes. These observations suggested that Pi acts as a strong chelator of the Be^{2+} cation.

Discussion

As stated previously, the effect of fluoroberyllates or fluoroaluminates on different enzymes were assumed to be related to the locking of a nucleoside diphosphate and a fluorometal mimicking a phosphate group in a nucleotide binding site of these enzymes. However, nothing was known about the occurrence of such complexes in solu-

tion. In addition, this model was criticized by Jackson (1988) who has pointed out that, in the presence of inorganic phosphate or ADP, the fluoroaluminates do not exist in the solution and hence cannot be responsible for the biological effects. We have carried out NMR experiments to test such an assertion. Indeed, we have shown, in agreement with Jackson (1988), that inorganic phosphate behaves as a strong chelator for beryllium and that low concentrations of this compound lead to the disappearance of the fluoroberyllates. As a consequence, great care must be taken to consider that competitive binding experiments between fluorometals and Pi are demonstrative of the biochemical analogy between these two compounds. On the contrary, in the presence of ADP, not only fluorometal species are still present in the solution but moreover ADP-fluorometal complexes are generated. The structures of the complexes consisting of ADP, beryllium and fluoride in solution were carefully investigated. This investigation was greatly facilitated by a parallel study in which ADP was replaced by pyrophosphate, a symmetrical compound assumed to mimic the polyphosphate chain of ADP. The validity of such an assumption was supported in part by the fact that Be^{2+} was recognized to interact only with the polyphosphate chain and not with the purine moiety of adenine nucleotides (Bock and Ash 1980).

A survey of the literature indicates that a number of complexes consisting of pyrophosphate and divalent cations have already been described. These complexes exhibit cyclical and symmetrical structures, namely, one atom of metal is associated either with one or two molecules of pyrophosphate with all the phosphate groups linked to the metal. With the following divalent cations: Sn^{2+}, V^{2+} or Cu^{2+}, complexes associate 2 molecules of PPi and one atom of metal (Mathieu et al. 1982; Gresser et al. 1986; Laurie et al. 1986), whereas cations such as Mo^{5+} and Mo^{6+} are known to generate equi-stoichiometric chelates with pyrophosphate (So et al. 1979; Geraldes and Castro 1986). Our results indicate unambiguously that in the absence of fluoride, Be^{2+} and pyrophosphate associate in three different types of complexes; two have cyclic structures (**Ia** and **Ib**), and the third one is an asymmetrical complex (**Ic**), in which only one out of the two phosphates of pyrophosphate interacts with Be^{2+} (see Scheme 1). These 3 different types of pyrophosphate-beryllium complex do exist simultaneously in solution containing equi-stoichiometric concentrations of beryllium and pyrophosphate.

In the case of ADP, a number of reports have described complexes in which one atom of a metal is linked to the four phosphate groups of two molecules of nucleoside diphosphates (Bock 1980; Shyy et al. 1985). In the present investigation, the high complexity of the ^{31}P spectra greatly impaired the precise identification of the structures of the ADP-Be complexes. However, it seems reasonable to assume that some of the ADP-Be complexes are made up with one atom of beryllium chelated by two molecules of ADP and structurally analogous to the $(PPi)_2$-Be complex observed (**Ia**), concomitantly with some asymmetrical monodentate ADP-Be complex (in which Be is linked to only one phosphate group) which might also be present in the solution (**Ic**). In the absence of additional data, we would favor the hypothesis that in the asymmetrical monodentate ADP-Be complex, Be^{2+} is linked only to the β-P. Structurally similar complexes in which the metal atom is linked only to β-P of ADP were observed with Co^{2+} cations (Cornelius et al. 1977). In accordance with this model, the comparison between the formulae **Ib** and **Ic** reveals that the magnetic environments of the β-P nuclei of these two compounds are rather similar whereas that of the α-P nuclei are significantly different. In other words, the α-P nuclei in the compounds **Ib** or **Ic** are expected to exhibit more different chemical shifts than the β-P nuclei. Thus, the following tentative assignment is proposed: the signals of the Be-bound β-P (formula **Ib** and **Ic**) are located in the broad signal P_1 (Fig. 1 b), the signals of the α-P of the compounds **Ic** and **Ib** are located in the broad signals P_2 and P_3 respectively. This assignment is consistent with the information drawn from 2D NOESY experiments and is also fairly well supported by the quantitative distribution of the peaks P_1, P_2 and P_3 in the absence of fluoride (in Fig. 3, the intensity of P_1 is close to the intensity of P_2+P_3). On the other hand, the complexation of beryllium or fluoroberyllates by the phosphate groups of different ligands at pH 8.0 probably depends on specific physico-chemical properties of these phosphate groups, as illustrated by the fact that nucleoside di and triphosphates chelate fluoroberyllates, whereas AMP, Ap_2A and ADPβS do not (data not shown).

As revealed by the present multinuclear NMR study, when fluoride was added to solutions containing beryllium and ADP or beryllium and pyrophosphate, two ternary complexes were generated in each case. The results of the ^9Be NMR studies and the striking homologies between the ^{19}F spectra recorded in the presence of either ADP or pyrophosphate strengthen the hypothesis that the structures of the two ADP-fluoroberyllate complexes may be similar to those of the two pyrophosphate-fluoroberyllate complexes. These new complexes are bidentate chelates, i.e., one atom of beryllium linked to one or two fluorides is chelated by the two phosphates of one molecule of ADP or pyrophosphate (**Id** and **Ie**). No other complex was observed. In particular, we were unable to detect a trifluorinated monodentate complex, namely a molecule in which one BeF_3 group is linked to the β-P of ADP (or to one phosphate group of PPi). However, it has been shown that the stoichiometry for the F : Be : ADP ratios is 3 : 1 : 1 respectively for the inhibited mitochondrial F_1-ATPase as well as in the case of the actin and tubulin proteins (Dupuis et al. 1989; Combeau and Carlier 1989; Issartel et al. 1991).

It is noteworthy that in the presence of millimolar concentrations of Mg^{2+}, the ADP-BeF_x species are displaced in favor of separated ADP-Mg and fluoroberyllate complexes. This result contrasts with the prediction of Jackson (1988) in the case of the fluoroaluminate species. Thus, under the conditions used in most biochemical experiments (with similar Mg^{2+} concentrations), the ternary ADP-BeF_x species are not detectable by NMR whereas the fluoroberyllate species are actually present. Rather than behaving as a preconstituted ATP analog in the

solution, ADP and BeF_x must bind independently to the target nucleotide binding site. Such an observation is in agreement with the reports on transducin where BeF_x combines with a GDP molecule already bound to the T α subunit (Bigay et al. 1985; Bigay et al. 1987). In the case of the F_1-ATPase where both ADP and BeF_x are added in solution, the inhibitory process must mimic the first step of ATP synthesis. BeF_x behaves like a free phosphate analog, but contrary to inorganic phosphate which shows poor affinity for F_1 in the presence of ADP, BeF_x would display a very high affinity for the enzyme. This would lead to the reversal of the last step of the ATP hydrolysis and block the enzyme with ADP + BeF_x bound in the catalytic sites. As for the structure of the species bound to the enzymes, the NMR approach did not prove to be adequate. ^{19}F-NMR spectra performed in the presence of as high as 200 μM F_1-ATPase fully inhibited by ADP + BeF_x did not show any resonance signal. But after thermal denaturation of the protein, a free fluoride peak was observed corresponding approximately to the stoichiometry of 2–3 fluorines per inhibiting species determined independently by potentiometric measurements (Dupuis et al. 1989).

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