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ARTICLE *in* BIOCHEMISTRY · OCTOBER 1992

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Band-3 Mediated Uptake of Beryllofluoride Complexes by Human Erythrocytes†

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Received May 11, 1992; Revised Manuscript Received July 16, 1992

ABSTRACT: Beryllium forms several multivalent fluoride complexes in aqueous solution; the relative concentration of each is governed by the relative concentrations of the constituent ions and pH. In ^{9}Be NMR spectra the ^{9}Be (spin = $^3/_2$) and ^{19}F (spin = $^1/_2$) spin coupling gave rise to an overlapping resonance triplet, quartet, and quintet of BeF_2, BeF_3^-, and BeF_4^{2-}, respectively. The low frequency shift of the quartet (0.31 ppm) and the quintet (0.62 ppm) from the triplet correlated with an increase in the number of $^{19}F^-$ ions in each complex. ^{19}F NMR spectra of the complexes showed that the spin-coupled quartet of each complex was progressively shifted to higher frequency with an increase in the number of F^- ions in the complex. Using ^{9}Be and ^{19}F NMR, the multiple equilibrium mixture of complexes was found to shift substantially to favor the BeF_3^- and BeF_4^{2-} with a relative increase of NaF concentration. The association constants for BeF_2, BeF_3^-, and BeF_4^{2-} at 25 °C were determined directly from the peak intensities of the spectra, and by a numerical fitting procedure for multiple spectra, and were 0.51 ± 0.17 mM^{-2}, 0.26 ± 0.03 mM^{-1}, and $1.0 \times 10^{-2} \pm 0.1 \times 10^{-2}$ mM^{-1}, respectively. ^{19}F NMR spectra of human erythrocytes to which Be^{2+} and F^- were added showed separate resonances from the intracellular populations of the complexes and these were shifted to higher frequency from their extracellular counterparts. Thus, the transmembrane uptake by erythrocytes could be detected without requiring the cells to be removed from their suspending medium. Preincubation of the erythrocytes with the anion-transport inhibitors 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS), and 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) resulted in complete blockage of the uptake of the complexes. The addition of butanol, up to 100 mM, did not enhance the uptake of the complexes when their transport had been inhibited by DIDS. Therefore, we conclude that the transmembrane uptake of beryllofluoride complexes is mediated by the anion transporter, band-3.

Sternweiss and Gilman (1982) reported the activation of adenylate cyclase by trace amounts of Be^{2+} and Al^{3+} in the presence of millimolar concentrations of F^-. Since then, a number of proteins, including G-proteins, microtubules, F-actin, and other phosphotransfer enzymes have been found to be subject to either activation or inhibition by complexes of beryllium fluoride or aluminium fluoride under in vitro conditions (Bigay et al., 1985, 1987; Combeau & Carlier, 1989; Dupuis et al., 1989; Issartel et al., 1991). A number of mechanisms have been proposed to describe the binding of the complexes to enzymes (Chabre, 1990). In enzyme reactions involving ATP and GTP, it is generally believed that the complexes achieve their effects by structurally mimicking the γ-phosphate of the nucleotide and forming bridging complexes with the enzymes. Issartel et al. (1991), using ^{9}Be and ^{19}F NMR, recently reported direct evidence of the formation of beryllofluoride complexes with ADP. However, there appear to be few reports of the biological effects of beryllofluoride or aluminofluoride complexes under in vivo conditions.

The formation of ternary complexes of beryllium–fluoride–hydroxy and aluminium–fluoride–hydroxy complexes in aqueous media have been reported (Goldstein, 1964; Mesmer & Baes, 1969; Martin, 1988; Anttila et al., 1991). The fluoride ion forms ionic bridges with the beryllium or aluminium ions; this has led to the suggestion of tetrahedral and hexacoordinate structures for beryllo- and aluminofluoride complexes, respectively. In the presence of fluoride ions, Be^{2+} and Al^{3+} form a variety of multivalent complexes given by the general formulae $Be_pF_q^{2p-q}$ or $Be_pF_q(OH)_r^{2p-(q+r)}$, and $Al_pF_q^{3p-q}$ or $Al_pF_q(OH)_r^{3p-(q+r)}$, the concentrations of the individual species being dependent on the relative concentrations of the constituent ions and pH (Martin, 1988; Anttila et al., 1991; Issartel et al., 1991).

The human erythrocyte is one of the simplest mammalian cells. Its most abundant membrane protein is band-3 with 0.8–1.2 million copies per cell. This 97-kDa (Passow, 1986) protein mediates transmembrane exchange of the majority of small inorganic anions (e.g., Cl^-, HCO_3^-, and HPO_4^{2-}). To achieve the inhibition of ATPases whose active sites are located on the cytoplasmic surface of the red cell membrane, Be^{2+}, Al^{3+}, or their fluoride complexes must be transported across the membrane. By taking advantage of the noninvasive nature of multinuclear NMR, we demonstrated, in the present study, that beryllofluoride complexes were rapidly transported into the erythrocytes and that this uptake was exclusively via band-3. This finding may help explain the apparently more effective inhibition of ATPases in intact cells when both ions are present compared with when either is present alone (Chabre, 1990; Morris et al., 1992).

MATERIALS AND METHODS

Chemicals. $BeSO_4$ and NaF were purchased from BDH Chemicals (Kilsyth, Vic., Australia). Erythrocyte anion-transport inhibitors, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) and 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), were from Sigma Chemical Co., St. Louis, MO, and Pfaltz and Bauer Co., Waterbury, CT, respectively. Trifluoroacetate was obtained from Merck Chemical Co., Munchen, Germany. Tris(hydroxymethyl)aminomethane was

† This work was supported by an Australian National Health and Medical Research Council grant to P.W.K. and an Australian Research Council Postdoctoral Fellowship to M.B.M. A.S.L.X. received a Consolidated Medical Research Ph.D. Scholarship from the University of Sydney.

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0006-2960/92/0431-9263$03.00/0

purchased from Sigma. D_2O (99.75%) was from the Australian Institute of Nuclear Science and Technology, Lucas Heights, NSW, Australia. All reagents were of AR grade.

Stock solutions of $BeSO_4$ and NaF were made up freshly and stored at 4 °C for no longer than 24 h. Appropriate portions of each solution were mixed to achieve the Be^{2+} and F^- concentrations required to form the relative fractions of each complex in the mixture. Where the solutions were incorporated into erythrocyte suspensions, NaCl was used as an osmotic support. The pH of the solutions was adjusted to the physiological range, as given in the text and the figure captions. Tris-HCl buffer (20 mM Tris titrated with 10 M HCl; 126 mM NaCl; pH 7.2–7.5) was filtered three times through a Millipore filter (0.45-μm pore) to remove paramagnetic complexes before it was used for suspending the cells.

DIDS and DNDS solutions (50 mM in H_2O) were stored at 4 °C in the dark (Frohlich & Gunn, 1981). Inhibition of the anion exchange of erythrocytes by DIDS (0.5 mM) and DNDS (1.0 mM) was achieved by incubating the erythrocytes in physiological media (Hc 40–50%) at 37 °C for not less than 10 min.

Erythrocyte Suspensions. Concentrated erythrocyte suspensions were supplied by the NSW Red Cross Blood Transfusion Service and were used within 3 days of collection from healthy donors. Routinely, the red cells were resuspended in an isotonic solution of Tris-HCl-buffered saline of at least three volumes. The buffy coat and the extracellular suspending fluid were then removed by aspiration after centrifuging the cell suspensions (3×, 2300*g*, 5 min at 4 °C). The cell suspensions were then gassed with humidified CO to convert oxyhemoglobin into carbonmonoxyhemoglobin, which is a relatively stable diamagnetic form. The cells were then washed in the same buffer before being used for the studies of the uptake of the beryllofluoride complexes.

NMR Experiments. ^{19}F NMR experiments were performed on a Bruker AMX-400 wb spectrometer, with a 5-mm dedicated ^{19}F probe tuned to 376.47 MHz (Bruker Analytische Messtechnik, Karlsruhe, Germany). "Fully relaxed" spectra were acquired, with preacquisition delay of five longitudinal relaxation times (T_1) and 90° pulse width, to examine the multiple equilibrium of complexes and the uptake of the complexes by erythrocytes. Chemical shifts were referenced to 0.000 ppm using 200 mM trifluoroacetate (in D_2O) in a coaxial reference capillary. Details of data averaging and spectral line-broadening are given in the figure captions. T_1 values of the complexes were determined by using the COMP-T_1 pulse program (Levitt & Ernst, 1983).

^9Be NMR spectra were acquired on the above spectrometer, but with a 10-mm multinuclear probe tuned to 56.23 MHz. "Fully relaxed" spectra were acquired with preacquisition delay of five T_1 values and 90° pulse width. T_1 relaxation times were measured similarly to those of ^{19}F. Details of the spectral data processing are given in the figure captions. At the present, there is no commonly accepted external and internal standard for the ^9Be NMR chemical shift. The chemical shift of 0.000 ppm was therefore set at 56.23 MHz which was the transmitter frequency; at the magnetic field used, the ^1H resonance frequency of the ^1H NMR reference compound TSP was 400.135416 MHz.

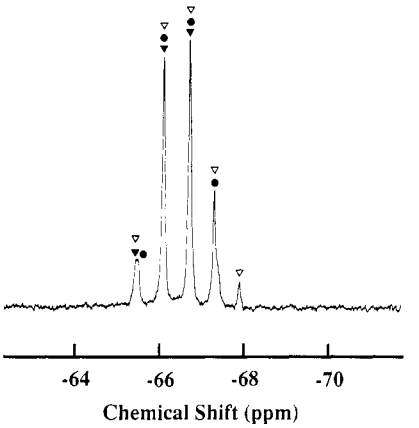

FIGURE 1: "Fully relaxed" ^9Be NMR spectrum of the beryllofluoride complexes formed by 20 mM $BeSO_4$ and 180 mM NaF (pH 7.78, 25 °C). The spectrum was acquired at 56.23 MHz; 2 Hz of spectral line broadening was applied to the free induction decay (FID) that was averaged from 32 transients, acquired with 30-s preacquisition delay, prior to Fourier transformation. The multiplets labeled with solid triangles, circles, and open triangles are the resonances of BeF_2, BeF_3^-, and BeF_4^{2-}, respectively. The chemical shifts of the quintet components of BeF_4^{2-} are –65.49 ppm, –66.13 ppm, –66.75 ppm, –67.35 ppm, and –67.95 ppm; those of BeF_3^- quartet are –65.49 ppm, –66.13, –66.75 ppm, and –67.35 ppm; those of the BeF_2 triplet are –65.49 ppm, –66.13 ppm, and –66.75 ppm, relative to 0.000 ppm set at 56.23 MHz.

Multiple Equilibrium of Beryllofluoride Complexes. The multiple equilibrium of beryllofluoride complexes can be represented by the reaction scheme

$$2F^- + Be^{2+} \leftrightarrow BeF_2 + F^- \leftrightarrow BeF_3^- + F^- \leftrightarrow BeF_4^{2-} \quad (1)$$

assuming that the formation of beryllofluorohydroxides is negligible at physiological pH (Martin, 1988). Thus, the association constants K_1, K_2, and K_3 for BeF_2, BeF_3^-, and BeF_4^{2-}, respectively, are given by

$$K_1 = [BeF_2]/([Be^{2+}][F^-]^2) \quad (2)$$

$$K_2 = [BeF_3^-]/([BeF_2][F^-]) \quad (3)$$

$$K_3 = [BeF_4^{2-}]/([BeF_3^-][F^-]) \quad (4)$$

where the brackets denote molar concentration. In accordance with the mass conservation of Be^{2+}, the original concentration, $[Be^{2+}]_o$, is given by

$$[Be^{2+}]_o = [Be^{2+}] + [BeF_2] + [BeF_3^-] + [BeF_4^{2-}] \quad (5)$$

By combining eq 2–4 with eq 5, we obtained the expessions for the equilibrium concentrations of the complexes:

$$[BeF_2] = K_1[Be^{2+}]_o[F^-]^2/D \quad (6)$$

$$[BeF_3^-] = K_1K_2[Be^{2+}]_o[F^-]^3/D \quad (7)$$

$$[BeF_4^{2-}] = K_1K_2K_3[Be^{2+}]_o[F^-]^4/D \quad (8)$$

where $D = (1 + K_1[F^-]^2 + K_1K_2[F^-]^3 + K_1K_2K_3[F^-]^4)$. Equations 6–8 were fitted simultaneously onto the data that expressed the concentration of each complex species as the function of free fluoride concentration. The fitting was by nonlinear least-squares regression (Osborne, 1976) on a Hewlett/Packard 9000/200 computer, and the analysis yielded the association constants for the three reactions.

RESULTS

(1) ^9Be NMR Studies. Beryllofluoride Complexes. Figure 1 shows the "fully relaxed" ^9Be NMR spectrum obtained from

a solution of 20 mM $BeSO_4$ and 180 mM NaF (pH 7.78); it shows the superimposed peaks from three different beryllofluoride complexes. The relative spectral intensities of the components of the multiplets were 1.0, 5.9, 13.7, 12.0, and 3.3, ranging from low to high frequency. These peaks correspond to the overlap of a quintet, a quartet, and a triplet resonating, respectively, at −66.75 ppm, −66.44 ppm, and −66.13 ppm relative to 0.000 ppm set at 56.23 MHz of the transmitter frequency routinely set on our spectrometer (in turn this was related to the absolute frequency of TSP in the ^1H NMR spectrum). In other words, these multiplets are assigned respectively to BeF_4^{2-}, BeF_3^-, and BeF_2. An increase in the number of F^- ions per complex results in a shift to low frequency of the multiplet. This phenomenon can be attributed to the increase in electron shielding of the "reporter" ^9Be atom (Granger, 1983; Issartel et al., 1991). The line widths of the components of the multiplets (~5 Hz) were almost identical to that of Be^{2+} (as $BeSO_4$) in free solution. Therefore, the formation of complexes with fluoride did not appear to affect the average local molecular motion sufficiently to produce a perceptible change in the line widths of the NMR spectra. In addition, the narrowness of the multiplets may be due to the axial symmetry along the Be^{2+} complexes (Issartel et al., 1991). The position of the multiple equilibrium of the complexes was changed by increasing the NaF concentration from 10 mM to 30 mM to 100 mM while the concentration of $BeSO_4$ was kept constant (4 mM, pH 7.2). It was evident that BeF_3^- was the predominant species in the presence of 30 mM NaF (spectrum not shown).

^9Be NMR spectra of erythrocytes suspended in isotonic buffer containing 15 mM $BeSO_4$ and 45 mM NaF showed no evidence of separate resonances from the complexes in the extracellular and cytoplasmic compartments. There was, however, an increase of ~2 Hz in the line width. Addition of the paramagnetic shifting reagent dysprosium tripolyphosphate (DyPPP, 1 mM $DyCl_2$:2.5 mM PPP, pH 7.46) resulted in the shifting of all multiplets by ~11 Hz to higher frequency; this was accompanied by broadening of the resonances by ~5 Hz. It was apparent that the concentration of DyPPP did not shift sufficiently the extracellular multiplets and thus allow the intracellular resonance(s) to be identified. A further increase of DyPPP concentration caused precipitation (possibly of DyPPP and beryllofluoride complexes). Nevertheless, the uptake of beryllofluoride complexes by the erythrocytes was demonstrated as follows: Packed cells that were incubated in isotonic solution containing 15 mM $BeSO_4$ and 45 mM NaF were incubated with 0.5 mM DIDS. Subsequent washing of the cells was aimed at removing the extracellular salts. A ^9Be NMR spectrum of a hemolysate prepared from these cells showed evidence of BeF complexes although the peaks were not well resolved (spectra not shown).

(2) ^{19}F NMR Studies. Beryllofluoride Complexes. Attempts to determine the equilibrium constants of the components in the multiple-equilibrium system using ^9Be NMR were hampered by the low sensitivity and spectral resolution obtainable with this nucleus. ^{19}F NMR, with much higher sensitivity and a large chemical shift range, proved to be a more efficient means to this end. The ^{19}F NMR spectrum of a solution of NaF and $BeSO_4$ showed well resolved quartets from the spin coupling of ^{19}F with ^9Be sharing the ionic bonds (Figure 2). Figure 3 shows the variation of the relative intensities of the peaks from the complexes with an increase in F^- concentration. The peak assignments were made on the basis of the principle of mass action whereby, with increasing F^- concentration, the concentration of the higher order

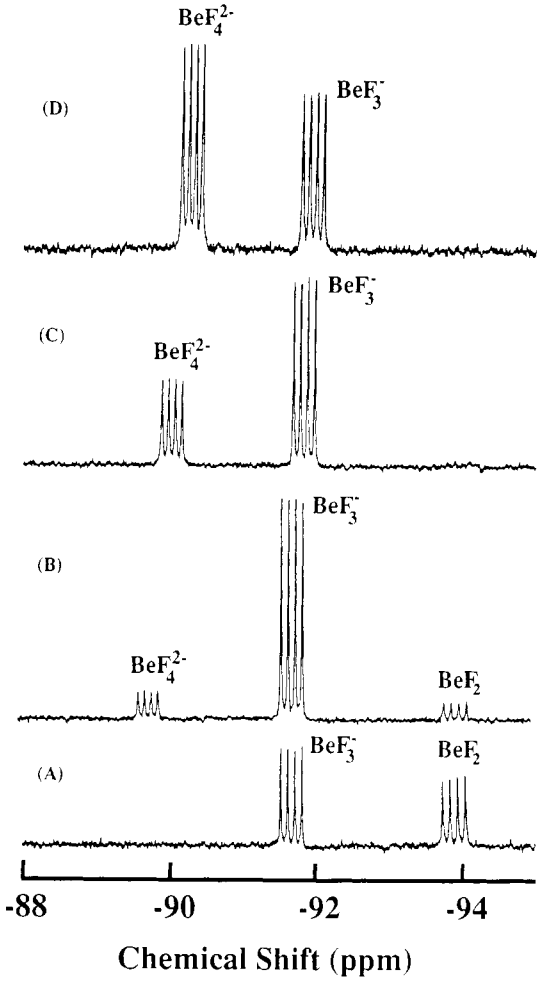

FIGURE 2: Stack plot of "fully relaxed" ^{19}F NMR spectra of beryllium fluoride complexes (pH 7.2, 25 °C) formed by 4 mM $BeSO_4$ and NaF ranging from 10 mM (A) to 30 mM (B) to 60 mM (C) and to 100 mM (D); 2 Hz of spectral line broadening was applied to the FID averaged from 12 transients prior to Fourier transformation. The chemical shift of the resonance multiplets of BeF_2, BeF_3^-, and BeF_4^{2-} and the respective concentrations are given in Table I. The ^9Be and ^{19}F spin coupling constants were measured to be 34 Hz, 37 Hz, and 35 Hz, respectively.

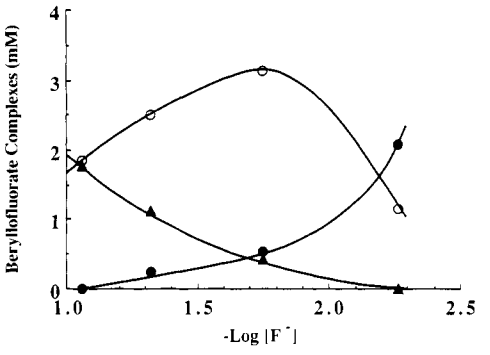

FIGURE 3: Equilibrium distributions of beryllofluoride complexes (25 °C, pH 7.2) as a function of the negative logarithm of free fluoride ion concentration, in the presence of 4 mM $BeSO_4$. The distribution curves were drawn by nonlinear least-squares regression of eqs 6–8 onto the data; the association constants were obtained from the regressions (Table II).

complexes increases. The assignments were consistent with those reported previously (Issartel et al., 1991). The concentrations of the complexes were determined directly from the spectral intensities at different initial concentrations of NaF according to the mass conservation law and are given in Table I. The association constants of BeF_2, BeF_3^-, and BeF_4^{2-}

Table I: ^{19}F NMR Chemical Shift (δ) and Equilibrium Distribution of Beryllofluoride Complexes Formed by 4 mM Be^{2+} (as $BeSO_4$) in the Presence of Various Concentrations of NaF at 25 °C

	BeF_2		BeF_3^-		BeF_4^{2-}		F^-	
$[NaF]_{total}$ (mM)	δ (ppm)	concn[a] (mM)	δ (ppm)	concn[a] (mM)	δ (ppm)	concn[a] (mM)	δ (ppm)	concn[a] (mM)
10.0	−93.90	2.1	−91.67	1.2	*b*	*b*	−43.58	2.4
30.0	−93.98	0.5	−91.73	3.1	−89.78	0.4	−43.60	17.8
60.0	*b*	0.2	−91.83	2.5	−90.00	1.1	−43.62	47.5
100.0	*b*	*b*	−91.94	1.9	−90.27	1.8	−43.65	87.3

[a] The concentrations of the complexes were determined from ^{19}F NMR spectral intensities of the complexes and the initial concentrations of NaF according to the mass conservation law. [b] Not detected; the chemical shift values were measured relative to that of 200 mM trifluoroacetate in a coaxial capillary.

Table II: Association Constants of the Beryllofluoride Complexes at 25 °C

complexes	BeF_2 (mM^{-2}) K_1	BeF_3^- (mM^{-1}) K_2	BeF_4^{2-} (mM^{-1}) K_3	ref
association constants[a]	~0.5	0.25 ± 0.05	0.009 ± 0.001	present study
association constants[b]	0.51 ● 0.17	0.26 ± 0.03	0.010 ± 0.001	present study
association constants[c]	630	0.631	0.031	Martin (1988)
association constants[d]	459	0.614	0.027	Mesmer and Baes (1969)

[a] The equilibrium association constants (±SD, $n = 3$ except for BeF_2) were calculated directly from the equilibrium concentrations of the complexes determined by ^{19}F NMR at pH 7.2. [b] The equilibrium association constants (±SD, $n = 3$) were given from nonlinear least-squares regression of eqs 6–8 on to the data of Figure 3. [c] The equilibrium association constants were determined at pH 7.4. [d] The equilibrium association constants were determined in acidic media in the presence of 1 M NaCl.

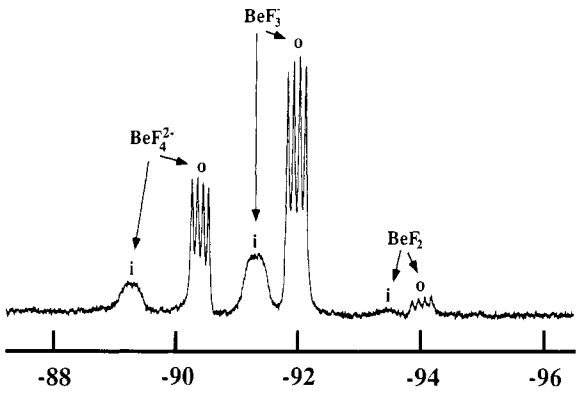

FIGURE 4: "Fully relaxed" ^{19}F NMR spectrum of beryllofluoride complexes incorporated in erythrocytes suspended in isotonic Tris-HCl (20 mM Tris) buffered saline (pH 7.45). Briefly, an aliquot of $BeSO_4$/NaF solution was added to the packed cells in Tris-HCl-buffered saline to achieve final concentrations of 4 mM $BeSO_4$ and 45 mM NaF and 43% hematocrit. The spectrum was acquired after transmembrane distribution equilibrium of the complexes at 25 °C; 2 Hz of spectral line broadening was applied to the FID averaged from 128 transients prior to Fourier transformation. Chemical shifts were referenced to trifluoroacetate at 0.000 ppm in a coaxial capillary. The chemical shift differences of the transmembrane BeF_4^{2-}, BeF_3^-, and BeF_2 were measured to be 1.115 ppm, 0.659 ppm, and ~0.53 ppm, respectively.

were then determined both directly from the spectral intensities and indirectly by simultaneously fitting eqs 6–8 onto the data shown in Figure 3 (Table II). The association constants calculated by both methods agreed within errors.

Uptake of Beryllofluoride Complexes by Erythrocytes. In suspensions of human erythrocytes, all three detectable beryllofluoride complexes gave separate ^{19}F NMR resonances from the intra- and extracellular populations (Figure 4). The broader peaks with unresolved multiplet components are from the intracellular populations of the complexes. The broadening of the lines of the intracellular species is consistent with more rapid transverse relaxation, which occurs as a result of the higher microviscosity that exists in the presence of ~5 mM carbonmonoxyhemoglobin inside the cells (Kuchel & Chapman, 1991).

The shift of the resonances from the intracellular species to higher frequency, relative to the extracellular resonances, is similar to that encountered with trifluoroacetate, F^-, and difluorophosphate (London & Gabel, 1989; Chapman & Kuchel, 1990; Xu et al., 1991). The shifts for intracellular F^- (not shown in the spectral plot of Figure 4), BeF_4^{2-}, BeF_3^-, and BeF_2, were 1.245 ppm, 1.115 ppm, 0.659 ppm, and 0.526 ppm, respectively. It is evident that the increase of the shift of intracellular resonances is correlated with the number of fluorine ions in each complex.

Erythrocytes were preincubated in a suspension with 1 mM DNDS at 25 °C for 10 min. ^{19}F NMR spectra acquired subsequently, after the addition of NaF and $BeSO_4$, showed no evidence of uptake of the complexes over an ~60-min incubation period. Figure 5 shows a graph of the ^{19}F NMR peak intensities of the resonances for each of the complexes, versus the time of incubation. No resonances representing intracellular beryllofluoride complexes were evident. Note that some free F^- had appeared in the erythrocytes. The permeability coefficients for the F^- influx and efflux were obtained by nonlinear least-squares regression of flux equations onto the data (see figure legends); the values were 2.9×10^{-8} cm s^{-1} and 7.7×10^{-8} cm s^{-1}, respectively. In a similar study with 0.5 mM DIDS (37 °C, 10 min), no evidence of resonances from intracellular species was found when spectra were recorded over a time course of ~30 min.

Butanol has been shown to alter membrane fluidity, and thus enhance the membrane permeability to solutes which traverse the membranes by simple diffusion across the lipid bilayer (Stein, 1986; Potts et al., 1989). In the present work, pretreatment of the erythrocytes with up to 100 mM butan-1-ol, after preincubation of the cells with 0.5 mM DIDS, revealed no uptake of F^- or of the complexes. However, at 150 mM butanol, which brought about a significant amount of hemolysis, a small resonance from "intracellular" BeF_4^{2-} was evident.

DISCUSSION

The progressive decrease in chemical shift of the resonances of the beryllofluoride complexes in the ^9Be NMR spectrum (Figure 1) with an increase in number of fluoride ions in the

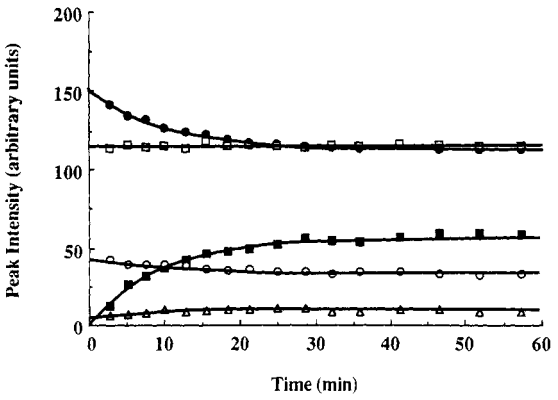

FIGURE 5: Plot of ^{19}F NMR relative spectral intensities of transmembrane fluoride and extracellular beryllofluoride complexes as a function of incubation time of DNDS-pretreated erythrocytes. An aliquot of packed cells pretreated with DNDS (1 mM) in buffer (pH 7.45), was diluted into an isotonic solution of complexes to achieve final concentrations of 4 mM $BeSO_4$ and 45 mM NaF with hematocrit 46%. "Fully relaxed" ^{19}F NMR spectra were acquired sequentially at 25 °C over a period of ~60 min. Solid circles and squares denote the intensities of extra- and intracellular fluoride. Open circles, squares, and triangles denote those of extracellular BeF_4^{2-}, BeF_3^-, and BeF_2, respectively. The efflux equation $[F^-]_{extracellular} = [F^-]_{total}\, k_1/(k_1 + k_{-1})\{1 + \exp[-(k_1 + k_{-1})t]\}$ and the influx equation $[F^-]_{intracellular} = [F^-]_{total}\, k_1/(k_1 + k_{-1})\{1 - \exp[-(k_1 + k_{-1})t]\}$ were fitted to the spectral intensities of F^- using nonlinear least-squares regression to give the influx and efflux rate constants k_1 and k_{-1}, respectively. The rate constants were used to obtain the membrane permeability for the influx (P_1) and efflux (P_{-1}) calculated as $P_1 = MCV\,(1 - H_c)k_1A^{-1}H_c^{-1}$ and $P_{-1} = MCVk_{-1}A^{-1}$, where MCV, H_c, and A are the mean cell volume, hematocrit, and mean cell surface area (143 μm^2; Sha'afi et al., 1967), respectively. The solid lines for the intra- and extracellular F^- peak areas were drawn from the regressions.

complexes provides evidence of increased shielding of the "reporter" ^9Be by F^-. The F^- increases electron density around the ^9Be and thus shifts the resonances to lower frequency (Granger, 1983). On the other hand, the progressive shift of the ^{19}F NMR quartets of BeF_3^- and BeF_4^{2-} to higher frequency with respect to BeF_2 (Figure 2 and Table I) is attributed to the shift of electron density to the more electronegative atom in the formation of the ionic bonds (Pauling, 1964; Granger, 1983). However, the contribution to the electron shielding of $^{19}F^-$ also comes from direct hydrogen bonding of the $^{19}F^-$ with solvent water (Kollman & Allen, 1972; Kirk & Kuchel, 1988; Xu et al., 1991). The ^{19}F NMR resonances of the complexes were shifted to lower frequency with an increase of NaF from 10 mM to 100 mM (Figure 2 and Table I). This can be attributed to an increase in diamagnetic susceptibility as the result of an increase in ionic strength.

The use of ^{19}F NMR spectroscopy not only allowed us to determine the multiple equilibrium of complexes but also to detect erythrocyte uptake of the complexes; this was made possible by their being well-resolved ^{19}F NMR resonances from the complexes in the extracellular and cytoplasmic compartments. Note that the association constants determined for BeF^{3-} and BeF_4^{2-} (Table II) were slightly smaller than those obtained by Mesmer and Baes (1969). The differences, however, are in the range expected for measuring metal–ligand binding, as observed generally for other studies using different techniques (Smith & Martell, 1976). In addition, the ionic strength used by Maesmer and Baes (1.0 M) is larger than that used in this study and may account for the discrepancy. It is also possible that an inability to distinguish between binary beryllofluoride complexes and ternary beryllofluorohydroxide complexes (if any) may contribute to the differences in the values of the association constants. In particular, the indirect bridging between ^{19}F and OH^- in a ternary complex is not expected to affect significantly the electronic shielding on ^{19}F. As a consequence, for example, the ^{19}F resonance from BeF_3^- would be expected to strongly overlap the resonance from $BeF_3(OH)^{2-}$. The overlapping of resonances would lead to an overestimate of the concentrations of the binary beryllofluoride complexes, and consequently an overestimate of the association constants, compared with those from previous studies (Mesmer & Baes, 1969; Martin, 1988). As our association constants were smaller than those in previous studies, an inability to distinguish between ternary and binary complexes is unlikely to be the source of the discrepancy.

In addition, Martin (1988) reported that the binding of OH^- to Be^{2+} became negligible when the concentration of fluoride was greater than 1 mM at pH 7.5. In the present study, the lowest concentration of fluoride was 10 mM. Thus, if we accept this point, it is reasonable to assume that the presence of complexes containing OH^- was negligible in our experiments.

The values of our equilibrium constants may be affected by the presence of Tris since the three hydroxyl groups on Tris may bind Be^{2+}. However, the binding of Tris to other group IIA cations, Mg^{2+} and Ca^{2+}, is known to be negligible (Gueffroy, 1981). There appear to be no reports on the binding of Tris to Be^{2+}, but on this basis it also would be expected to be negligible.

The association constant for BeF_2 (Table II) appears to be significantly smaller than that determined by Mesmer and Baes (1969). In calculating the association constant (K_1) for BeF_2 (eq 2), the concentration of free Be^{2+} was determined from the total concentration of beryllium ions added and the concentrations of the complexes present, according to the mass conservation law (eq 5). It was shown in a simulated calculation that a ±20% variation of $[BeF_2]$, estimated from ^{19}F spectral intensities, would give rise to a K_1 ranging from 0.14 mM^{-2} to 2.90 mM^{-2}, if the average concentration of 2.1 mM (Table I) for BeF_2 at total $[F^-]$ of 10 mM was used. Similarly, if the true concentrations of both BeF_2 and BeF_3 were 20% larger than the measured values of 2.1 mM and 1.2 mM, respectively, the K_1 value would rise to 154 mM^{-2}. An uncertainty of 20% in the concentrations for both BeF_3 and BeF_2 is in keeping with the relatively low signal-to-noise ratios for the ^{19}F resonances of these species when using 10 mM F^-. In turn, these errors give rise to large changes in the calculated concentration of free Be^{2+} and hence the value of K_1. This may be the reason for the large discrepancy between the value of K_1 calculated here and that obtained by Mesmer and Baes (1969). The association constants for the formation of BeF_2 and BeF_3 will be less affected since their calculation does not depend directly on the concentration of free Be^{2+}.

Figure 4 shows the ^{19}F NMR spectrum of the beryllofluoride incorporated into an erythrocyte suspension; the resonances of the intracellular populations were shifted to higher frequency compared with the corresponding extracellular resonances. This phenomenon is closely similar to that of trifluoroacetate (London & Gabel, 1989), F^- (Chapman & Kuchel, 1990), difluorophosphate, and monofluorophosphate (Xu et al., 1991). The above observations point to a common physical basis for the shifting; viz. the disruption of the direct hydrogen bonding of H_2O to ^{19}F by intracellular hemoglobin (Xu et al., unpublished data). This gives rise to a decrease of electron shielding of the ^{19}F atom and thus shifts the intracellular resonances to higher frequency.

DNDS and DIDS are known to bind to the explasmic substrate binding "pocket" of the anion-transport protein,

band-3, via noncovalent and covalent bonding, respectively (Cabantchik & Rothstein, 1974; Knauf, 1979; Passow, 1986). This results in, respectively, competitive or noncompetitive inhibition of band-3-mediated anion exchange (Frohlich, 1982; Frohlich & Gunn, 1987), while there appears to be no effect on simple diffusion of small neutral solutes through the membrane (e.g., dimethyl methylphosphonate; Potts et al., 1989). Figure 5 shows the sensitive inhibition of uptake of beryllofluoride complexes by DNDS. The small decrease of the relative spectral peak intensity of extracellular BeF_4^{2-} and the concomitant increase of that of BeF_2 was probably due to a shift of the multiple equilibrium as a small fraction of the free F^- entered the cells. The lack of uptake by the erythrocytes that had been pretreated with DNDS, or DIDS followed by butanol treatment, confirmed the insignificance of the pathway of simple diffusion in the uptake of the complex(es). In view of the large van der Waals volume of dimethyl methylphosphonate (64.9 $cm^3\ mol^{-1}$) compared with those of BeF_3^- (27.7 $cm^3\ mol^{-1}$) and BeF_4^{2-} (30.4 $cm^3\ mol^{-1}$) calculated according to Bondi (Bondi, 1964; Bigay et al., 1987; Lof, 1987), the lack of simple diffusion is more likely to be attributed to the charge repulsion between the charged complexes and the negatively charged membrane rather than to the sizes of the complexes. It is worth noting that uptake by simple diffusion of the supposedly neutral species, BeF_2, was also completely inhibited in the presence of the stilbenedisulfonates. This may be due to the polar nature of the complex which may prevent its diffusion through the membrane in spite of its small van der Waals volume (17.0 $cm^3\ mol^{-1}$) relative to that of dimethyl methylphosphonate.

In conclusion, it was demonstrated in this study that the appearance of beryllofluoride complexes in the erythrocytes is probably mediated by band-3. We propose that the monovalent anionic BeF_3^- and F^- are the two species principally involved in the facilitated uptake and that the other species arise inside as the result of re-establishment of the multiple equilibrium in the cytoplasmic compartment. This multiple equilibrium exchange has been show, from our preliminary studies using magnetization transfer, to be within the subminute time scale. In other words, the uptake of the complexes by erythrocytes was too rapid to be monitored by a conventional time course.

ACKNOWLEDGMENT

Brian Bulliman is thanked for assistance with the computing.

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Registry No. BeF_4^{2-}, 18539-20-3; BeF_3^-, 19181-26-1; BeF_2, 7787-49-7.