Characterization of Transmembrane Chemical Shift Differences in the ^{31}P NMR Spectra of Various Phosphoryl Compounds Added to Erythrocyte Suspensions†

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ABSTRACT: Trimethyl phosphate, dimethyl methylphosphonate, diethyl methylphosphonate, trimethylphosphine oxide, and the hypophosphite, phenylphosphinate, and diphenylphosphinate ions all contain the phosphoryl functional group. When added to an intact erythrocyte suspension at 20 °C, each of the compounds gave rise to separate intra- and extracellular ^{31}P NMR resonances, and the separation between the two resonances of each compound varied with the mean cell volume. The differences between the intra- and extracellular chemical shifts were shown to be primarily attributable to the effects of hemoglobin. The presence of hemoglobin inside the cell gave rise to a significant difference in the magnetic susceptibilities of the two compartments. In addition, it exerted a large susceptibility-independent chemical shift effect, the magnitude of which was dependent upon the chemical structure of the phosphoryl compound involved. A number of other intra- and extracellular components were also shown to cause chemical shift variations, smaller than those arising from hemoglobin but nonetheless significant. The cell volume dependence of the transmembrane chemical shift differences therefore reflected not only the cell volume dependence of the intracellular hemoglobin concentration but also the changing concentration of the other solutes in the two compartments. In addition to their cell volume dependence, the transmembrane chemical shift differences varied with temperature. In the case of the nonelectrolytes this reflected not only the temperature dependence of the mechanism(s) responsible for the susceptibility-independent shift effects but also the temperature dependence of the rates at which the compounds traversed the cell membrane.

It has been shown that a number of compounds containing the phosphoryl functional group, when added to an erythrocyte suspension, give rise to intra- and extracellular ^{31}P NMR resonances that differ in their chemical shifts. This phenomenon has a number of useful applications. The observation of separate resonances for the intra- and extracellular populations of a compound enables the application of various NMR techniques to measure the rate at which the compound crosses the cell membrane. For example, the saturation-transfer technique has been used to measure the equilibrium exchange rate of the nonelectrolyte dimethyl methylphosphonate (Kirk & Kuchel, 1986) and to identify the pathway by which it permeates the membrane (Potts, Kirk, and Kuchel, unpublished results). Similarly, Labotka and Omachi (1987, 1988) have made use of the transmembrane chemical shift differences observed for a number of substituted phosphorus oxyacids to characterize their membrane transport properties and thereby gain valuable information regarding the specificity of the erythrocyte anion transport protein. The separation of the intra- and extracellular resonances of the univalent hypophosphite ion has enabled its use as a ^{31}P NMR probe of red cell membrane potential (Kirk et al., 1988) and as a probe of intracellular viscosity (Price, Kuchel, and Cornell, unpublished results). Furthermore, the cell volume dependence of the separation between the intra- and extracellular resonances of dimethyl methylphosphonate has been shown to provide a convenient method for monitoring changes in mean cell volume throughout the course of a ^{31}P NMR experiment (Kirk & Kuchel, 1985; Raftos et al., 1988).

The physical basis of the observed transmembrane chemical shift differences is not at all straightforward. We have recently demonstrated that in oxygenated cell suspensions the effective magnetic susceptibility of the intracellular compartment is significantly different from that of the external compartment (Kirk & Kuchel, 1988a). The susceptibility difference causes the intracellular chemical shift of a compound to be somewhat less than the corresponding extracellular shift. However, the difference is too small to account fully for the magnitude of the transmembrane shift differences observed for most compounds. Moreover, the effect is a general one and, as such, does not account for the observation that the separation between the intra- and extracellular resonances depends upon the chemical structure of the compound involved.

In the work presented here we have made use of eight phosphoryl compounds, a number of which gave rise to transmembrane ^{31}P NMR chemical shift differences that are substantially larger than those that have been reported previously. The transmembrane shift differences varied with mean cell volume and with temperature. Magnetic susceptibility effects certainly contributed to the observed transmembrane chemical shift differences, but in most cases were not solely (nor even mainly) responsible. Components of both the intra- and extracellular solutions were shown to affect (to different extents) the chemical shifts of a number of the phosphoryl compounds in a manner that could not be accounted for in terms of magnetic susceptibility. The phenomenon is therefore not attributable to any single effect of any single cellular component but, rather, to a complex interplay of the effects of both intra- and extracellular solutes on the ^{31}P NMR chemical shifts as well as the effects of the membrane transport properties of the compounds.

Experimental Procedures

Materials. Trimethyl phosphate [TMP;[1] $(CH_3O)_3PO$;

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Fluka AG, Buchs, West Germany], triethyl phosphate [TEP; $(CH_3CH_2O)_3PO$; BDH Chemicals Ltd., Poole, U.K.], dimethyl methylphosphate [DMMP; $(CH_3O)_2CH_3PO$; Ega Chemie, Steinheim, West Germany], diethyl methylphosphonate [DEMP; $(CH_3CH_2O)_2CH_3PO$; Aldrich, Milwaukee, WI], and trimethylphosphine oxide [TMPO; $(CH_3)_3PO$; Alfa Products, Danvers, MA] are electroneutral compounds, readily soluble in water. The monovalent hypophosphite (HP; $H_2PO_2^-$), phenylphosphinate, [PP; $(C_6H_5)HPO_2^-$], and diphenylphosphinate [DPP; $(C_6H_5)_2PO_2^-$] ions are the conjugate bases of hypophosphoric acid, phenylphosphinic acid, and diphenylphosphinic acid, respectively (Aldrich). These acids all have a single pK_a of around 1, and the concentrations of the un-ionized species are therefore negligible at physiological pH values.

Solutions. In all experiments the hypophosphite, phenylphosphinate, and diphenylphosphinate anions were added to samples in the form of an equimolar aqueous solution, adjusted to a pH of ~7.3 with NaOH (10 M). When added to suspensions of intact cells, the equimolar solution was diluted so as to have an osmolality of ~300 mOsm kg^{-1}, which corresponded to a concentration of about 48 mM for each of the three ions. The isoosmotic addition of the electrolytes to cell suspensions ensured that the addition of the solution (containing the relatively impermeant sodium ion) caused minimal pertubation of the mean cell volume. In experiments other than those involving intact erythrocytes the electrolytes were added in a more concentrated form, with the equimolar concentrations of the three ions being typically around 750 mM.

Preliminary studies showed that the five nonelectrolytes under investigation all permeate the erythrocyte membrane very rapidly. These compounds were therefore added to all samples (including intact cell suspensions) in the form of a concentrated equimolar mixture (1.84 M). The high membrane permeability of these compounds ensured that the addition of a small volume of the concentrated mixture had little effect on the cell volume.

All solutions were made up within the 24-h period preceding each experiment.

Erythrocyte Suspensions. Fresh erythrocytes from normal donors were obtained from the Red Cross Blood Transfusion Service (Sydney, NSW, Australia) and washed five times in isotonic saline (154 mM NaCl). Glucose was added to a concentration of 10 mM. The cells were then suspended at low hematocrit (Ht) and bubbled gently with humidified carbon monoxide for 30 min at room temperature so as to convert intracellular hemoglobin to the stable diamagnetic form [(carbonmonoxy)hemoglobin]. Following carbonmonoxygenation, the cells were packed by centrifugation (Ht > 0.90). Subsequent experimental manipulations are described in the appropriate figure legends.

Hematocrits were measured by using a microhematocrit centrifuge. A 2% correction was applied to the measured hematocrit to account for trapped extracellular solution (Dacie & Lewis, 1975). Mean cell volumes were calculated from the corrected hematocrit and cell count which was measured by using a Coulter counter (Model ZF, Coulter Electronics, Dunstable, U.K.). In all cases the mean cell volume and intracellular hemoglobin concentrations (under physiological conditions) were within the normal range.

Lysates. Concentrated red cell lysates were prepared from cells washed five times in hypertonic saline (280 mM NaCl). Glucose was added to a concentration of 5 mM, and then the cells were suspended at low hematocrit and bubbled gently with humidified carbon monoxide for 30 min at room temperature. Following carbonmonoxygenation the suspension was centrifuged (8000*g*, 20 min). The supernatant solution was removed, and then the packed cells were lysed by repeated sonication (Branson Sonifier B-15, Danbury, CT). The fraction of the total lysate volume accessible to water was determined gravimetrically as has been described previously (Savitz et al., 1964).

Purified Hemoglobin. Hemoglobin was purified according to a method similar to that described by Gary Bobo (1967). Fresh cells were washed five times in hypertonic saline (280 mM NaCl) and then packed by centrifugation (8000*g*, 20 min). The supernatant solution was removed, and the packed cells were transferred to a separating funnel, to which was then added an equal volume of diethyl ether. The funnel was shaken thoroughly in order to ensure full lysis of the cells and maximum extraction of the membrane components into the ether phase. The mixture was kept overnight at 4 °C. The lower (aqueous) phase was isolated and then centrifuged (8000*g*, 20 min), and the uppermost portion (containing residual ether) was discarded. The remaining solution was subjected to rotary evaporation under reduced pressure (room temperature, 45 min) so as to remove all traces of ether. The solution was then dialyzed for 45 h at 4 °C against distilled water (5 × 2 L) containing low concentrations of antibiotics (2.5 mg L^{-1} amphotericin B, 27 mg L^{-1} penicillin G, and 50 mg L^{-1} streptomycin sulfate). Following the final dialysis step the hemoglobin was reconcentrated by using Aquacide II (Calbiochem, La Jolla, CA). Denatured protein was removed by centrifugation (10000*g*, 30 min), and the hemoglobin solution was then converted to the diamagnetic carbonmonoxy form by exposure to humidified carbon monoxide in a rotary tonometer at room temperature for 20 min.

NMR Methods. Broad-band proton-decoupled ^{31}P NMR spectra were acquired at 161.9 MHz by using a Varian XL400 spectrometer operating in the Fourier transform mode. The NMR sample tubes (10-mm outer diameter) were spun at ~10 Hz throughout spectral accumulation. NMR sample temperatures were measured by using an ethylene glycol capillary as has been described (Bubb et al., 1988). High-power decoupling has been shown to cause significant heating of biological samples (Bubb et al., 1988). The degree of heating varies with sample composition (ionic strength), and it was therefore necessary to measure and adjust the temperature of each sample individually. In all experiments other than that in which the temperature was deliberately varied, the temperature control was set so as to give an NMR sample temperature of 20 ± 1 °C.

The large proton–phosphorus coupling constant in the hypophosphite ion ($J_{H-P} \simeq 520$ Hz) gave rise to a number of small (residual coupling) resonances symmetrically distributed about the main ^{31}P NMR resonance. These resonances were absent from the undecoupled spectrum, and the possibility of their being due to impurities may therefore be discounted. A similar through smaller effect was noted in the case of the phenylphosphinate ion.

NMR spectra were acquired by using short excitation pulses (nutation angle $\simeq 30°$) with a recycle time of 1–2 s. Unless specified otherwise, chemical shifts were *measured* relative to that of an external reference compound [usually DEMP

[1] Abbreviations: TMP, trimethyl phosphate; TEP, triethyl phosphate; DMMP, dimethyl methylphosphonate; DEMP, diethyl methylphosphonate; TMPO, trimethylphosphine oxide; HP, hypophosphite ion; PP, phenylphosphinate ion; DPP, diphenylphosphinate ion; Ht, hematocrit; Hb, hemoglobin; ATP, adenosine 5′-triphosphate; DPG, 2,3-diphosphoglycerate.

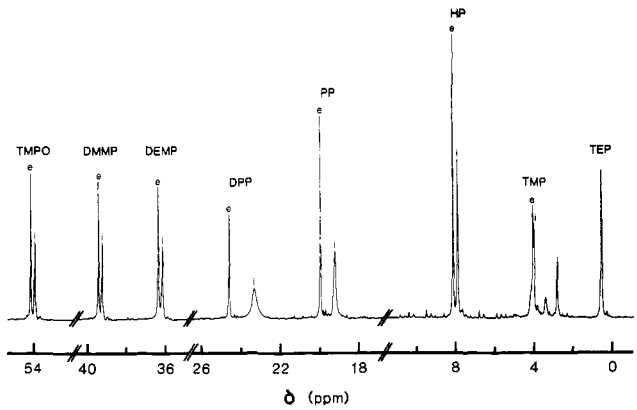

FIGURE 1: ^{31}P NMR spectrum of carbonmonoxygenated erythrocytes to which had been added TMP, TEP, DMMP, DEMP, TMPO, HP, PP, and DPP, each at a concentration of 10 mM with respect to the total sample volume. The cells were prepared as described under Experimental Procedures and then diluted with isotonic sucrose solution so that the final hematocrit (following the addition of the phosphoryl compounds) was 0.56. With the exception of TEP, the intracellular resonances (i) of each compound appear at a lower frequency than the extracellular resonances (e). The intra- and extracellular resonances of TEP are averaged to a single peak by the fast exchange of TEP across the cell membrane (Kirk & Kuchel, 1988a). The two ^{31}P NMR resonances of intracellular DPG appear at 4.17 and 3.42 ppm, and that of intracellular inorganic phosphate appears at 2.78 ppm.

(10% v/v) in ethylene glycol] sealed within a coaxial capillary and are *quoted* relative to that of 85% phosphoric acid. The error in the measured shifts was estimated to be 0.004 ppm.

Analytical Methods. The concentration of hemoglobin in lysate and purified hemoglobin samples was measured by using the cyanomethemoglobin assay of Van Kampen and Zijlstra (1961). The quantitative conversion of (carbonmonoxy)-hemoglobin to cyanomethemoglobin was ensured by allowing the hemoglobin–reagent solutions to stand overnight prior to spectrophotometric measurement.

The concentrations of Na^+ and K^+ in isolated extracellular solutions and cell lysates were measured by using a flame photometer (Corning-EEL, U.K.)

Molecular Volumes. The van der Waals volumes of TMP, TEP, DMMP, and DEMP were calculated as described by Bondi (1964). The van der Waals radii of the phosphorus, carbon, oxygen, and hydrogen atoms were taken to be 1.80, 1.70, 1.50, and 1.20 Å, respectively (Bondi, 1964). The covalent bond lengths used in calculations were as follows: P=O, 1.44 Å; P–O, 1.57 Å; P–C, 1.82 Å (Corbridge, 1966); C–O, 1.43 Å; C–C, 1.54 Å; C–H (methylene), 1.07 Å; C–H (methyl), 1.10 Å (Kennard, 1983).

RESULTS

Chemical Shift Effects in Cell Suspensions. Figure 1 shows the ^{31}P NMR spectrum arising from an isotonic suspension of carbonmonoxygenated erythrocytes to which had been added equal concentrations (10 mM) of TMP, TEP, DMMP, DEMP, TMPO, HP, PP, and DPP. All the compounds other than TEP are seen to have given rise to NMR signals consisting of two separate resonances. Addition of a Mn^{2+}–albumin complex (100 μM final concentration) to the extracellular solution resulted in the diminution of the higher frequency resonance of each compound while leaving the lower frequency resonance unaffected. Lysing the cells caused each pair of resonances to coalesce. The higher frequency resonance therefore corresponds, in each case, to the extracellular population of the compound and the lower frequency resonance to the intracellular population. In the case of TEP, it has been demonstrated that the intra- and extracellular chemical shifts

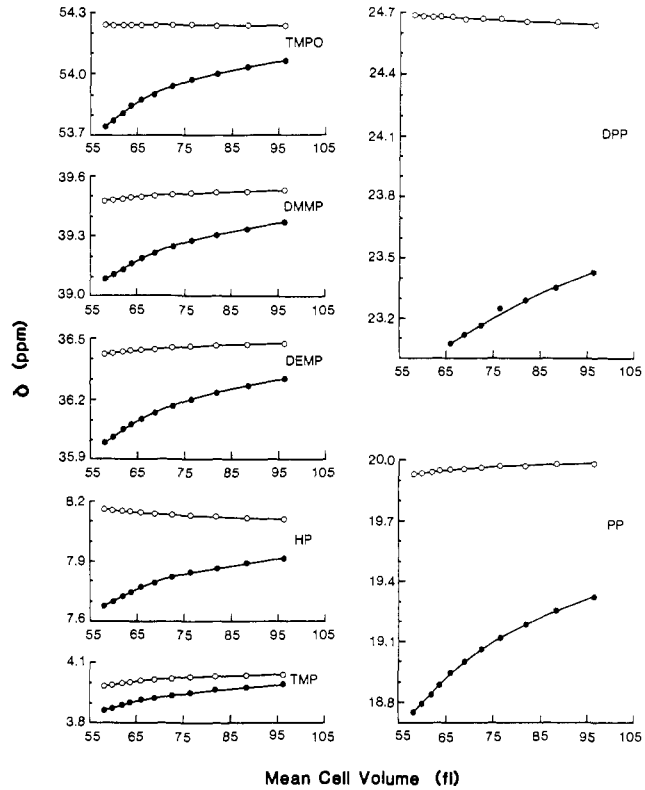

FIGURE 2: Variation of intra- and extracellular chemical shifts with mean cell volume. Closed symbols denote intracellular shifts, and open symbols denote extracellular shifts. Each sample was prepared by the addition of an equimolar solution of the eight phosphoryl compounds (1.9 mL, 48 mM) to cells suspended in isotonic saline (6.2 mL, Ht $\simeq$ 0.90) followed by the addition of a sucrose solution (0.90 mL) that ranged in concentration from 0 to 2.78 M. The final, equimolar concentration of the eight phosphoryl compounds was 10 mM with respect to the total sample volume. The use of sucrose rather than an electrolyte to manipulate the osmotic pressure ensured minimal variation of ionic strength between samples and therefore minimal variation in the effects of the decoupler on the sample temperature. Chemical shifts were measured relative to an external reference compound and are quoted relative to that of 85% phosphoric acid. The lines were fitted visually.

of the compound *are* significantly different from one another; however, the two resonances are averaged by the fast exchange of the compound between the two compartments (Kirk & Kuchel, 1988a).

Manipulation of the extracellular osmotic pressure by the addition of varying concentrations of sucrose caused both the intra- and extracellular chemical shifts of each compound to vary. Figure 2 shows the intra- and extracellular chemical shifts (measured relative to that of an external reference compound in a coaxial capillary) as a function of the mean cell volume. Figure 3 shows the cell volume dependence of the transmembrane chemical shift difference ($\Delta\delta$) for all seven compounds giving rise to separate intra- and extracellular resonances. In all cases, $\Delta\delta$ increased as the cell volume was reduced.

Figure 4 shows the variation of the transmembrane chemical shift differences with temperature. As the temperature was increased, the intra- and extracellular resonances of first TMP and then DEMP coalesced. The corresponding spectra of these two compounds are shown in Figure 5.

Extracellular Chemical Shift Effects. It has been demonstrated that when an oxygenated lysate prepared from packed cells is diluted, by varying amounts, with supernatant solution isolated from the cells prior to lysis (and therefore having approximately the same ionic strength as the lysate), the variation of the ^{31}P NMR chemical shift of TEP may be

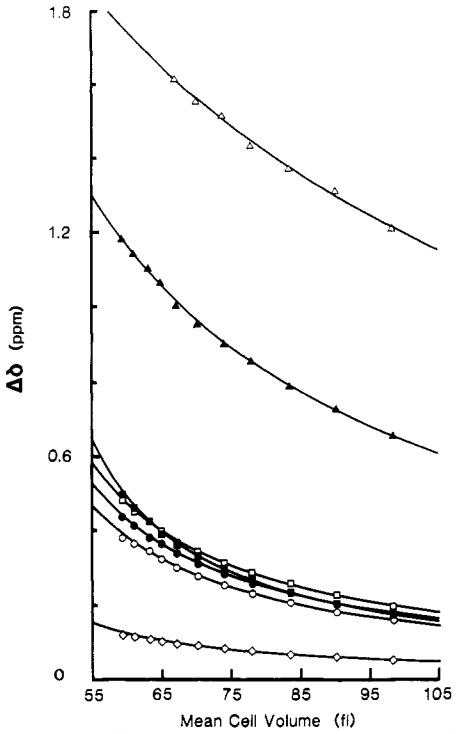

FIGURE 3: Variation of the transmembrane chemical shift differences ($\Delta\delta$) with mean cell volume. Samples were prepared as described in the legend to Figure 2. The symbols for the different compounds are as follows: ◊, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP. The data for each compound were fitted by least-squares regression to the equation $\Delta\delta = 1/[E(\text{mean cell volume}) + F]$. E and F are constants and took the following values: TMP, $E = 0.2878$, $F = -9.188$; DMMP, $E = 0.0951$, $F = -3.101$; DEMP, $E = 0.0854$, $F = -2.815$; TMPO, $E = 0.0970$, $F = -3.797$; HP, $E = 0.0768$, $F = -2.501$; PP, $E = 0.0175$, $F = -0.197$; DPP, $E = 0.0066$, $F = 0.174$.

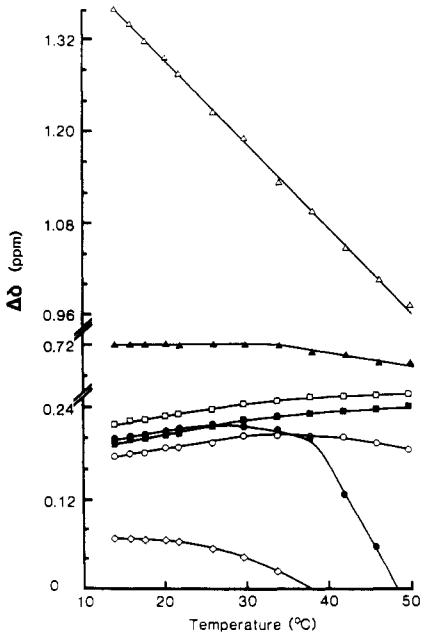

FIGURE 4: Variation of the transmembrane chemical shift differences ($\Delta\delta$) with temperature. The sample was prepared as described in the legend to Figure 1. Symbols: ◊, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP. The curves were fitted visually.

fully accounted for in terms of the magnetic susceptibility of hemoglobin (Kirk & Kuchel, 1988a). In similar experiments (results not shown) it was demonstrated that the same is true when the experiment is repeated with a carbonmonoxygenated lysate. Similarly, it has been shown that, in aqueous solutions,

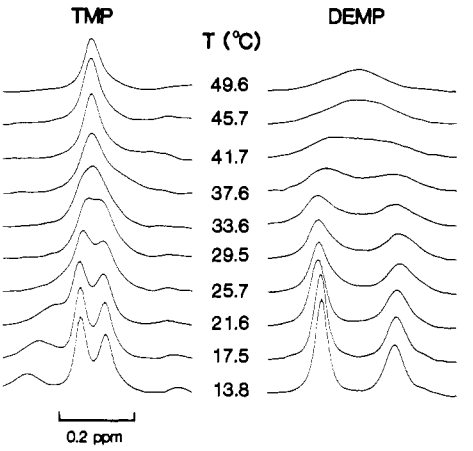

FIGURE 5: Temperature dependence of the ^{31}P NMR signals of TMP and DEMP arising from an isotonic erythrocyte suspension. The sample was prepared as described in the legend to Figure 1.

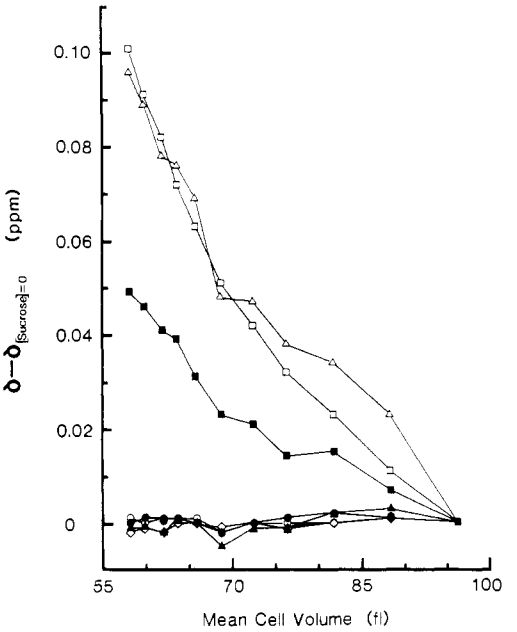

FIGURE 6: Chemical shift effects in isolated extracellular solutions. Immediately following the experiment giving rise to Figures 2 and 3 the cell samples were centrifuged and the extracellular solutions were isolated. The chemical shifts in the solutions are plotted as a function of the mean cell volume in the samples from which the solutions were isolated. The ^{31}P NMR chemical shifts in the solutions were measured relative to that of (internal) TEP, and the observed shift variations are therefore independent of bulk susceptibility effects. Chemical shifts are quoted relative to their value in the solution isolated from the suspension having the highest mean cell volume ([sucrose] = 0) in order to facilitate comparison of the data. Note that the chemical shifts of TMP, DMMP, DEMP, and PP were invariant with sucrose concentration and that the four data sets therefore coincide with one another. Symbols: ◊, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP.

the variation of the chemical shift of TEP with NaCl concentration (and therefore with ionic strength) is negligible [<0.01 ppm (M NaCl)$^{-1}$; Costello et al., 1976]. The invariability of the chemical shift of TEP with ionic strength or hemoglobin concentration (once susceptibility effects have been accounted for) implies that TEP is a suitable internal chemical shift reference. Variations of ^{31}P NMR chemical shifts *relative to that of internal TEP* represent shift effects that are not attributable to magnetic susceptibility effects and which must, therefore, have their origin elsewhere.

Following the acquisition of the spectra giving rise to Figures 2 and 3 the extracellular solutions were isolated from the cells

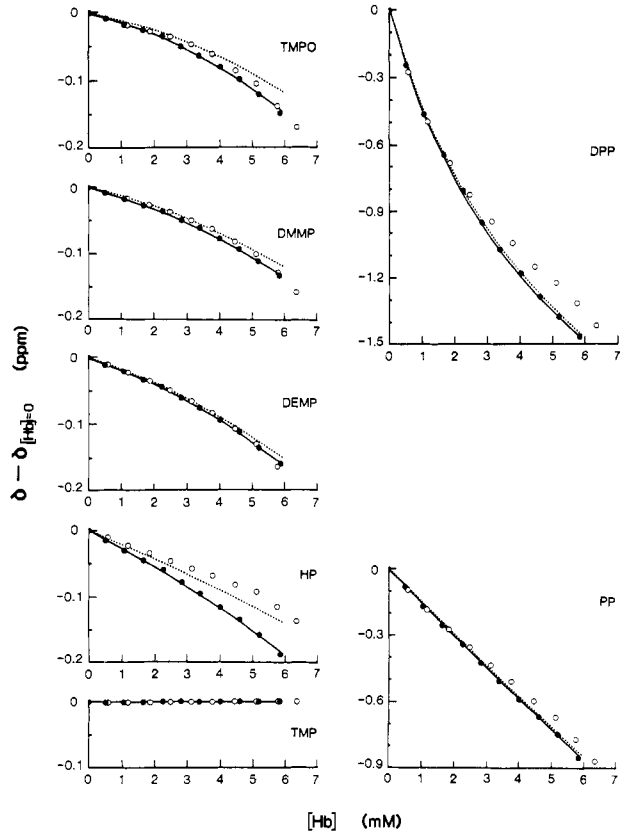

FIGURE 7: Effects of cell lysate (open symbols) and purified hemoglobin (closed symbols) on ^{31}P NMR chemical shifts. Concentrated cell lysates and purified hemoglobin solutions were prepared as described under Experimental Procedures. Each was diluted by varying amounts in distilled water, and the eight phosphoryl compounds were added, each to a concentration of 10 mM. The concentration of lysate is expressed in terms of the measured hemoglobin concentration. Chemical shifts were measured relative to that of TEP and are quoted relative to their value in the absence of hemoglobin ($\delta_{[Hb]=0}$). The solid lines (with the exception of that for DPP) were drawn by using quadratic equations ($\delta - \delta_{[Hb]=0} = B[Hb]^2 + C[Hb]$) fitted to the purified hemoglobin data. The coefficients of the fitted equations are as follows: TMP, $B = -1.95 \times 10^{-5}$, $C = 1.43 \times 10^{-4}$; DMMP, $B = -1.58 \times 10^{-3}$, $C = -0.0129$; DEMP, $B = -1.92 \times 10^{-3}$, $C = -0.0157$; TMPO, $B = -2.24 \times 10^{-3}$, $C = -0.0112$; HP, $B = -1.11 \times 10^{-3}$, $C = -0.0247$; PP, $B = 2.11 \times 10^{-3}$, $C = -0.1564$. The curve describing the variation of δ^{DPP} with purified hemoglobin concentration was fitted visually. The broken lines represent simulations of the variation of chemical shifts with lysate concentration, based on the assumption that both the hemoglobin and the intracellular ions exert the same shift effects when together in the lysate as was observed in the separate purified hemoglobin and model intracellular ion solutions (eq 3). Note that the ordinate scale for the two aromatic compounds (PP and DPP) differs from that of the nonaromatic compounds.

by centrifugation, and a further series of spectra was acquired. Figure 6 shows the chemical shifts of the phosphoryl compounds in the isolated extracellular solutions as a function of the mean cell volume in the suspensions from which the solutions were isolated. The shifts were *measured* relative to that of TEP and are *quoted* relative to those in the solution isolated from the suspension having an extracellular sucrose concentration of 0 M ($\delta_{[sucrose]=0}$) so as to facilitate comparison of the data. The chemical shifts of TMP, DMMP, DEMP, and PP were seemingly unaffected by the increasing sucrose concentration whereas those of TMPO, HP, and DPP increased.

Intracellular Chemical Shift Effects. Figure 7 shows the effects of cell lysate and purified hemoglobin on the chemical shifts of the phosphoryl compounds. The chemical shifts were measured relative to that of TEP so as to eliminate chemical shift effects arising from variations in the magnetic susceptibilities of the samples. The shift of each compound is quoted relative to its value in the absence of hemoglobin ($\delta_{[Hb]=0}$). Neither the purified hemoglobin nor the lysate had any effect on the chemical shift of TMP. The chemical shifts of the other six compounds decreased as the concentration of hemoglobin increased in both the purified hemoglobin and lysate solutions. In most cases, however, the purified hemoglobin caused the resonances to be shifted to lower frequency than did the lysate at any given hemoglobin concentration.

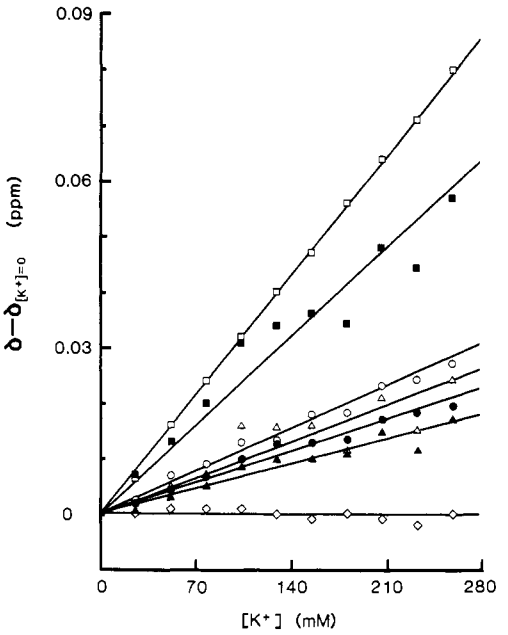

FIGURE 8: Effect of intracellular ions on ^{31}P NMR chemical shifts. A model solution containing the major intracellular ions was constituted from KCl (258.0 mM), NaCl (13.1 mM), $MgCl_2$ (4.2 mM), Na_2ATP (4.2 mM), and Na_5DPG (12.1 mM) so that the final proportions of K^+, Na^+, Mg^{2+}, ATP, and DPG were approximately the same as those in the concentrated cell lysate giving rise to Figure 7. A series of samples was prepared by diluting the model solution by varying amounts in distilled water and then adding an equimolar solution of the eight phosphoryl compounds so as to give a final concentration of 10 mM of each. The concentration of ions present in the model solutions is expressed in terms of the concentration of K^+. Chemical shifts were measured relative to that of TEP and are quoted relative to their value in the absence of intracellular ions ($\delta_{[K^+]=0}$). The symbols for the different compounds are as follows: ◇, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP. The straight lines were fitted by linear regression ($\delta - \delta_{[K^+]=0} = A[K^+]$). The slopes ($A$) of the fitted lines take the following values: TMP, $A = 0$; DMMP, $A = 1.11 \times 10^{-4}$; DEMP, $A = 8.17 \times 10^{-5}$; TMPO, $A = 2.28 \times 10^{-4}$; HP, $A = 3.07 \times 10^{-4}$; PP, $A = 6.48 \times 10^{-5}$; DPP, $A = 9.37 \times 10^{-5}$.

The cell lysate giving rise to Figure 7, in undiluted form, had a hemoglobin concentration of 6.47 mM (with respect to the total lysate volume) and a fractional water volume (α_L) of 0.640. The concentration of K^+ in the undiluted lysate (per liter of water) was measured to be 172 mM and that of Na^+ was 55 mM. The ratio of Na^+ to K^+ concentrations was higher than is normally quoted for fresh erythrocytes due to the presence of a small amount of extracellular (hypertonic) saline in the packed cells prior to lysis. From the literature values of normal hemoglobin, Mg^{2+}, ATP, and DPG levels in fresh human erythrocytes (Pennell, 1974; Beutler, 1984), the Mg^{2+}, ATP, and DPG concentrations in the undiluted cell lysate (per liter of water) were estimated as 2.8, 2.8, and 8.1 mM, respectively.

Figure 8 shows the effects of a solution comprised of the major intracellular ions (excluding hemoglobin) on the ^{31}P

NMR chemical shifts of the phosphoryl compounds under investigation. K^+, Na^+, Mg^{2+}, ATP, and DPG were combined to give concentrations in the same proportions as in the concentrated cell lysate. The concentration of the intracellular ions is expressed in terms of the concentration of K^+. The chemical shifts were again measured relative to that of TEP and are quoted relative to their value in the absence of the intracellular ions ($\delta_{[K^+]=0}$). With the exception of TMP, the chemical shifts of the phosphoryl compounds *increased* relative to that of TEP as the concentration of intracellular ions was increased. The straight lines were fitted by using linear regression ($\delta - \delta_{[K^+]=0} = A[K^+]$).

The solid lines in Figure 7 (with the exception of that for DPP) were drawn by using quadratic equations ($\delta - \delta_{[Hb]=0} = B[Hb]^2 + C[Hb]$) fitted to the purified hemoglobin data (closed symbols) for each of the compounds. The solid line describing the variation of δ^{DPP} with purified hemoglobin concentration was fitted visually. The broken lines represent an attempt to simulate the variation of the phosphoryl chemical shifts with cell lysate concentration (open symbols) by adding together their dependence on purified hemoglobin concentration and their dependence on the concentration of the other major intracellular ions in the absence of hemoglobin. The simulations take account of the dependence of both the amount of intracellular ions present and the fractional water volume on the proportion of lysate present in the sample. The fractional water volume of each sample (α) may be written in terms of the hemoglobin concentration ([Hb]):

$$\alpha = ([Hb]/[Hb]_L)(\alpha_L - 1) + 1 \quad (1)$$

where α_L is the fractional water volume and $[Hb]_L$ is the hemoglobin concentration in the undiluted lysate. The concentration of the intracellular ions in the lysate (expressed in terms of the concentration of K^+ per liter of water) is therefore given by the expression

$$[K^+] = (\alpha_L/\alpha)[K^+]_L([Hb]/[Hb]_L) = \frac{\alpha_L[K^+]_L([Hb]/[Hb]_L)}{([Hb]/[Hb]_L)(\alpha_L - 1) + 1} \quad (2)$$

where $[K^+]_L$ represents the concentration of ions (per liter of water) in the concentrated cell lysate and $\alpha_L[K^+]_L$ is therefore the concentration of the ions with respect to the total lysate volume.

If both the hemoglobin and the intracellular ions exerted the same effect on the phosphoryl chemical shifts when together in the lysate as was observed in the purified hemoglobin and model ion solutions, then simple addition of the two effects should yield the observed dependence of the chemical shifts on lysate concentration. Thus, the variation of chemical shifts (measured relative to TEP and quoted relative to their value in the absence of lysate) with lysate concentration (expressed in terms of the lysate hemoglobin concentration) should be described by the equation

$$\delta - \delta_{[Hb]=0} = A\left[\frac{\alpha_L[K^+]_L([Hb]/[Hb]_L)}{([Hb]/[Hb]_L)(\alpha_L - 1) + 1}\right] + B[Hb]^2 + C[Hb] \quad (3)$$

where A is the coefficient describing the linear dependence of the chemical shifts on the ion concentrations in the model solutions (Figure 8) and B and C are the coefficients describing the quadratic dependence of the chemical shifts on hemoglobin concentration in the purified hemoglobin solutions (Figure 7, solid lines). The broken lines in Figure 7 (with the exception of that for DPP) were drawn by using eq 3. In the case of DPP, for which the variation of chemical shift with purified hemoglobin could not be properly described by a quadratic equation, the quadratic expression in eq 3 (i.e., $B[Hb]^2 + C[Hb]$) was replaced by the curve fitted visually (Figure 7).

Discussion

Extracellular Chemical Shift Effects. From Figures 6–8 it is evident that the components of neither the extracellular solution nor the cell cytoplasm caused any variation in the ^{31}P NMR chemical shift of TMP relative to that of TEP (which served, in these experiments, as an internal chemical shift reference). This invariance suggests that, as with TEP, the chemical shift of TMP in such solutions varies solely in response to variations in magnetic susceptibility.

Figure 2 shows the variation of intra- and extracellular chemical shifts with mean cell volume. As the cell volume was reduced, the chemical shift of the extracellular TMP resonance decreased; the implication is, therefore, that the decrease in the mean cell volume was accompanied by a decrease in the bulk magnetic field strength in the external compartment. The extracellular magnetic field strength is influenced by the composition (susceptibility) of both the intra- and extracellular compartments as well as the hematocrit (Fabry & San George, 1983; Labotka, 1984; Kirk & Kuchel, 1988a). The decrease in the extracellular field strength, with decreasing cell volume, therefore reflects the combined effects of the increasing extracellular concentration of (diamagnetic) sucrose, the increasing concentration of intracellular components [particularly (carbonmonoxy)hemoglobin], and the decreasing hematocrit.

The cell volume dependence of the *extracellular* chemical shifts of DMMP, DEMP, and PP in the intact cell suspensions was virtually identical with that of TMP, and the same was true in the *isolated* extracellular solutions (Figure 6). Like TMP, the chemical shift behavior of the extracellular resonances of these three compounds may therefore be accounted for in terms of the bulk magnetic field strength in the extracellular solution. The same is *not* true, however, for the chemical shift behavior of TMPO, HP, and DPP. In the cell suspensions (Figure 2) the chemical shifts of the extracellular resonances of TMPO, HP, and DPP *increased* as the mean cell volume was reduced by an increase in the extracellular sucrose concentration. Similarly, in the isolated extracellular solutions (Figure 6) the chemical shifts of all three increased relative to that of TEP as the sucrose concentration increased. Variation in chemical shifts relative to that of TEP is unrelated to general magnetic susceptibility effects. The explanation for the variation of the chemical shifts of TMPO, HP, and DPP with the changing chemical composition of the solution must therefore lie elsewhere.

Intracellular Chemical Shift Effects. As the mean cell volume was reduced, the *intracellular* resonances of all seven compounds giving rise to separate intra- and extracellular resonances moved to lower frequency (Figure 2). The migration to lower frequency of the intracellular TMP resonance infers that the magnetic field strength in the internal compartment decreased as the cells were shrunk. This decrease was primarily a consequence of the increasing concentration of diamagnetic (carbonmonoxy)hemoglobin inside the cell but also reflects the increasing concentration of the other (less diamagnetic) intracellular components as well as the decreasing susceptibility of the extracellular solution.

The chemical shifts of the intracellular resonances of the other six compounds decreased (with decreasing cell volume)

by a much greater amount than that of TMP (Figure 2). The same trend is seen in the cell lysate experiment (Figure 7, open symbols) in which the chemical shift of TMP (relative to that of TEP) remained invariant with lysate concentration while the shifts of the other six compounds decreased by varying amounts. It is evident from these experiments that one or more of the intracellular components caused the phosphoryl resonances (other than that of TMP) to shift to lower frequency by a mechanism that is unrelated to bulk susceptibility effects. From the purified hemoglobin experiment (Figure 7, closed symbols) it is apparent that hemoglobin was the component primarily responsible for the susceptibility-independent effects. In most cases, however, there was a significant difference between the lysate and purified hemoglobin data, indicating that the shift effects in the lysate cannot be *fully* explained in terms of the hemoglobin alone.

From Figure 8 it is evident that the principal intracellular ions exerted a *direct* (susceptibility-independent) effect on the chemical shifts of the phosphoryl compounds (other than TMP); the resonances moved to higher frequency as the concentration of ions was increased. The chemical shift variations were much less than those seen with hemoglobin but are, nonetheless, significant. The broken lines in Figure 7 represent an attempt to simulate the chemical shift effects observed in the lysate by adding together the direct effect of hemoglobin on chemical shifts and the direct effect of the principal intracellular ions. Qualitatively, the simulations all predict that the shifts to lower frequency seen in the lysate should be less than those in the purified hemoglobin, as was indeed observed. Quantitatively, there is, in most cases, a small difference between the simulations and the lysate data. The discrepancies may be due to a number of factors. It is possible that one of the many compounds present in the lysate at very low concentrations and (consequently) omitted from the model intracellular ion solutions caused significant variations in the phosphoryl chemical shifts. Another, more likely, explanation is that in addition to their *direct* effects on chemical shifts the intracellular ions exerted an *indirect* effect by influencing the manner in which hemoglobin affected the chemical shifts. It should be recognized that because the purified hemoglobin was dialyzed against distilled water, the ionic strength in the hemoglobin samples was less than that in the corresponding lysate samples. Ionic strength may affect the structure of hemoglobin as well as the possible interaction of the phosphoryl compounds (particularly those bearing a net charge) with the protein; either effect may be responsible for the discrepancies between the curves.

Temperature Effects. The temperature dependence of the transmembrane chemical shift differences (Figures 4 and 5) provides some insight into the membrane transport properties of a number of these compounds. The NMR saturation-transfer technique has been shown to provide a convenient means of measuring the equilibrium exchange of DMMP across the cell membrane (Kirk & Kuchel, 1986), and recently the technique has been used to show that this compound permeates the membrane principally by simple diffusion through the lipid bilayer (Potts, Kirk, and Kuchel, unpublished results). TMP, TEP, DEMP, and TMPO are, like DMMP, small nonelectrolytes, and it is perhaps reasonable to expect that these too should cross the membrane by simply dissolving in and diffusing through the bilayer.

As has been shown previously, the failure of the intra- and extracellular populations of TEP to give rise to separate ^{31}P NMR resonances (despite the significant differences in the effective magnetic susceptibilities of the intra- and extracellular compartments) is a consequence of its fast exchange across the cell membrane (Kirk & Kuchel, 1987). In the case, of TMP, at temperatures below about 35 °C, the difference in the susceptibility of the two compartments *did* result in a significant separation between the intra- and extracellular resonances. However, at temperatures above 35 °C it too was in fast exchange on the NMR time scale and only a single (weighted average) resonance is observed. As has been discussed, the intrinsic intra- and extracellular chemical shifts of TEP differ by the same amount as those of TMP. Assuming that the ^{31}P NMR T_2 values for the two compounds are similar, then the observation of separate intra- and extracellular resonances for TMP (at temperatures below 35 °C) and only a single resonance for TEP suggests that TEP crosses the cell membrane faster than TMP (Sandström, 1982). This is entirely consistent with the hypothesis that such compounds cross the cell membrane by simple diffusion. The simple diffusion process is currently understood in terms of the partitioning of the compound from the aqueous phase into the bilayer, followed by diffusion through the lipid phase (Lieb & Stein, 1986a,b; Walter & Gutknecht, 1986). In the simplest possible model, the membrane permeability coefficient (P) for the compound is given by the expression

$$P = KD_{mem}/\lambda \quad (4)$$

where K is the membrane–water partition coefficient, D_{mem} is the diffusion coefficient of the compound within the membrane, and λ is the thickness of the membrane [eq 2.5, Lieb and Stein (1986a)].

The apolar solvent *n*-hexadecane provides a good model for the partitioning properties of the red cell membrane. Lieb and Stein (1986a) have demonstrated that a plot of the logarithm of the red cell membrane permeability coefficients (corrected for the effects of molecular volume on D_{mem}) against the logarithm of the hexadecane–water partition coefficient for a range of compounds yields a straight line of unit slope (r = 0.98). Within a homologous series of compounds the addition of a methylene group (CH_2) causes the hexadecane–water partition coefficient to increase approximately 4-fold (Lieb & Stein, 1986a). TEP has three more methylene groups than TMP, and it might therefore be expected to have a hexadecane–water partition coefficient (and therefore a membrane–water partition coefficient) some 4^3 (i.e., 64) times greater than that of TMP.

The van der Waals volume of TEP (99.6 cm^3 mol^{-1}) is 1.43 times greater than that of TMP (69.5 cm^3 mol^{-1}). For the human red cell membrane, the membrane diffusion coefficient is found to be related to the van der Waals volume (V) of the compound by the expression [eq 2.7, Lieb and Stein (1986a)]

$$\log [D_{mem}\ (\text{cm}^2\ \text{s}^{-1})] = -4.8861 - 0.0516\ [V\ (\text{cm}^3\ \text{mol}^{-1})] \quad (5)$$

The membrane diffusion coefficient of TMP will therefore be some 35.7 times higher than that of TEP. While this, in itself, will tend to favor the transport of TMP over that of TEP, the 64-fold difference in their membrane–water partition coefficients will result in the equilibrium exchange rate for TEP being almost twice that for TMP (eq 4).

A similar analysis can be applied in the case of DMMP and DEMP. DEMP has two more methylene groups than DMMP, and it might therefore be expected to have a membrane–water partition coefficient roughly 4^2 (i.e., 16) times that of DMMP. The van der Waals volumes of DEMP and DMMP are 84.7 and 64.7 cm^3 mol^{-1}, respectively, and the membrane diffusion coefficient of DMMP will therefore be some 10.8 times higher

than that of DEMP (eq 5). As before, however, the diffusional advantage of the smaller molecule does not compensate for the much greater solubility of the larger molecules in the bilayer, and the equilibrium exchange rate for DEMP will be some one and a half times that for DMMP (eq 4).

The effects of the different transport rates for DMMP and DEMP are again evident in the temperature dependence of the transmembrane chemical shift differences (Figure 4). The intra- and extracellular resonances of DEMP coalesced at around 48 °C, indicating the onset of the fast-exchange condition. At this temperature the transmembrane exchange of DMMP was still sufficiently slow for separate intra- and extracellular peaks to be resolved, though at temperatures above about 33 °C, $\Delta\delta^{DMMP}$ decreased with increasing temperature and the resonances broadened, indicating the onset of the intermediate-exchange condition. If the ^{31}P NMR T_2 values for the two compounds are similar, then the onset of the fast-exchange condition for DEMP at a temperature at which the transport of DMMP falls within the intermediate-exchange regime infers that the membrane permeability of DEMP is greater than that of DMMP.

Summary. From the experiments reported here it is apparent that the observed transmembrane ^{31}P NMR chemical shift differences and their variation with cell volume may be attributed primarily (though not entirely) to the action of hemoglobin. The presence of [oxy- or (carbonmonoxy)-] hemoglobin inside the cell gave rise to a significant difference in the magnetic susceptibilities of the internal and external compartments, and the difference (as reflected in the difference between the intra- and extracellular chemical shifts of TMP) increased as the mean cell volume was reduced and the intracellular hemoglobin concentration thereby increased. In addition to its effects on bulk susceptibility, hemoglobin caused large shifts to lower frequency of the ^{31}P NMR resonances of DMMP, DEMP, TMPO, HP, PP, and DPP while leaving those of TMP and TEP unaffected. As with susceptibility effects, the magnitude of these susceptibility-independent effects increased with increasing hemoglobin concentration and, therefore, with decreasing mean cell volume. A number of other intra- and extracellular solutes have been shown to cause (susceptibility-independent) variations in the phosphoryl chemical shifts; however, the magnitude of these variations was, in most cases, much less than those arising from the effects of hemoglobin.

In this paper we have identified the various shift effects that are operating in a cell suspension and that combine to give the observed transmembrane chemical shift differences. The actual physical basis of these effects remains to be elucidated. In the following paper (Kirk & Kuchel, 1988b) we describe a series of experiments that have led to a hypothesis for the mechanism by which hemoglobin exerts a susceptibility-independent effect on phosphoryl chemical shifts.

Registry No. TMP, 512-56-1; TEP, 78-40-0; DMMP, 756-79-6; DEMP, 683-08-9; TMPO, 676-96-0; HP, 15460-68-1; PP, 16002-14-5; DPP, 18357-17-0; DPG, 138-81-8; ATP, 56-65-5; Na, 7440-23-5; K, 7440-09-7; Mg, 7439-95-4.

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