Physical Basis of the Effect of Hemoglobin on the ^{31}P NMR Chemical Shifts of Various Phosphoryl Compounds†

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ABSTRACT: The marked difference between the intra- and extracellular ^{31}P NMR chemical shifts of various phosphoryl compounds when added to a red cell suspension may be largely understood in terms of the effects of hemoglobin on the ^{31}P NMR chemical shifts. The presence of [oxy- or (carbonmonoxy)-] hemoglobin inside the red cell causes the bulk magnetic susceptibility of the cell cytoplasm to be significantly less than that of the external solution. This difference is sufficient to account for the difference in the intra- and extracellular chemical shifts of the two phosphate esters trimethyl phosphate and triethyl phosphate. However, in the case of the compounds dimethyl methylphosphonate, diethyl methylphosphonate, and trimethylphosphine oxide as well as the hypophosphite, phenylphosphinate, and diphenylphosphinate ions, hemoglobin exerts an additional, much larger, effect, causing the ^{31}P NMR resonances to shift to lower frequency in a manner that cannot be accounted for in terms of magnetic susceptibility. Lysozyme is a protein structurally unrelated to hemoglobin and was shown to cause similar shifts to lower frequency of the resonances of these six compounds; this suggests that the mechanism may involve a property of proteins in general and not a specific property of hemoglobin. The effect of different solvents on the chemical shifts of the eight phosphoryl compounds provided an insight into the possible physical basis of the effect. The ^{31}P NMR chemical shifts of the two phosphate esters were entirely insensitive to the nature of the solvent whereas those of the other six compounds showed a very strong solvent dependence which is understood, primarily, in terms of the number and strength of hydrogen bonds formed between the solvent molecules and the phosphoryl oxygen atoms. It is therefore proposed that, in addition to magnetic susceptibility effects, hemoglobin exerts its influence on phosphoryl chemical shifts by disrupting the hydrogen bonding of the phosphoryl group to solvent water.

When added to a suspension of intact red cells, a number of compounds containing the phosphoryl functional group give rise to separate intra- and extracellular ^{31}P NMR resonances, the separation between which increases as the mean cell volume is decreased. The phenomenon has been shown to have a number of applications in the study of the red cell by ^{31}P NMR spectroscopy (Kirk & Kuchel, 1984, 1985; Labotka & Omachi, 1987, 1988; Raftos et al., 1988; Kirk et al., 1988), and it is therefore important that the physical basis of the effect be properly understood.

It has been demonstrated that when intracellular hemoglobin is in the fully diamagnetic (oxy or carbonmonoxy) state, the red cell cytoplasm is more diamagnetic than the extracellular solution and that the magnetic field strength in the internal compartment is therefore lower than that in the external compartment (Kirk & Kuchel, 1988a). This effect certainly contributes to the observed transmembrane chemical shift differences but, in most cases, is not solely (nor even mainly) responsible. In the preceding paper (Kirk & Kuchel, 1988b) it was demonstrated that, in addition to magnetic susceptibility effects, both intra- and extracellular solutes influence, to different extents, the chemical shifts of a number of the phosphoryl compounds. Hemoglobin in particular was seen to cause large (susceptibility-independent) shifts to lower frequency of most of the phosphoryl resonances and was therefore identified as being primarily responsible for the observed transmembrane shift differences and their dependence on the mean cell volume. In this paper we have attempted to elucidate the actual mechanism(s) by which hemoglobin exerts its substantial susceptibility-independent influence on the phosphoryl chemical shifts.

Experimental Procedures

Materials. The phosphoryl compounds under study were obtained as reported in the preceding paper (Kirk & Kuchel, 1988b). Diethyl [(ethoxycarbonyl)methyl]phosphonate [$(C_2H_5O)_2P(O)CH_2CO_2C_2H_5$] was obtained from Aldrich, Milwaukee, WI. Egg white lysozyme was obtained from Sigma Chemical Co. (St Louis, MO). The various solvents used in the study of the effects of solvent on ^{31}P NMR chemical shifts were all of analytical reagent grade.

Solutions. The hypophosphite, phenylphosphinate, and diphenylphosphinate anions were added to samples in the form of a concentrated aqueous, equimolar solution (~750 mM), adjusted to a pH of ~7.3 with NaOH (10 M). The five nonelectrolytes were added in the form of a concentrated, equimolar mixture (1.84 M). All solutions were made up within the 24-h period preceding each experiment. Purified (carbonmonoxy)hemoglobin solutions were prepared and the hemoglobin concentrations measured as described in the preceding paper (Kirk & Kuchel, 1988b). Lysozyme solutions were prepared by dissolving crystalline egg white lysozyme (2.5 g) in an equimolar aqueous solution of the eight phosphoryl compounds (20 mM, 5.0 mL) to which had been added low concentrations of antibiotics (2.5 mg L^{-1} amphotericin B, 27 mg L^{-1} penicillin G, and 50 mg L^{-1} streptomycin sulfate). The lysozyme solution was subsequently combined in varying proportions with the original equimolar solution to yield a number of samples (0.5 mL) having a range of lysozyme concentrations and a fixed concentration of the eight phosphoryl compounds. The final lysozyme concentrations were

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estimated from the absorbance at 280 nm ($A_{280}^{1\%}$ = 25.32; Bruzzesi et al., 1965).

NMR Methods. Broad-band proton-decoupled ^{31}P NMR spectra were obtained as described in the preceding paper (Kirk & Kuchel, 1988b). The ^{31}P NMR chemical shift of TEP has been shown to be entirely insensitive to the factors (other than magnetic susceptibility) giving rise to transmembrane chemical shift differences in intact red cell suspensions (Kirk & Kuchel, 1988a). TEP thus served as an effective internal chemical shift reference in experiments in which the influence of factors other than bulk sample susceptibility on chemical shifts was examined.

In the study of the effects of solvent on the ^{31}P NMR chemical shifts of the nonelectrolyte phosphoryl compounds (including that of TEP) the very large variation in the magnetic susceptibilities of the different solvents necessitated the use of a spherical/cylindrical reference capillary (Wilmad Glass Co., Inc., Buena, NJ) as described by Frei and Bernstein (1962). The capillary was filled with a reference solution of diethyl [(ethoxycarbonyl)methyl]phosphonate (0.75 M) in acetone. Acetone has a much smaller diamagnetic susceptibility than most of the other solvents used in the study, and the marked difference between the magnetic susceptibility of the reference solution and those of the different solvent samples ensured that the ^{31}P NMR signals arising from the cylindrical and spherical portions of the capillary were well separated from one another. The ^{31}P NMR chemical shifts of the phosphoryl compounds in the different solvent samples were measured relative to that of diethyl [(ethoxycarbonyl)methyl]-phosphonate in the spherical portion of the capillary. The use of a spherical "external" reference capillary eliminates the contribution of sample susceptibility to the measured chemical shifts (Lagodzinskaya & Klimenko, 1982). A certain amount of error is introduced by imperfections in the spherical interface (Lagodzinskaya & Klimenko, 1982); however, any such error was judged to be small relative to the very large chemical shift variations observed between solvents.

Results

Effect of Protein Solutions on Chemical Shifts and Line Widths. Figure 1 shows the effects of increasing concentrations of lysozyme and (carbonmonoxy)hemoglobin on the ^{31}P NMR chemical shifts of TMP,[1] DMMP, DEMP, TMPO, HP, PP, and DPP. The shifts were measured relative to that of internal TEP, thus correcting for chemical shift variations arising from the large diamagnetic susceptibilities of the two proteins, and are quoted relative to their value in the absence of protein ($\delta_{[protein]=0}$). The chemical shift of TMP remained invariant with either protein concentration, whereas both lysozyme and hemoglobin caused the chemical shifts of the other six compounds to decrease. Each curve, with the exception of that describing the variation of δ^{DPP} with hemoglobin concentration, was drawn using a quadratic equation fitted to the data by linear regression ($\delta - \delta_{[protein]=0} = A[protein]^2 + B[protein]$). The data representing the variation of δ^{DPP} with hemoglobin concentration could not be properly described by a simple quadratic function and were fitted visually.

Figure 2 was derived from the same experiment as Figure 1 and shows the effects of the two proteins on the line widths of the ^{31}P NMR resonances of all eight phosphoryl compounds.

[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; Hb, hemoglobin.

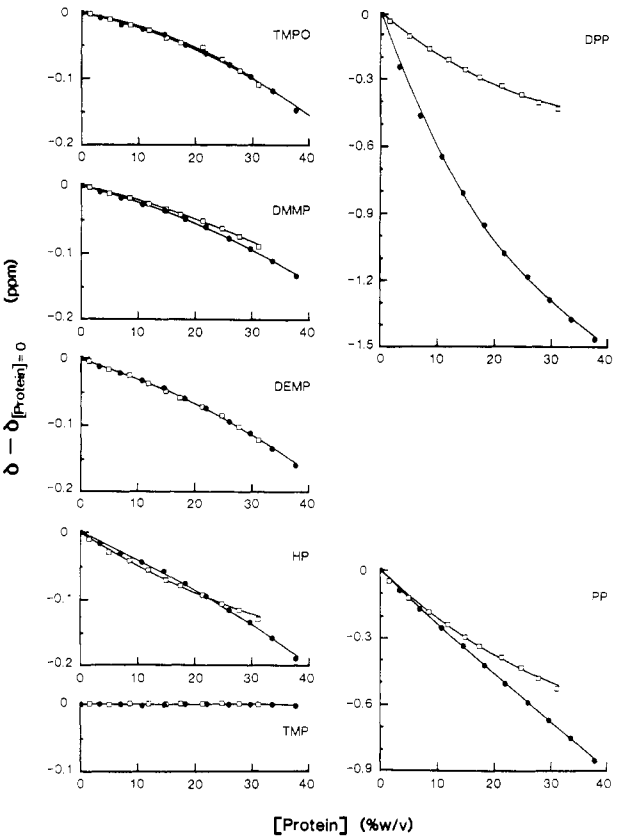

FIGURE 1: Effects of lysozyme (open symbols) and purified hemoglobin (closed symbols) on ^{31}P NMR chemical shifts. The lysozyme samples were prepared as described under Experimental Procedures. The concentrated hemoglobin solution [prepared as described in the preceding paper (Kirk & Kuchel, 1988b)] was diluted by varying amounts in distilled water, and the eight phosphoryl compounds were added, each to a concentration of 10 mM. Chemical shifts were measured relative to that of TEP and are quoted relative to their value in the absence of protein ($\delta_{[protein]=0}$). The curves were drawn by using quadratic equations fitted to the data ($\delta - \delta_{[protein]=0} = A[protein]^2 + B[protein]$). The coefficients of the equations fitted to the lysozyme data took the following values: TMP, $A = -8.7 \times 10^{-6}$, $B = 0.3 \times 10^{-3}$; DMMP, $A = -3.29 \times 10^{-5}$, $B = -1.8 \times 10^{-3}$; DEMP, $A = -4.33 \times 10^{-5}$, $B = -2.5 \times 10^{-3}$; TMPO, $A = -6.05 \times 10^{-5}$, $B = -1.5 \times 10^{-3}$; HP, $A = 4.38 \times 10^{-5}$, $B = -5.4 \times 10^{-3}$; PP, $A = 2.19 \times 10^{-4}$, $B = -2.34 \times 10^{-2}$; DPP, $A = 2.38 \times 10^{-4}$, $B = -2.08 \times 10^{-2}$. The coefficients of the equations fitted to the hemoglobin data took the following values: TMP, $A = -4.7 \times 10^{-7}$, $B = 0$; DMMP, $A = -3.81 \times 10^{-5}$, $B = -2.0 \times 10^{-3}$; DEMP, $A = -4.61 \times 10^{-5}$, $B = -2.4 \times 10^{-3}$; HP, $A = -5.40 \times 10^{-5}$, $B = -1.7 \times 10^{-3}$; HP, $A = -2.66 \times 10^{-5}$, $B = -3.8 \times 10^{-3}$; PP, $A = 5.08 \times 10^{-5}$, $B = -2.43 \times 10^{-2}$. The line representing the variation of δ^{DPP} with hemoglobin concentration was fitted visually. Note that the ordinate scale for the two aromatic compounds (PP and DPP) differs from that of the nonaromatic compounds.

Effects of Solvent on Chemical Shifts. Table I shows the ^{31}P NMR chemical shifts of the five nonelectrolyte phosphoryl compounds when dissolved (each at a concentration of 50 mM) in a variety of solvents. The chemical shifts were measured relative to that of diethyl [(ethoxycarbonyl)methyl]-phosphonate present in the spherical portion of an external spherical/cylindrical capillary; this sample configuration eliminates the contribution of the different magnetic susceptibilities of the various solvents to chemical shift variations (Frei & Bernstein, 1962). The shifts are quoted relative to their value in water. The chemical shift of TMPO varied over a range of 56.2 ppm, that of DMMP over a range of 18.4 ppm, that of DEMP over a range of 19.7 ppm, that of TMP over a range of 1.5 ppm, and that of TEP over a range of 1.6 ppm. In Figure 3 the chemical shifts of TEP, TMP, DMMP, and DEMP are plotted against the corresponding shift of TMPO

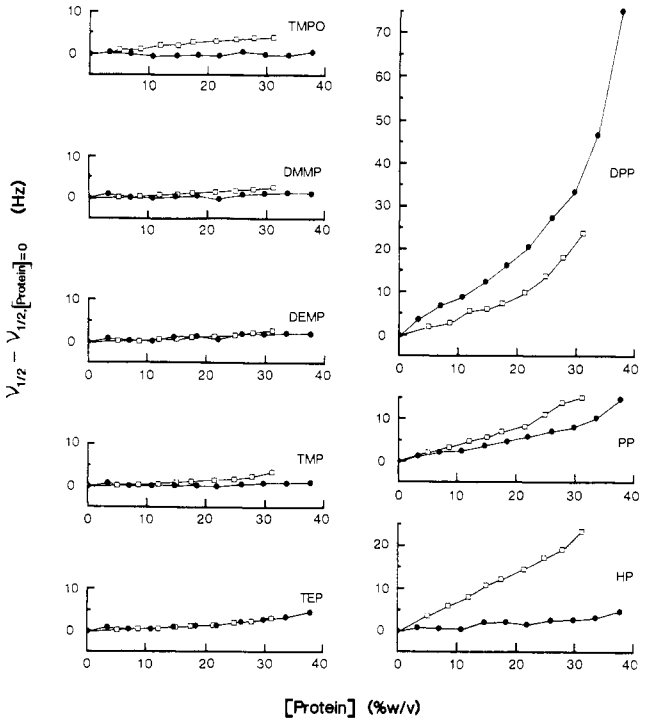

FIGURE 2: Effects of lysozyme (open symbols) and purified hemoglobin (closed symbols) on ^{31}P NMR line widths. The data were derived from the same experiment as Figure 1. The ordinate shows the measured width at half-height ($\Delta\nu_{1/2}$) from which had been subtracted the corresponding line width in the absence of protein ($\Delta\nu_{1/2,[protein]=0}$).

Table I: Effects of Solvent on the ^{31}P NMR Chemical Shifts of the Five Nonelectrolyte Phosphoryl Compounds (Each at a Concentration of 50 mM)[a]

	$\delta - \delta_{H_2O}$ (ppm)				
solvent	TEP	TMP	DMMP	DEMP	TMPO
heptane	0.1	−0.2	−7.3	−7.9	–[b]
cyclohexane	0	−0.2	−7.3	−7.8	–[b]
benzene	−0.1	−0.4	−6.6	−7.1	−20.5
carbon tetrachloride	−0.2	−0.5	−6.5	−7.1	−19.7
acetone	−0.8	−0.9	−6.3	−6.6	−17.7
chloroform	−0.6	−0.7	−4.8	−5.1	−13.2
tert-butyl alcohol	−1.5	−1.5	−5.4	−5.6	−10.1
isobutyl alcohol	−1.3	−1.4	−5.0	−5.2	−8.8
2-propanol	−1.3	−1.4	−4.9	−5.1	−8.6
1-pentanol	−1.0	−1.1	−4.4	−4.7	−7.4
1-butanol	−1.1	−1.2	−4.4	−4.7	−7.3
1-propanol	−1.1	−1.2	−4.3	−4.6	−7.2
ethanol	−1.2	−1.2	−4.3	−4.5	−7.0
methanol	−1.0	−1.0	−3.4	−3.6	−5.3
water	0	0	0	0	0
deuterium oxide	0.1	0	0	0	0
acetic acid	−1.3	−1.3	−2.4	−2.6	0.5
sulfuric acid	−0.4	−0.4	11.1	11.8	35.7

[a] The shifts were measured relative to that arising from a spherical "external" reference and are quoted relative to that in water (δ_{H_2O}). [b] Insoluble.

in the different solvents. The straight lines were fitted to the data by linear regression, and the slopes provide a quantitative measure of the sensitivity of the chemical shift of each compound to the nature of the solvent (Maciel & Natterstad, 1965). For TEP the line has a slope of 0.003 ± 0.011, for TMP 0.008 ± 0.010, for DMMP 0.345 ± 0.020, and for DEMP 0.325 ± 0.019. The corresponding line for TMPO has (by definition) a slope of 1.000.

The complete insensitivity of the ^{31}P NMR chemical shift of TEP to solvent effects confirms its suitability as an internal chemical shift reference.

As with DMMP, DEMP, and TMPO the ^{31}P NMR chemical shifts of the three ionized phosphoryl compounds showed a marked dependence on the nature of the solvent. Figure 4 shows the chemical shift behavior of both charged and uncharged phosphoryl compounds as water was replaced by

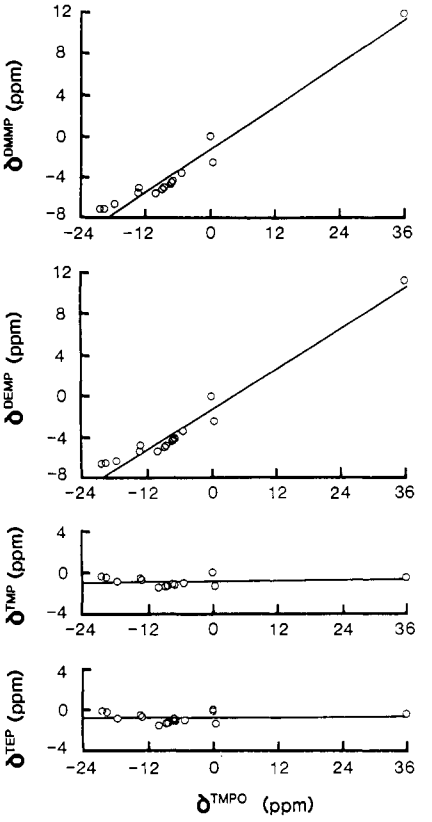

FIGURE 3: Chemical shifts of TEP, TMP, DMMP, and DEMP as a function of the corresponding shifts of TMPO in a range of different solvents. The data were taken from Table I, and the straight lines were fitted by linear regression. Chemical shifts are quoted relative to that in water. The slopes of the lines for TEP, TMP, DMMP, and DEMP are 0.003 ± 0.011, 0.008 ± 0.010, 0.345 ± 0.020, and 0.325 ± 0.019, respectively.

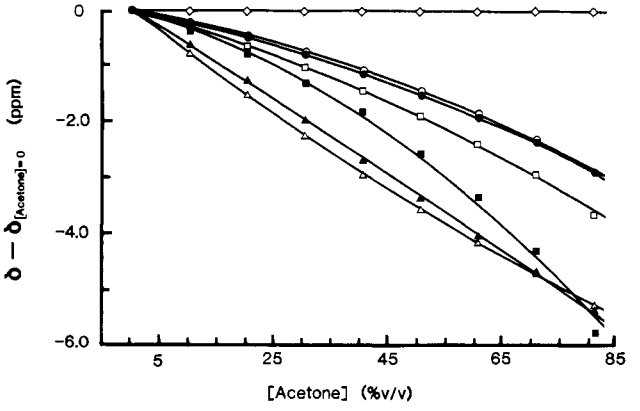

FIGURE 4: Effects of acetone on ^{31}P NMR chemical shifts. A series of samples was prepared having a fixed equimolar concentration (8 mM) of the eight phosphoryl compounds and varying proportions of acetone and water. The chemical shifts were measured relative to that of TEP and are quoted relative to that in the absence of acetone ($\delta_{[acetone]=0}$). The symbols for the different compounds are as follows: ◇, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP. The curves were drawn by using quadratic equations fitted to the data ($\delta - \delta_{[acetone]=0} = C[\text{acetone}]^2 + D[\text{acetone}]$). The coefficients of the fitted equations have the following values: TMP, $C = 3.3 \times 10^{-6}$, $D = -0.5 \times 10^{-3}$; DMMP, $C = -2.24 \times 10^{-4}$, $D = -1.76 \times 10^{-2}$; DEMP, $C = -1.82 \times 10^{-4}$, $D = -2.09 \times 10^{-2}$; TMPO, $C = -5.04 \times 10^{-4}$, $D = -2.73 \times 10^{-2}$; HP, $C = -2.08 \times 10^{-4}$, $D = -2.76 \times 10^{-2}$; PP, $C = -4.32 \times 10^{-5}$, $D = -6.4 \times 10^{-2}$; DPP, $C = 1.79 \times 10^{-4}$, $D = -8.00 \times 10^{-2}$.

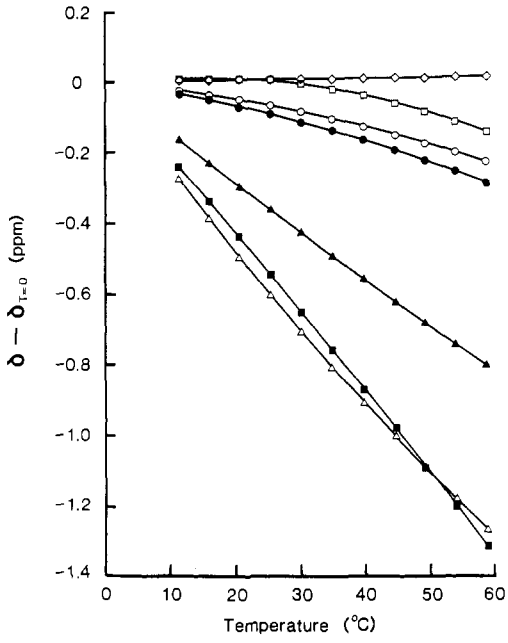

FIGURE 5: Effects of temperature on the ^{31}P NMR chemical shifts of the phosphoryl compounds in aqueous solution. Each of the phosphoryl compounds was present at a concentration of 8 mM. The chemical shifts were measured relative to that of TEP and are quoted relative to their (extrapolated) value at 0 °C ($\delta_{T=0}$). The symbols for the different compounds are as follows: ◇, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP. The curves were drawn by using quadratic equations fitted to the data [$\delta - \delta_{T=0} = E(T)^2 + F(T)$]. The coefficients of the fitted equations have the following values: TMP, $E = -8.8 \times 10^{-7}$, $F = 0.3 \times 10^{-3}$; DMMP, $E = -3.45 \times 10^{-5}$, $F = -1.8 \times 10^{-3}$; DEMP, $E = -3.54 \times 10^{-5}$, $F = -2.8 \times 10^{-3}$; TMPO, $E = -2.55 \times 10^{-5}$, $F = -2.1 \times 10^{-2}$; HP, $E = -8.21 \times 10^{-5}$, $F = 2.5 \times 10^{-3}$; PP, $E = 1.99 \times 10^{-5}$, $F = -1.49 \times 10^{-2}$; DPP, $E = 7.33 \times 10^{-5}$, $F = -2.61 \times 10^{-2}$.

acetone as the solvent. The shifts were measured relative to that of TEP and are quoted relative to that in the absence of acetone ($\delta_{[acetone]=0}$). The chemical shift of TMP was unaffected by the increasing concentration of acetone whereas those of the other six compounds decreased by varying amounts. Each curve was drawn by using a quadratic equation fitted to the data by nonlinear regression ($\delta - \delta_{[acetone]=0} = C[acetone]^2 + D[acetone]$).

Effect of Temperature on Chemical Shifts. Figure 5 shows the temperature dependence of the chemical shifts of the phosphoryl compounds in aqueous solution. These shifts were again measured relative to that of TEP and are quoted relative to their (extrapolated) value at 0 °C ($\delta_{T=0}$).

Discussion

Effect of Protein Solutions on Chemical Shifts and Line Widths. (Carbonmonoxy)hemoglobin has a large diamagnetic susceptibility, and its presence inside the intact erythrocyte causes the magnetic susceptibility of the intracellular compartment to be less than that of the external compartment. The difference in the effective magnetic susceptibilities of the two compartments is sufficient to account for the difference between the intra- and extracellular ^{31}P NMR chemical shifts of the two phosphate esters, TMP and TEP. However, the transmembrane chemical shift differences observed for the remaining six phosphoryl compounds under investigation are substantially larger than can be accounted for by bulk susceptibility effects alone. In the preceding paper it was demonstrated that in addition to a general susceptibility effect hemoglobin exerts a direct (susceptibility-independent) effect on the chemical shifts of these six compounds and that, in most cases, it is *this* effect that is primarily responsible for the observed transmembrane chemical shift differences and their dependence on the mean cell volume.

From Figure 1 it is evident that lysozyme, like hemoglobin, exerts a susceptibility-independent effect on the ^{31}P NMR chemical shift of the majority of the phosphoryl compounds. In the case of the nonelectrolytes the lysozyme and hemoglobin had very similar effects on the phosphoryl chemical shifts. In the case of the three electrolytes there were significant differences in the effects of the two proteins. Furthermore, the general character of the dependence of the chemical shifts of the electrolytes on protein concentration was (in most cases) quite different from that seen for the nonelectrolytes. Figure 2 provides some insight into the origin of this difference. The line width ($\Delta\nu_{1/2}$) of an NMR resonance is inversely proportional to the transverse relaxation time (T_2) of the nuclear population giving rise to the resonance [e.g., Dwek (1973)]. The binding of a small molecule to a macromolecule may cause a substantial reduction of the T_2 of the nuclei at the bound site and, therefore, an increase in the line width. If there is fast chemical exchange between the bound and free species, then the nuclei at the two sites will give rise to a single resonance representing a weighted average of that in each environment (Dwek, 1973). The fast-exchange binding of a small molecule (such as the phosphoryl compounds under investigation) to a macromolecule (such as a protein) may therefore cause the NMR resonance arising from the small molecule to broaden.

The quantitative interpretation of NMR line broadening data in terms of binding constants is not at all straightforward and generally requires a knowledge of the chemical shifts of the nuclei at the free and bound sites as well as some knowledge of the rate of exchange between sites (Lanir & Navon, 1971; Dwek, 1973). Furthermore, the effects of solution viscosity on the line width must be properly understood; an increase in viscosity causes a decrease in the T_2 of the free molecule and a consequent increase in the line width. Nevertheless, at a qualitative level, the broadening of the resonance(s) arising from a small molecule in the presence of an increasing concentration of protein would suggest (providing due account has been taken of the effect of the protein on the solution viscosity) that the small molecule is binding to the protein.

Figure 2 shows the effects of lysozyme and hemoglobin on the ^{31}P NMR line widths of the eight phosphoryl compounds. The line widths of all five nonelectrolytes increased slightly as the protein concentration was increased, and with the possible exception of TMPO, there was little difference between the effects of lysozyme and hemoglobin. The broadening of the resonances of the nonelectrolytes was not large (compared to that seen for the electrolytes), and certainly some, if not all, of the line-width increase must be attributed to the effect of the two proteins on the viscosity of the solution.

In the case of the HP ion, an increasing concentration of hemoglobin had the same slight effect on the ^{31}P NMR line width as was seen for the nonelectrolytes. However, the HP resonance broadened dramatically in the presence of an increasing concentration of lysozyme. In contrast to the nonaromatic compounds, the line widths of the aromatic PP and DPP ions increased markedly with increasing hemoglobin concentration, the latter much more so than the former. Lysozyme was also seen to cause substantial broadening of the resonances of the two aromatic ions.

Although one must be cautious in interpreting such results, the large effect of lysozyme on the HP line width and the large effects of both lysozyme and hemoglobin on the line width of

PP and DPP would seem to indicate that the HP ion undergoes significant binding to lysozyme and that the two aromatic ions, PP and DPP, bind significantly to both proteins.

These conclusions cast some light on the chemical shift behavior of the three electrolytes shown in Figure 1. The curves describing the variation of the chemical shifts of the nonelectrolytes DMMP, DEMP, and TMPO are all concave downward (i.e., the coefficient, A, of the squared term in the fitted quadratic equation is negative). The same is true for the curve describing the variation of the HP chemical shift with hemoglobin concentration. However, in those cases in which the phosphoryl compounds are thought to undergo significant binding to the protein, the opposite is true. The curves describing the variation of the chemical shift of HP with lysozyme concentration and the variation of the chemical shifts of PP and DPP with lysozyme and hemoglobin concentration are all concave upward (i.e., the coefficient, A, in the fitted quadratic equation is positive). The putative binding of the charged phosphoryl compounds to the proteins is seemingly reflected in the character of the variation of the ^{31}P NMR chemical shifts with protein concentration.

The striking similarity between the (susceptibility-independent) effects of lysozyme on the chemical shifts of TMP, DMMP, DEMP, and TMPO and the corresponding effects of hemoglobin would suggest that both proteins exerted their influence on the chemical shifts of the nonelectrolytes via a common mechanism. In other words, the effect of hemoglobin on the nonelectrolyte chemical shifts was not a consequence of any unique property of the hemoglobin molecule but was more likely related to a property of proteins in general. The same mechanism was probably operating to affect the chemical shifts of the electrolytes; however, the variation of the chemical shifts of PP and DPP with hemoglobin concentration and the variation of the chemical shifts of HP, PP, and DPP with lysozyme concentration were evidently complicated by binding effects.

The question remains as to the nature of the general mechanism by which the proteins cause the ^{31}P NMR resonance of the phosphoryl compounds (other than TMP and TEP) to shift to lower frequency. Any such mechanism must account for the complete lack of a susceptibility-independent chemical shift effect in the case of TMP and TEP, despite the obvious structural similarity of these compounds to a number of other compounds (particularly DMMP and DEMP) that show a very strong dependence of chemical shift on hemoglobin concentration. The effect of solvent on the ^{31}P NMR chemical shifts provides some insight into the phenomenon involved.

Effects of Solvent and Temperature on Chemical Shifts. In Table I the solvents are ordered on the basis of their effects on the chemical shift of TMPO. The five solvents uppermost in the table lack any "acidic" hydrogen atom, capable of participating in a hydrogen bond. The remaining solvents all possess a hydrogen atom able to either engage in hydrogen bond formation or, in the case of the strong acid H_2SO_4, effect full proton transfer. The chemical shift ranges of TMPO, DMMP, and DEMP in the "aprotic" solvents were 2.8, 1.0, and 1.3 ppm, respectively. The corresponding shift ranges of the three compounds in the "protic" solvents were 48.9, 15.9, and 16.9 ppm, respectively.

The same trend has been noted for the solvent dependence of the ^{31}P NMR chemical shift of triphenylphosphine oxide (Maciel & James, 1964) as well as the ^{13}C NMR chemical shift of the carbonyl groups of acetone (Maciel & Ruben, 1963) and a number of other ketones and esters (Maciel & Natterstad, 1965). In the aprotic solvents, the phosphoryl (or carbonyl) resonances appeared to lower frequency, and variations in the nature of the solvent gave rise to only small chemical shift variations. In the protic solvents the resonances appeared to higher frequency, and variations in the nature of the solvent gave rise to much larger chemical shift variations than were seen for the aprotic solvents.

The basis of the solvent dependence of the chemical shifts of phosphoryl (and carbonyl) compounds in protic solvents has been discussed in some detail (Maciel & James, 1964; Maciel & Natterstad, 1965). The dependence is primarily understood in terms of the equilibrium

$$XYZP{=}O + HA \rightleftarrows XYZP{=}O\cdots HA \rightleftarrows XYZP{=}OH^+ + A^-$$

where X, Y, and Z are the substituents on the phosphoryl group, HA is the protic solvent molecule, and the dotted line denotes a hydrogen bond. The formation of a hydrogen bond at the phosphoryl oxygen atom results in electron deshielding at the phosphorus atom and causes the corresponding ^{31}P NMR resonance to be shifted to higher frequency. Full protonation of the phosphoryl group causes even more extensive deshielding at the phosphorus nucleus and results in a further shift to higher frequency. The equilibrium is fast on the NMR time scale, and the different phosphoryl species in solution therefore give rise to only a single, weighted-average resonance, the chemical shift of which is determined by the position of the equilibrium.

Of the various solvents tested, only sulfuric acid is a sufficiently strong acid to actually protonate the phosphoryl group to any significant extent. In the other protic solvents the concentration of the cationic phosphoryl species is negligible and chemical shift variations are therefore primarily due to variation in the *number* and *strength* of solvent–solute hydrogen bonds (Maciel & James, 1964).

Using ultraviolet spectroscopy, Balasubramanian and Rao (1962) found that the tendencies for a range of proton–donor solvents to form hydrogen bonds with a number of carbonyl compounds increased in the order: chloroform < *tert*-butyl alcohol < 2-propanol < ethanol < methanol < water ≃ deuterium oxide. The order is the same as that for these solvents in Table I, which would seem to confirm the primary role of hydrogen bonding in determining the chemical shifts of TMPO, DMMP, and DEMP in protic solvents.

If the slopes of the lines in Figure 3 are taken as a measure of the sensitivity of the ^{31}P NMR chemical shifts of the various compounds to the nature of the solvent, then both DMMP and DEMP show approximately one-third the sensitivity of TMPO to solvent effects whereas both TMP and TEP are entirely insensitive. Obviously, the nature of the substituents on the phosphoryl group is critical in determining the solvent sensitivity of the ^{31}P NMR chemical shifts. In the case of TMPO the phosphoryl group is substituted with three alkyl groups; in the case of DMMP and DEMP there are two alkoxyl groups and only one alkyl group bonded to the phosphorus atom; in the case of TMP and TEP all three substituents are alkoxyl groups.

Maciel and Natterstad (1965) found a very similar trend in the solvent dependence of the ^{13}C NMR chemical shifts of a range of carbonyl compounds. The sensitivity of the shifts to solvent effects was greatly reduced when an alkyl group or hydrogen atom was replaced with an alkoxyl group.

There are two possible explanations for the effects of the phosphoryl substituents on the solvent sensitivity of the phosphoryl chemical shifts. The substitution of an alkoxyl group for an alkyl group might reduce the tendency of the compound to engage in hydrogen bonding. Alternatively (or

additionally), it might reduce the extent to which hydrogen bonding at the phosphoryl oxygen influences the electron shielding at the phosphorus nucleus. As was pointed out by Maciel and Natterstad (1965), the alkoxy groups exert an electron-withdrawing inductive influence whereas the alkyl groups exert an electron-donating inductive effect. Replacement of an alkyl group with an alkoxyl group on the phosphorus atom results in an inductive withdrawal of electrons from the phosphoryl group and a consequent decrease in the ability of the phosphoryl oxygen to engage in hydrogen bond formation. Using both ^1H NMR and infrared spectroscopy, Gramstad and co-workers have measured the association constants for the hydrogen bonding of various proton donors to a number of phosphoryl compounds including TEP and DMMP (Gramstad & Mundheim, 1972; Gramstad & Olsen, 1974; Futsaeter & Gramstad, 1980). The association constant for TEP was consistently lower than that for DMMP, but in no case was the former less than 20% of the latter. While this difference is sure to be of some significance, it is unlikely to account fully for the very different solvent dependences of the ^{31}P NMR chemical shifts of the two compounds. It therefore seems likely that the reduction in the chemical shift solvent dependence that accompanies the substitution of an alkoxy group for an alkyl group reflects a reduction in the *extent* to which hydrogen bonding affects the phosphoryl chemical shift.

Of the five aprotic solvents used in the solvent-effects study, only acetone is miscible with water. Figure 4 shows the variation of the ^{31}P NMR chemical shifts of both charged and uncharged phosphoryl compounds as solvent water was replaced by acetone. The protons of acetone are incapable of participating in hydrogen bonds whereas those of water have an overwhelming tendency to do so. The chemical shift variations seen in Figure 4 may therefore be understood as being primarily a consequence of the disruption (by acetone) of the hydrogen bonds formed between the water protons and the phosphoryl oxygen atoms.

An increase in temperature is known to disrupt the formation of hydrogen bonds (O'Reilly, 1974). The decrease in the chemical shifts of the various phosphoryl compounds (other than TMP) relative to that of TEP as the temperature was increased (Figure 5) is again consistent with the degree of hydrogen bonding being a major determinant of the chemical shifts of these compounds in aqueous solution.

Hydrogen Bond Disruption as a Basis for the Effect of Hemoglobin on Chemical Shifts. Apart from bulk magnetic susceptibility effects, hemoglobin had no effect on the ^{31}P NMR chemical shifts of either TEP or TMP. As is seen from Figure 1, however, it caused the chemical shifts of the other six phosphoryl compounds to decrease. From Table I and Figures 3 and 4 it is evident that disruption of the formation of hydrogen bonds at the phosphoryl oxygen atoms had no effect on the chemical shift of either TEP or TMP but caused the chemical shifts of the other six compounds to decrease. When added to biological samples, the phosphoryl compounds will tend to form hydrogen bonds with solvent water. It would therefore seem reasonable to postulate that hemoglobin exerts its effects on the chemical shifts of the various compounds by perturbing the hydrogen bonding between the phosphoryl oxygen atoms and the hydrogen atoms of water.

There are a number of ways in which hemoglobin might perturb hydrogen bonding. If hemoglobin, like acetone, somehow causes a *general* disruption of solute–solvent hydrogen bonds (i.e., if the variation of the chemical shifts with both hemoglobin and acetone concentration reflect no more than the sensitivities of the chemical shift of each compound

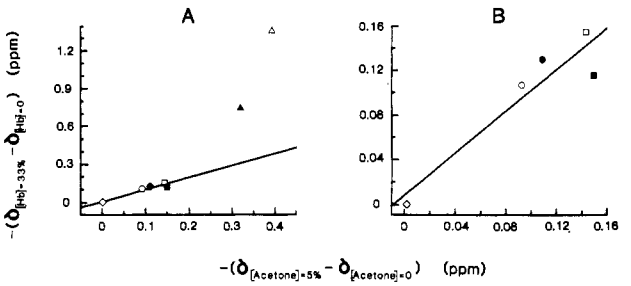

FIGURE 6: Correlation between the effect of acetone and the effect of hemoglobin on phosphoryl chemical shifts. The negative of the ^{31}P NMR chemical shift of each of the phosphoryl compounds in hemoglobin solutions having a hemoglobin concentration of 33% w/v (from Figure 1) is plotted as a function of the negative of the chemical shift of the compounds in a dilute (5% v/v) acetone solution (from Figure 4). The line was fitted by linear regression to the five points corresponding to the nonaromatic compounds $[-(\delta_{[Hb]=33\%w/v} - \delta_{[Hb]=0}) = 0.943\ [-(\delta_{[acetone]=5\%v/v} - \delta_{[acetone]=0})] + 0.008;\ r = 0.93]$. Panel A shows all seven points. Panel B shows just those representing the nonaromatic compounds. Symbols: ◊, TMP; ○, DMMP; ●, DEMP; ■, TMPO; □, HP; ▲, PP; △, DPP.

to factors affecting hydrogen bonding), then one might expect a linear correlation between the effects of hemoglobin (at physiological concentrations) and the effects of acetone (at low concentrations) on the ^{31}P NMR chemical shifts. In Figure 6 the negative of the chemical shift of each of the phosphoryl compounds in a hemoglobin solution having a hemoglobin concentration of 33% w/v (from Figure 1) is plotted as a function of the negative of the chemical shift of the compound in a dilute (5% v/v) acetone solution (from Figure 4). The line was fitted by linear regression to the five points corresponding to the nonaromatic compounds (r = 0.93). The most striking feature of Figure 6A is the deviation of the points corresponding to the aromatic compounds from the fitted line. PP, with a single phenyl group, falls well above the fitted line, and DPP, with two phenyl groups, even more so. Hemoglobin exerts a much stronger effect on the chemical shifts of the two aromatic compounds than would be expected if the sole factor determining the magnitude of the effect was the sensitivity of the phosphoryl chemical shifts to hydrogen bond disruption.

Figure 2 casts some light on the probable origin of the deviation of the aromatic compounds from the line fitted to the nonaromatic compounds. As has been discussed, the marked increase in the line width of the DPP resonance and, to a lesser extent, the PP resonance with increasing hemoglobin concentration indicates that the two aromatic compounds are binding to the hemoglobin whereas there is little evidence that any of the nonaromatic compounds are binding to any significant extent. The (fast-exchange) binding of a small molecule to a macromolecule can in itself cause changes in the chemical shift of the resonances arising from the small molecule (Dwek, 1973). However, in the case of the PP and DPP ions it is not necessary to postulate any direct effect of binding on the phosphoryl chemical shifts. The three univalent ions DPP, PP, and HP form a homologous series; DPP has two phenyl groups, PP has one phenyl group, and HP has no phenyl groups. Figure 2 suggests that DPP binds to hemoglobin to a greater extent than does PP while there is little evidence to suggest that HP binds to any significant extent. The implication is, therefore, that the phenyl groups are important in the binding process, and this would suggest that the aromatic ions are binding (via their phenyl groups) at a hydrophobic site (or sites) on the hemoglobin molecule. There would be a natural tendency for such sites to exclude solvent water, and the binding process is therefore likely to disrupt

the hydrogen bonds that exist between the phosphoryl compounds and water in free solution. Any such disruption would lead to a large shift to lower frequency of the phosphoryl resonances, as is indeed observed.

In comparison with the points corresponding to the two aromatic compounds, the points corresponding to the five nonaromatic compounds appear to show quite a good linear correlation (Figure 6A). However, it is clear from Figure 6B that there *is* significant scatter about the fitted line. The scatter may well relate to the fact that the effect of acetone on the chemical shifts is not entirely due to hydrogen bonding effects. There is likely to be a small but significant contribution of polar and van der Waals interactions to the observed dependence of the various chemical shifts on acetone concentration (Figure 4), and there is no suggestion that hemoglobin undergoes the same polar and van der Waals interactions with the phosphoryl compounds as acetone. The alternative explanation is, however, that the scatter seen in Figure 6B represents differences in the extent to which hemoglobin disrupts the hydrogen bonding of the nonaromatic compounds and this leads to the question as to the nature of the specific mechanism by which the disruption occurs.

If, as is thought to be the case for the two aromatic compounds, the disruption results from the binding of the phosphoryl compounds to the hemoglobin, then the extent to which hemoglobin affects the phosphoryl chemical shifts would depend on not only the sensitivity of the shift to hydrogen bonding but also the extent of binding. However, any such binding would have to be nonspecific, with the compounds having a similar affinity for lysozyme.

It is conceivable that the proteins might exert their effect on the phosphoryl chemical shifts by simply competing with solvent water for hydrogen bonds at the phosphoryl oxygen atoms. Such an explanation could only be valid if the protein–phosphoryl hydrogen bonds are significantly weaker than the water–phosphoryl hydrogen bonds so that the chemical shifts of the phosphoryl compound are much less when hydrogen bonded to the protein than when hydrogen bonded to water.

An alternative explanation may relate to the effects of proteins in general on the properties of water. Proteins in aqueous solution are surrounded by "water of hydration" that exhibits physical properties quite different from those of free water. Gary Bobo (1967) measured the fraction of the total water in human erythrocytes and hemoglobin solutions that is accessible to a range of uncharged solutes and interpreted his results in terms of the extent to which the various nonelectrolytes were able to penetrate the water of hydration. The ability to penetrate the hydration phase was shown to depend upon the size, shape, and hydrogen bonding properties of the molecule involved and was also found to increase with increasing temperature. The different nonelectrolyte (and electrolyte) phosphoryl compounds of interest in this study vary in size, shape, and hydrogen bonding properties, and they might therefore be expected to penetrate, to different extents, the water of hydration surrounding a protein in solution. If the penetration of these compounds into the hydration phase causes disruption of hydrogen bonding at the phosphoryl oxygen atoms, then such a phenomenon could indeed explain the general effect of proteins on the phosphoryl chemical shifts; the magnitude of the shift effect would reflect not only the sensitivity of the shift to hydrogen bond disruption but also the ability of the compound to penetrate the hydration phase.

Summary. It should be clear from the above discussion [and that in the preceding paper (Kirk & Kuchel, 1988b)], that the physical basis for the differences in the intra- and extracellular chemical shifts of the various phosphoryl compounds studied is not straightforward. The significant difference between the magnetic susceptibilities of the two compartments affects the chemical shifts of all compounds equally; it is sufficient to explain the difference between the intra- and extracellular chemical shifts of TMP and TEP but accounts for only part of the shift differences noted for the other six compounds. The susceptibility-independent component of the observed transmembrane shift differences may be understood in terms of the effects of hydrogen bonding on the ^{31}P NMR chemical shifts. Hemoglobin is thought to cause the resonance frequencies of the compounds (other than TMP and TEP) to decrease by perturbing the hydrogen bonding between the phosphoryl oxygen atoms and solvent water. The physical basis of the perturbation is probably derived from a property of proteins in general (rather than a specific property of hemoglobin) and may involve either the binding of these compounds to the protein or the penetration of the compounds into the ordered water of hydration surrounding the proteins. In the case of the two aromatic ions there is evidence to suggest that these compounds bind to hemoglobin, possibly via a hydrophobic effect. This interaction gives rise to much larger chemical shift variations than are seen for the nonaromatic compounds and accounts for the large line widths seen for the intracellular resonances of the aromatic ions in intact cell suspensions (Kirk & Kuchel, 1988b).

Registry No. TMP, 512-56-1; TEP, 78-40-0; DMMP, 756-79-6; DEMP, 683-08-9; TMPO, 676-96-0; HP, 6303-21-5; PP, 89713-30-4; DPP, 18357-17-0.

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Activity of Soybean Lipoxygenase in the Absence of Lipid Hydroperoxide†

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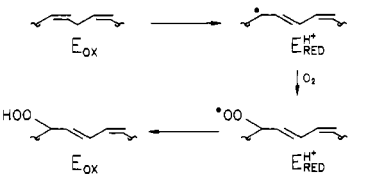
ABSTRACT: Soybean lipoxygenase was assayed under conditions such that the concentration of the enzyme was in excess of the concentration of the substrate, arachidonic acid. Under these conditions, the concentration of lipid hydroperoxides present as contaminants in the substrate was negligible relative to the enzyme concentration, and the concentration of lipid hydroperoxide product could be determined accurately. The ferric form of the enzyme was observed to be fully active and to catalyze the oxidation of arachidonic acid at a near-diffusion-controlled rate, 1.4×10^7 M^{-1} s^{-1} at 0 °C, at concentrations of lipid hydroperoxides as low as 5% of the enzyme concentration. From this, it can be concluded that the higher oxidation states that would be accessible by oxidation of Fe(III) by hydroperoxide are not required for catalysis by soybean lipoxygenase. Surprisingly, the activation of the ferrous form of the enzyme was also observed at insignificantly low lipid hydroperoxide concentrations. This activation presumably involves oxidation of the ferrous to the ferric form of the enzyme and must be more facile than has hitherto been reported. This result may rationalize previous reports that the ferrous and the ferric forms of the enzyme are both active.

The kinetics and mechanism of soybean lipoxygenase have been extensively studied, and the available information has been collected in several recent reviews (Ludwig et al., 1987; Petersson et al., 1987; Schewe et al., 1986; Kühn et al., 1986a; Lands, 1984; Veldink et al., 1984). The enzyme is a monomer with a single iron per active site, which is neither a heme nor an iron–sulfur center. The iron is considered to be the oxidant that oxidizes the 1,4-diene of a polyunsaturated fatty acid to a pentadienyl radical intermediate (Scheme I). The pentadienyl radical is then trapped by oxygen to give a lipid hydroperoxide as the final product. For example, when the substrate is arachidonic acid as in this study, the product is 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (15-HPETE).[1] The hydroperoxide product appears to play a key role in this reaction. The iron in the isolated enzyme is almost entirely in the ferrous (Fe^{II}) state and is oxidized by lipid hydroperoxides to the ferric (Fe^{III}) state. This oxidation to the ferric form is generally considered to be necessary for enzyme activity. Several careful investigations have shown that the distinction between the ferrous and the ferric forms of the enzyme cannot always be made, however. The ferric enzyme can exhibit a lag period comparable to that of the ferrous enzyme (de Groot et al., 1975a), which suggests that the ferric form still requires lipid hydroperoxide for activity. In addition, it has been demonstrated that the reticulocyte lipoxygenase has a continuous requirement for lipid hydroperoxide during turnover (Kühn et al., 1986b). On the basis of these observations, it has been proposed that the iron in the active form of the enzyme may be in a higher oxidation state, for example, ferryl iron as in the cytochrome P-450's (Ludwig et al., 1987). Spectral evidence for these higher oxidation states as intermediates has been provided for prostaglandin H synthase, which catalyzes a similar reaction but which is a hemoprotein (Lambier et al., 1985; Kulmacz et al., 1987).

Scheme I



A major impediment to clarifying the role of lipid hydroperoxide in the lipoxygenase reaction is the fact that the unsaturated fatty acid substrates are susceptible to autoxidation. One must expect, therefore, that the substrate would invariably be contaminated with lipid hydroperoxide, perhaps at immeasurably low concentrations that would nonetheless be significant when compared to the enzyme concentration. In order to avoid this complication, we have investigated the activity of soybean lipoxygenase under conditions such that the enzyme concentration is in excess of the substrate and the concentration of lipid hydroperoxides is demonstrably insignificant.

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[1] Abbreviations: 15-HPETE, 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13-(*E*)-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5(*Z*),8(*Z*),11(*Z*),13-(*E*)-eicosatetraenoic acid; HPLC, high-pressure liquid chromatography.

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