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MODULATION OF 2,3-DIPHOSPHOGLYCERATE ^{31}P-NMR RESONANCE POSITIONS BY RED CELL MEMBRANE SHAPE

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Summary

Na^+ transport in the red cells of the dog is dependent on cell volume, a 20% change in cell volume leading to a 25-fold increase in apparent Na^+ flux; the effect is dependent upon metabolic energy. We have found that swelling and shrinking dog red cells causes a shift in the ^{31}P-NMR peak of 2,3-diphosphoglycerate, which is present in dog red cells at 5.5 mM. Control experiments indicate that the 2,3-diphosphoglycerate resonance peak shifts may not be attributed to: interaction with hemoglobin, changes in cell pH, ionic strength, diamagnetic susceptibility or small changes in the Mg^{2+}/2,3-diphosphoglycerate ratio. Experiments with chlorpromazine and pentanol which alter red cell membrane area by a mechanism different from osmotic swelling suggest that 2,3-diphosphoglycerate interacts with a binding site in the cell that is dependent upon the physical condition of the dog red cell membrane.

2,3-Diphosphoglycerate is present in red cells in an unusually high concentration and can readily be observed by ^{31}P-nuclear magnetic resonance in intact red cells as shown by Moon and Richards [1] and Henderson et al.[2] for rabbit and human red cells. 2,3-Diphosphoglycerate is also present at 5.5 mM concentration in dog red cells which exhibit an interesting anomaly in Na^+ transport that has been linked to phosphoglycerate metabolism by Elford and Solomon [3]. Na^+ transport in dog red cells depends markedly on the volume of the cell, a 20% decrease in cell volume leading to a 25-fold increase in apparent Na^+ flux across the membrane [3,4]. Elford and Solomon [3] have suggested that the volume effect is mediated by the concentration of 3-phosphoglycerate, since agents which increased the concentration of this substrate decrease the dependence of Na^+ flux on cell volume. This raised a general question: does the state of the dog red cell membrane modulate the interaction of phosphorylated metabolic intermediates with membrane-bound

enzymes? Consequently, we have studied the effect of dog red cell volume on the ^{31}P-NMR spectrum of 2,3-diphosphoglycerate, a direct precursor of 3-phosphoglycerate. Our results indicate an interaction between 2,3-diphosphoglycerate and an unknown site in the red cell that is dependent upon the physical state of the membrane and is not related to the 2,3-diphosphoglycerate interaction with hemoglobin.

The NMR experiments were carried out on a Varian XL-100-15 Fourier transform spectrometer operating at 40.5 MHz for ^{31}P observation. The 16 K Varian 620 computer is interfaced with a Computer Operations tape deck for storage of data. 90° rf pulses of 32 μs were routinely used. Acquisition times of 0.3—0.5 s allowed accumulation of 120—200 transients per min; 1000—10 000 transients were required for observation of 2,3-diphosphoglycerate. Digital filtering with time constants of 0.075—0.2 s was applied to the accumulated free induction decay to improve signal-to-noise ratios. Broad band proton decoupling (1500 Hz band width; 3 W) was used. The spectrometer was locked on the deuterium signal of $^{2}H_2O$ using pulsed lock mode. Samples were observed non-spinning. They contained red cells at 50% hematocrit suspended in buffer of the following composition (mM): 150, NaCl/5, KCl/17, Tris/20% $^{2}H_2O$, modified by the addition of 200 mM sucrose to shrink the cells, unless otherwise specified. Dog red cells were normally observed within 12 h of their removal from the animals.

Both of the 2,3-diphosphoglycerate NMR resonances change position with red cell volume as shown in Fig. 1, in which the resonances are identified according to Moon and Richards [1]. The separation we have observed between the two resonances in dog red cells is 32 Hz in reasonable agreement with the 28 Hz found in human red cells by Henderson et al. [2]. The left hand portion of the figure shows the results when sucrose is added to the isosmolar medium. As the cells shrink the resonances move downfield from a phosphoric acid external standard. The right hand portion of the figure shows a similar change in resonance position when the cells are shrunken by the addition of choline chloride or swollen by the removal of NaCl from the medium. Results for cell swelling with NaCl were essentially similar to those with choline chloride as are the sucrose ones when converted to relative cell volume. Thus, the change in resonance positions is independent of the nature of the solute causing the change of cell volume.

Heustis and Raftery [5] have shown that binding of 2,3-diphosphoglycerate to hemoglobin produces a change in resonance position. Thus, it was first necessary to see whether the results in Fig. 1 could be attributed to an interaction of 2,3-diphosphoglycerate with hemoglobin. Since 2,3-diphosphoglycerate binding to the oxygenated and carbon monoxygenated forms of hemoglobin is minimal, studies were made of the effect of oxygen and carbon monoxide. When additional oxygen was passed through the red cell suspension prior to measurement, the resonance of phosphate-3 was shifted upfield by 1.6 Hz and the resonance of phosphate-2 was shifted upfield by 0.8 Hz. When the red cell was treated with carbon monoxide prior to measurement, the resonances shifted upfield by 0.2 and 0.4 Hz respectively for phosphates-2 and -3 in normal cells and 0.3 and 0.2 Hz for cells shrunken by the addition of 200 mM sucrose. Since these shifts are small compared with those shown

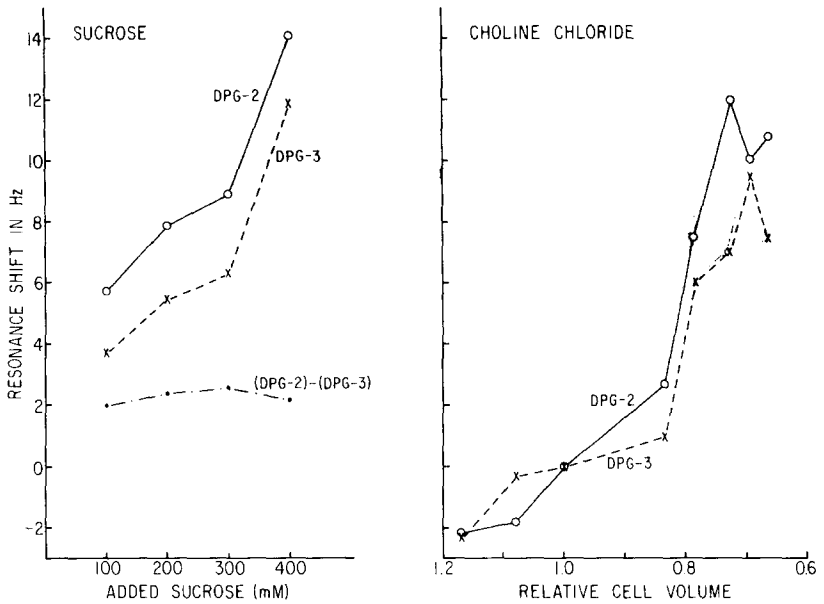

Fig.1. Shift in ^{31}P-NMR peaks of 2,3-diphosphoglycerate (DPG) with dog red cell volume. The left hand section shows the effect of adding sucrose to a medium isosmolar with dog blood (in one experiment, typical of seven) and the right hand section shows the resonance shifts as a function of relative cell volume. The cells were suspended in buffer and either NaCl was removed to swell the cells, or choline chloride was added to shrink the cells.

in Fig. 1, it is concluded that the effect we observe does not reflect the interaction with hemoglobin. Furthermore, Heustis and Raftery [5] have shown that one resonance shifts almost twice as much as the other when 2,3-diphosphoglycerate is bound to hemoglobin. To the contrary, changes in cell volume cause both resonances to shift by about the same amount, as shown in the difference curve at the bottom of the sucrose data in Fig. 1.

Moon and Richards [1] have shown that a pH shift from pH 6.5 to 7.5 causes a shift of about 5—10 Hz in the difference between the 2-phosphate and the 3-phosphate resonances in carbon monoxide-treated rabbit red cell hemolysates. Our experiments were carried out on whole red cells from a different species, and we have therefore measured the effect of pH directly. For this purpose the buffer pH was shifted by about one unit, from pH 7.7 to 6.8. Duhm [6] has shown that under normal conditions the cellular pH in human red cells tracks the extracellular pH to within 0.1—0.2 pH units. Since the Donnan equilibrium for Cl^- in the dog is the same as in man, an external pH change of 1.1 units should produce an intracellular change of about 1 unit. This pH change caused the 2-phosphate resonance to shift by 0.5 Hz for normal dog red cells and 0.1 Hz for cells shrunken with 200 mM sucrose. For the 3-phosphate resonance the shifts were 0.1 and 0.3 Hz. Thus, the chemical shifts in Fig. 1 may not be attributed to changes in red cell pH.

When the dog red cell shrinks or swells, the concentration of each of the impermeant solutes also changes; some or all of these concentration changes could be responsible for the chemical shift. The cell water volume is

markedly different at the two extremes in Fig. 1. Addition of 200 mM sucrose causes the red cell volume to decrease to 77% of control (400 mM, 67%) and the cell water volume to decrease to 67% of control (400 mM, 53%). A control experiment was carried out to study the effect of ionic strength by examining how KCl concentration affects the 2,3-diphosphoglycerate ^{31}P-NMR spectrum in free solution. 2,3-Diphosphoglycerate was dissolved in a buffer of the following composition: 5 mM NaCl/25 mM Tris/20% $^{2}H_2O$ plus KCl which varied from 100 to 300 mM. This corresponds to a cell volume excursion from 124 to 72% of normal. The pH was adjusted to 7.4 for each sample. The change in the KCl concentration from 100 to 250 mM shifted the 3-phosphate resonance by 1.1 Hz; raising the KCl concentration to 300 mM caused an additional shift of 1.1 Hz. Since these shifts are much smaller than the 8—10 Hz shifts in Fig. 1 over the same volume range, it is unlikely that the cell-shrinking effect may be attributed to changes in cellular ionic strength. This conclusion was extended in experiments in which the cell membranes in a 2 ml sample of packed red cells were disrupted with 0.5% Triton X-100. Normal buffer was then added in steps of 0.5 ml up to a total of 2.5 ml. This produced small shifts* in the resonances in the direction opposite to that observed when whole cells swell. Between the first and last addition, the 2-phosphate resonance shifted by 2 Hz and the 3-phosphate resonance by 1.5 Hz.

It was also possible that changes in diamagnetic susceptibility could contribute significantly to the effect we have observed. To test this, cells were incubated for 3 h with 25 mM phosphate (plus NaCl) to increase intracellular phosphate. After washing to remove extracellular phosphate, the cells were shrunken with 200, 300 and 400 mosM sucrose. The phosphate resonance shifted only slightly with cell volume; the total excursion between control and 400 mosM sucrose amounted* to 2 Hz. Since this is small compared with the shifts in Fig. 1, the resonance shift produced by cell swelling may not be attributed to changes in diamagnetic susceptibility.

The effect of Mg^{2+} on the 2,3-diphosphoglycerate spectrum was also measured in free solution. Since both Mg^{2+} and 2,3-diphosphoglycerate permeate the red cell membrane slowly, if at all, changes in cell volume should not affect the concentration ratio of these two constituents. For this reason the Mg^{2+} concentration was altered by ± 20% for the control experiment. The control solution consisted of: 5 mM $MgCl_2$ /90 mM NaCl/ 10 mM KCl/5 mM 2,3-diphosphoglycerate/100 mM Tris, pH 7.4/20% $^{2}H_2O$. Reducing the Mg^{2+} concentration to 4 mM shifted the 3-phosphate resonance upfield by 0.2 Hz and increasing it to 6 mM shifted it 0.2 Hz in the same direction. Hence it seems unlikely that the effect of cell shrinking on the spectrum may be attributed to a change in the Mg^{2+}/2,3-diphosphoglycerate concentration ratio in the intracellular free water volume.

The concentration of all the cell solutes varies in a similar manner and it is not possible to make direct measurements such as those above for each solute. Instead, we have altered the physical state of the red cell membrane

*These spectra were measured on a JEOL FX 60 NMR spectrometer (operating at 24.15 MHz for ^{31}P) and the observed shifts have been multiplied by 100/60 to make them comparable with the measurements on the Varian XL-100-15 machine.

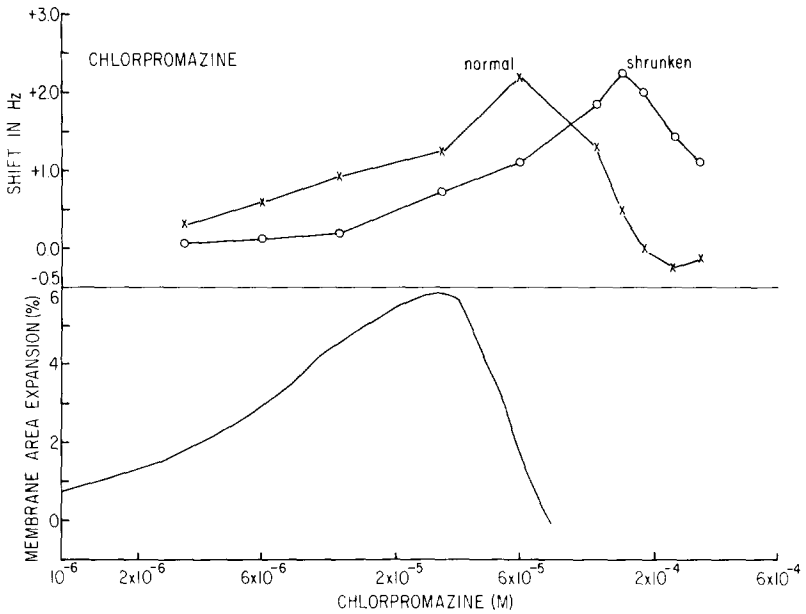

Fig. 2. Shift in ^{31}P-NMR resonance of the 2-phosphate of 2,3-diphosphoglycerate with increasing amounts of chlorpromazine in one experiment typical of two. The bottom curve shows membrane area redrawn from Seeman et al. [7].

by another mechanism. Seeman and his colleagues have studied the effects of a wide variety of anesthetics on red cell properties. In the course of these studies they have shown [7] that the dissolution of anesthetics in the red cell membrane frequently casuses an increase in membrane area. In the case of chlorpromazine the human red cell membrane area passes through a maximum at about 6% expansion, as shown in the bottom section of Fig. 2. The top portion of Fig. 2 shows that the addition of chlorpromazine also caused the 2-phosphate resonance to shift through a maximum, analogous to the effect on membrane area. Since the area data were obtained on human red cells and the NMR data on dog red cells, the difference in maxima is probably not significant, particularly because the red cell membrane lipids in the two species differ appreciably [8].

Fig. 2 shows that the 2-phosphate resonance in the shrunken red cells also shifts through a similar maximum, even further displaced from the maximum in membrane area. A similar but smaller shift was observed in the 3-phosphate resonance.

To make sure that the shifts in Fig. 2 were of a general nature rather than specific to chlorpromazine, similar experiments were carried out with another anesthetic, 1-pentanol, whose effect on red cell area had also been measured by Seeman et al. The results of these experiments, shown in Fig. 3, are similar to those with chlorpromazine for normal cells though there are differences in the behavior of shrunken cells.

The measurements of cell area were made on spherical human red cell ghosts so that the cell volume also increases with the area. In the case of the biconcave disc which the normal red cell approximates, Jacobs [9] has

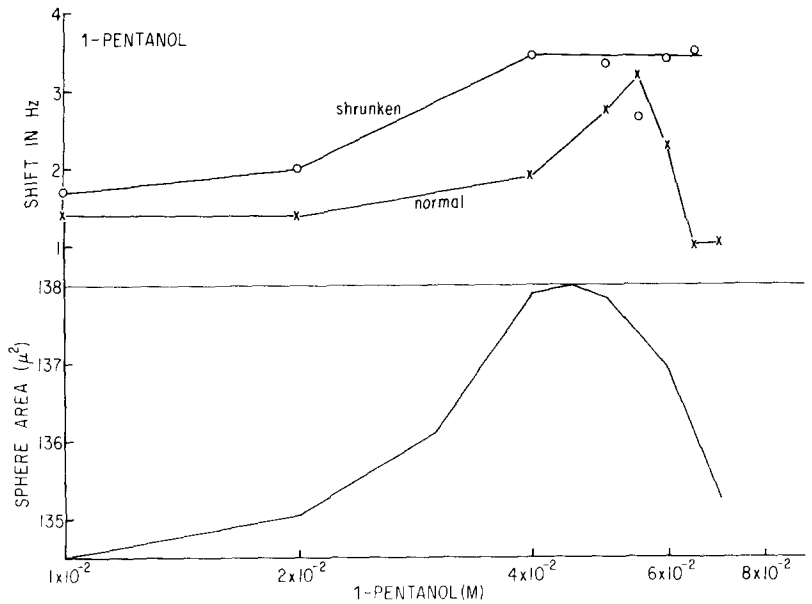

Fig.3. Shift in ^{31}P-NMR resonance of the 2-phosphate of 2,3-diphosphoglycerate with increasing amounts of 1-pentanol in one experiment, typical of two. The bottom curve shows membrane area redrawn from Seeman et al. [7].

shown that red cell area is independent of cell volume in the range between the biconcave disc and the sphere and Sha'afi et al. [10] have argued that this also applies when cells are shrunken. Thus we should not expect that the changes in membrane area produced in the biconcave disc by chlorpromazine and 1-pentanol would necessarily be accompanied by any significant change in volume in the range around the normal volume. Furthermore, Weed and Chailley [11] have pointed out that chlorpromazine causes cups to form in human red cell membranes, whereas alcohols such as ethanol and butanol cause spikes to form. With low drug concentrations, the surface modifications of either kind do not produce a change in volume. Hence the changes in resonance positions in Figs. 2 and 3 appear to be due to physical changes in the cell membrane rather than being mediated through changes in cell volume.

The results with the anesthetics, taken together with those in which red cell volume was changed by osmotic pressure, lead us to conclude that there is an interaction between 2,3-diphosphoglycerate and a binding site that is dependent upon the physical condition of the dog red cell membrane. This finding is of particular relevance to cation transport since in this species similar alterations in osmotic pressure cause profound changes in Na^+ transport which appear to be closely related to phosphoglycerate metabolism.

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