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The Effects of Hypothermia on the Intracellular pH of Erythrocytes Studied Using ^{31}P NMR and Endogenous Compounds

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Summary: The effects of hypothermia on the intracellular pH of human erythrocytes were studied non-invasively using ^{31}P NMR spectroscopy and the endogenous phosphorus-containing compounds glycerate 2,3-bisphosphate and inorganic phosphate. Specifically, the pH dependence of the ^{31}P NMR chemical shifts of these compounds was used to measure the intracellular pH at 25 and 37 °C. The possibility of a non-pH-dependent change on the chemical shifts of the 2-P and 3-P resonances of glycerate 2,3-bisphosphate due to the presence of paramagnetic deoxy-haemoglobin (i. e., a pseudo-contact interaction) was investigated and found to have negligible effect under the present experimental conditions. The most probable reasons for this are that the deoxy-haemoglobin concentration was too small and/or the glycerate 2,3-bisphosphate does not get sufficiently close to the paramagnetic centre to be affected. The change in intracellular pH with temperature was consistent with that predicted by the alphastat hypothesis.

Introduction

A number of invasive methods have been used to determine the intracellular pH (pH_i) of erythrocytes, including pH microelectrodes, pH-dependent dyes and weak acid or base distribution methods. ^{31}P NMR, however, provides a convenient means of non-invasively determining intracellular pH, as well as of measuring other intracellular variables such as the energy status of the cell in one measurement (1–7). NMR may also be used to probe cytoplasmic viscosity, transport and diffusion in erythrocytes (8–12). The chemical shift of the ^{31}P NMR resonance of many phosphorus-containing species has a pH-dependence. This dependence forms the basis of the NMR method. Of the endogenous phosphorus species only the phosphorus resonances of ATP, inorganic phosphate (P_i) and glycerate 2,3-bisphosphate are sufficiently NMR visible. The ATP resonances are not suitable for probing pH since their chemical shifts are sensitive to Mg^{2+} concentrations (13, 14) and the resonances are also small and quite broad. As a pH probe, P_i is the most suitable since its dissociation constant is close to physiological pH, but its chemical shift can be difficult to determine in the ^{31}P NMR spectrum since its resonance is very small and may be superimposed upon by the 2-P signal of glycerate 2,3-bisphosphate. It can be confused with extra-erythrocytic P_i. Glycerate 2,3-bisphosphate is unique to the erythrocyte, but its chemical shift is also sensitive to binding to deoxy-haemoglobin and Mg^{2+} (15). It is possible that a pseudo-contact shift mechanism (16) contributes to the change in glycerate 2,3-bisphosphate chemical shift upon binding to haemoglobin. A possible complication arising when attempting to measure pH at different temperatures is that the pseudo-contact shift has a temperature-dependence proportional to inverse temperature (17). The presence of met-haemoglobin and especially deoxy-haemoglobin cause a susceptibility difference between the intracellular and extracellular media which affects the measured chemical

shifts. *Fabry & San George* (18) showed that the phosphorus resonances of glycerate 2,3-bisphosphate broaden as the deoxy-haemoglobin concentration increases, and that the resonances shift downfield (19) in comparison to being in an oxy-haemoglobin solution. Internal reference compounds have been proposed, such as triethyl phosphate (20), which would circumvent the problems of susceptibility. Similarly, *Fabry & San George* (18) have suggested matching the intracellular and extracellular paramagnetic susceptibility by the addition of paramagnetics to the extracellular solution.

Due to reasons such as accidental hypothermia and the use of hypothermia in cardiac surgery (21, 22), it is important to understand the effects of hypothermia on the intracellular pH of erythrocytes. This may lead to better pH management in patients during hypothermia. In the present study the pH_i of erythrocytes were measured at 25 and 37 °C using only endogenous compounds and ^{31}P NMR in order to determine the rate of pH change. The possibility of a pseudo-contact shift interaction affecting the chemical shifts of glycerate 2,3-bisphosphate was also investigated.

Materials and Methods

Blood samples

Fresh venous blood samples were obtained from healthy donors. The blood was drawn into tubes containing heparin.

Haemolysate preparation

Blood was washed by centrifugation (1500 *g*; 5 min) three times in isotonic saline. The supernatant was removed, leaving the cell pellet with a haematocrit of about 0.8. The cells were then sonicated at ≈ 30 W for 20 s using a model 450 sonifier (Branson Sonic Power Co., Danbury, CT, USA).

The pH of haemolysates used in determining the pH standard titration curves were adjusted to different values (pH 6.9–7.7) with small amounts (with respect to the lysate volume) of HCl (0.1 mol/l) and NaOH (0.1 mol/l). The pH values of the haemolysate samples were measured with a Suntex model 2000A pH meter (Taipei, Taiwan, ROC) in combination with a glass-calomel combination electrode (Broadley James Corp. CA, USA) immediately before and after NMR measurement.

Haemolysate samples used in determining pseudo-contact shift effects on the measured chemical shifts were prepared as above, except that after the second saline wash the blood sample was divided into two equal parts. One half of the sample was then resuspended in saline at low haematocrit (< 0.2), gently bubbled with carbon monoxide (CO) for 5 min and then centrifuged to high haematocrit (≈ 0.8). The other half of each blood sample was washed in saline again as before. Finally, the haematocrit values of both 'halves' of the sample were carefully adjusted to within 1% of each other.

Erythrocyte preparation

The fresh venous blood samples were centrifuged (1500 *g*; 10 min) at 4 °C. The plasma and buffy coat were discarded. The pellet cells (haematocrit ≈ 0.85) were divided into two parts (i. e., one for each temperature studied), stored in crushed ice and measured within three hours. haematocrit values were determined using a Hawksley microhaematocrit centrifuge (Hawksley, UK) operated for 8 min.

NMR measurements

^{31}P NMR measurements were performed on a Bruker MSL 300 spectrometer at 121.5 MHz. The temperature was checked using ethylene glycol (23). Typical acquisition conditions were: spectral width 6 kHz digitized into 8 k data points; pulse width, 20 µs (i. e., ≈ 90° pulse); recycle delay 5 s. Each spectrum from which the chemical shifts were measured was the average of at least 80 transients. Samples (3 ml) were dispensed into 10-mm (o. d.) NMR tubes. An inner coaxial capillary containing 850 g/l H_3PO_4 was used as an external chemical shift reference (0 ppm). The samples were put into a water bath at the desired temperature for 5 minutes before NMR measurement.

pH_i determination

For a compound with one dissociable proton, the phosphorus chemical shift of the particular species is given by the *Henderson-Hasselbalch* equation,

$$pH = pK_a + \log_{10} [\delta_{obs} - \delta_1]/[\delta_2 - \delta_{obs}]$$

where pK_a is the dissociation constant, δ_{obs} is the observed chemical shift and δ_1 and δ_2 are the chemical shifts of the acid and base forms of the species, respectively. pH_i was determined from the chemical shifts of the 2-P and 3-P resonances of glycerate 2,3-bisphosphate and P_i.

Results

NMR spectra

A typical ^{31}P NMR spectrum of intact erythrocytes at high haematocrit (≈ 0.80) is given in figure 1. With time the intracellular P_i resonance increases in inten-

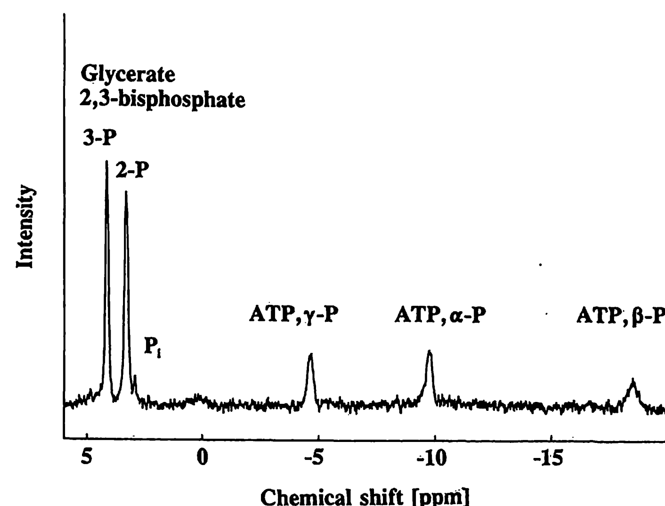

Fig. 1. ^{31}P NMR spectrum of freshly drawn venous blood at 37 °C. Before NMR measurement some of the plasma was removed to increase the haematocrit to about 0.85. Since this preparation was performed at 37 °C the P_i resonance is larger than that normally observed. The spectrum is presented with a line-broadening of 2 Hz to increase the apparent signal-to-noise. The chemical shift is relative to 85% H_3PO_4 (resonance not shown).

sity, while those of ATP and glycerate 2,3-bisphosphate decrease. Since the sample was adjusted to high haematocrit the extracellular P_i concentration is small and, consequently, so is its NMR resonance (not distinguishable from the baseline in the present spectrum). The broad hump at about 0 ppm results from phospholipids.

pH titrations

pH versus chemical shift calibration curves determined in CO-untreated lysates at 25 and 37 °C are given in figure 2. The *Henderson-Hasselbalch* equation was regressed onto each of the data sets and the apparent values for pK_a, δ_1 and δ_2 are given in table 1. The values are termed apparent since the measured chemical shifts have dependencies in addition to changes in pH (see Introduction and Discussion). By

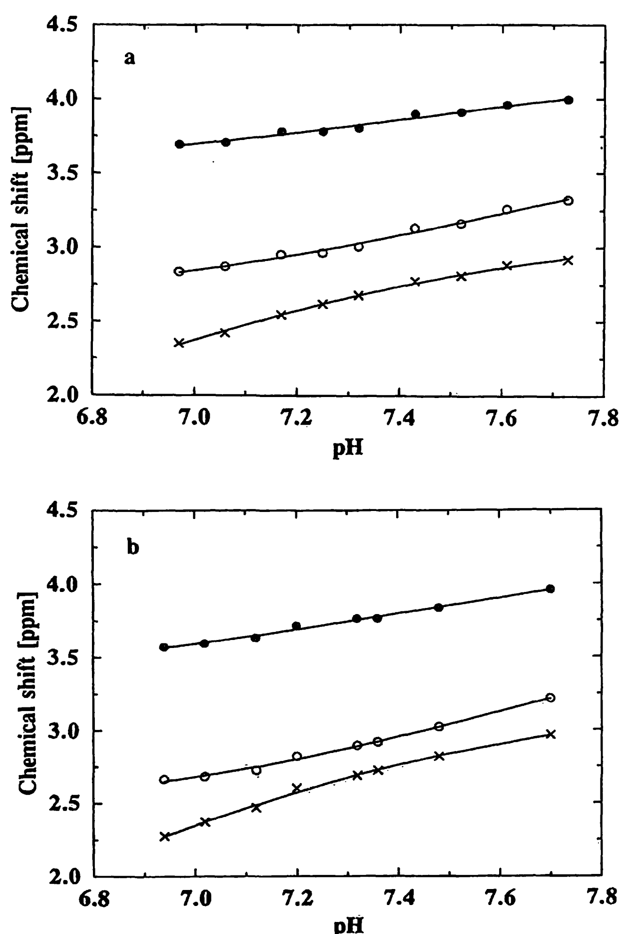

Fig. 2. pH versus chemical shift for endogenous phosphate compounds (●: 3-P resonance of glycerate 2,3-bisphosphate; o: 2-P resonance of glycerate 2,3-bisphosphate; x: P_i) at (a) 25 °C and (b) 37 °C in lysates prepared from CO-untreated cells. The solid lines represent regression of the *Henderson-Hasselbalch* equation through each data set. From comparison of the two plots it can be seen that the chemical shift with respect to pH of the P_i resonance is invariant with respect to temperature.

Tab. 1. Results of regression of the *Henderson-Hasselbalch* equation onto the pH titration data conducted on lysates from CO-treated and CO-untreated cells at 25 and 37 °C. Before lysing both cell samples had a haematocrit of 0.78. Since the values given are apparent, error limits are not given.

Phosphorus compound	T (°C)	Quantity		
		δ_1 (ppm)	δ_2 (ppm)	pK_a
P_i	25	1.21	3.14	6.81
	37	1.03	3.22	6.82
2-Glycerate 2,3-bisphosphate	25	2.59	3.92	7.63
	37	2.40	3.96	7.66
3-Glycerate 2,3-bisphosphate	25	3.49	4.27	7.44
	37	3.31	4.26	7.38

comparing the titration curves for the endogenous compounds at 25 and 37 °C it can be seen that the titration curve for P_i is, within experimental error, temperature invariant. However, by comparing figures 2(a) and (b) it can be seen that at 37 °C the chemical shifts of the 2-P and 3-P resonances of glycerate 2,3-bisphosphate are consistently about 0.15 and 0.1 ppm lower, respectively, than at 25 °C at the same pH.

Determination of pseudo-contact shift effects on glycerate 2,3-bisphosphate chemical shifts

To determine if there were any effects on the measured glycerate 2,3-bisphosphate chemical shifts due to pseudo-contact effects from the paramagnetic haem groups of deoxy-haemoglobin, CO-treated and CO-untreated lysate 'pairs' were prepared from four blood samples, as described above. The CO binds very tightly to the haemoglobin, displacing glycerate 2,3-bisphosphate in the process (24). The chemical shifts of the endogenous phosphorus species were measured at 25 and 37 °C; the results are given in table 2. The average pH change with temperature measured with a pH electrode was, within experimental error, the same for both CO-treated and CO-untreated blood. For individual species in both the CO-treated and CO-untreated lysates the differential chemical shift ($\Delta\ \delta$) between that measured at 25 and 37 °C was, within experimental error, the same. Note that although the $\Delta\ \delta$ determined for the CO-treated and CO-untreated lysates results from different values of pH since the CO bubbling procedure lowers the lysate pH, $\Delta\ \delta$ corresponds to a similar pH change in both the CO-treated and CO-untreated samples (see fig. 2). Thus, glycerate 2,3-bisphosphate is subject to little, if any, pseudo-contact shift from deoxy-haemoglobin.

Tab. 2. pH and chemical shift variation with temperature in CO-treated and CO-untreated lysates.
Comparison of measured chemical shifts (ppm) of endogenous phosphorus compounds in lysates. The pH values given in the table were determined using a pH electrode.

Sample	T (°C)	pH	Phosphorus compound		
			3-glycerate 2,3-bisphosphate	2-glycerate 2,3-bisphosphate	P_i
− CO	25	7.42 ± 0.01	4.01 ± 0.08	3.22 ± 0.10	2.79 ± 0.05
	37	7.32 ± 0.01	3.85 ± 0.08	2.99 ± 0.10	2.67 ± 0.04
	Δ δ	0.10	0.16	0.23	0.12
+ CO	25	7.33 ± 0.02	3.74 ± 0.01	2.90 ± 0.01	2.59 ± 0.02
	37	7.25 ± 0.02	3.60 ± 0.01	2.67 ± 0.01	2.49 ± 0.02
	Δ δ	0.08	0.14	0.23	0.10

Tab. 3. Temperature dependence of pH in erythrocyte and lysate samples.
In the lysate samples the difference in pH between 25 and 37 °C was determined using a pH electrode in addition to the determination using the chemical shifts of the phosphorus compounds. Note that the errors quoted are standard errors. The p value resulting from using *Student*'s paired t-test to compare the pH values determined at the two temperatures was less than 0.05 in all cases.

Sample	T (°C) dpH/dT	pH -meter	Phosphorus compound		
			3-glycerate 2,3-bisphosphate	2-glycerate 2,3-bisphosphate	P_i
Lysate	25 °C	7.42 ± 0.01	7.74 ± 0.27	7.58 ± 0.13	7.46 ± 0.09
	37 °C	7.32 ± 0.01	7.49 ± 0.15	7.45 ± 0.11	7.29 ± 0.04
	dpH/dT	0.008	0.021	0.011	0.014
Normal	25 °C	na	7.69 ± 0.09	7.61 ± 0.05	7.42 ± 0.07
	37 °C	na	7.48 ± 0.06	7.42 ± 0.08	7.19 ± 0.03
	dpH/dT	na	0.018	0.016	0.019

Effect of temperature on pH_i of intact erythrocytes

Measurements of the chemical shifts of the 2-P and 3-P resonances of glycerate 2,3-bisphosphate and P_i in blood from 9 normal donors were made at 25 and 37 °C. The samples used for the temperature studies were divided into two portions; one for each temperature. Each portion was kept at 4 °C until just prior to measurement. In this way the ATP, glycerate 2,3-bisphosphate and oxygen concentrations should be similar for all of the measurements at each temperature. The chemical shifts of the 2-P and 3-P resonances of glycerate 2,3-bisphosphate and P_i were related to pH_i using the titration curve (see fig. 2). The mean pH_i values at 25 and 37 °C determined using glycerate 2,3-bisphosphate and P_i are given in table 3. From table 3 it can be seen that the mean pH_i decreases with increasing temperature. The data obtained at the two temperatures had some overlap and *Student*'s paired *t*-test was used to compare these data. The *t*-test gave $p < 0.05$ for all pairs of data and so the difference in pH at the two temperatures was deemed significant. The calculated values of dpH/dT are also given in table 3.

Discussion

We chose to use only endogenous and not exogenous species in our pH measurements to avoid the possibility of disturbing the metabolism of the erythrocytes. The influence of factors apart from pH-effects on the chemical shift of the endogenous phosphorus species can be seen from the difference in the values we obtained for δ_1, δ_2 and pK_a and those obtained by *Robitaille* and co-workers in free solution (7). Glycerate 2,3-bisphosphate was in poorer agreement with the literature values that P_i probably as a result of binding to haemoglobin. Because the chemical shifts measured are relative to a standard in a solvent of different diamagnetic susceptibility (i. e., 85% H_3PO_4)

geometry effects are important. Thus, to obtain the true chemical shift a further correction needs to be made regarding the orientation of the cylindrical sample tube to the main magnetic field (25, 26). For example, chemical shift values measured in water relative to 85% H_3PO_4 in a cylindrical tube should be corrected by −0.73 ppm if measured in a superconducting magnet (25). Since it was not possible to account quantitatively for factors (including diamagnetic susceptibility) other than pH that affect the chemical shift of the endogenous phosphorus species, the titration curve for determining the correlation between chemical shift and pH was performed in a concentrated lysate and not in free solution. It should be noted that within experimental error the same correction would apply for all of the samples; thus, we report uncorrected chemical shift values.

The temperature dependence of the glycerate 2,3-bisphosphate titration curves is probably due to differences in glycerate 2,3-bisphosphate binding to haemoglobin. Further, the chemical shift of both the 2- and 3-P resonances is known to be differentially altered by the binding of glycerate 2,3-bisphosphate to haemoglobin (19). The results given in table 2 show that gassing the blood with CO causes the intracellular pH to become more acid (by almost 0.1 pH unit). Therefore, to determine the physiologically relevant pH the cells must not be gassed as part of the measuring procedure. From the similarity in chemical shift changes of the glycerate 2,3-bisphosphate resonances with temperature in both the CO-treated and CO-untreated lysates it can be inferred that there is no pseudo-contact shift effect operating on the glycerate 2,3-bisphosphate resonances. The most probable reasons for the lack of pseudo-contact shifts are that the deoxy-haemoglobin concentration was too small and/or the glycerate 2,3-bisphosphate was not sufficiently close to the haem centre to be affected. By comparing the measured chemical shifts between CO-treated and CO-untreated lysates of similar pH (see tab. 2) it can be seen that the CO-treated lysates show more consistently than their CO-untreated counterparts, most likely as a result of smaller susceptibility differences and the glycerate 2,3-bisphosphate no longer binding to the haemoglobin. Thus, while glycerate 2,3-bisphosphate is not a good pH probe molecule in venous blood it would be better in arterial blood since the greater oxygen content inhibits glycerate 2,3-bisphosphate binding to haemoglobin. In arterial blood as well there will be a smaller susceptibility difference between the intracellular and extracellular spaces due to the lower deoxy-haemoglobin concentration.

By comparing the pH_i values obtained with glycerate 2,3-bisphosphate and P_i in table 3 it can be seen that the values obtained with P_i are closer to those obtained with the pH electrode. The higher pH_i values obtained with the glycerate 2,3-bisphosphate resonances than with the P_i resonance probably result from binding differences, since in the intact cell the haemoglobin has approximately a 15% higher concentration than in the lysates used to calibrate the chemical shift to the pH. It is likely that slight magnetic susceptibility differences resulting from the difference in haemoglobin concentrations between the cytoplasm of the intact cells and the lysate and magnetic field gradients inside the red cells (18) result in our measured intracellular pH values being higher than values obtained using other methods (e.g., (6, 27)). However, our study shows that while endogenous phosphates may not be suitable for determining the absolute pH value in venous blood, they are useful in determining the differential pH with temperature.

Our results (see tab. 3) for dpH/dT for the erythrocyte cytoplasm are in the range of 0.016 to 0.019 unit/°C (25−37 °C), while previous studies using non-NMR techniques over similar temperature ranges have given the value to be 0.0145 unit/°C (27−37 °C) (28, 29). The value of dpH/dT is known to decrease with increasing temperature. The value for plasma is less than for blood at 0.0118 unit/°C (29). The dpH/dT values we obtained for intact cells are consistent with alphastat pH-regulation (21, 22), in which pH is regulated by keeping the fractional dissociation of the imidazole moiety of histidine constant. According to this theory, as temperature increases arterial pH will fall $\approx$ 0.015 pH unit/°C.

In summary we have shown that it is possible to measure dpH/dT for the erythrocyte cytoplasm with ^{31}P NMR, using only endogenous phosphate compounds in conjunction with an external reference. By comparing data obtained in CO-treated and CO-untreated lysates we have shown that the ^{31}P resonances of glycerate 2,3-bisphosphate are not subject to pseudo-contact shift effects from deoxy-haemoglobin. Our data on the temperature dependence of pH from intact normal erythrocytes is consistent with that predicted by the alphastat hypothesis and previous measurements.

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